



Volume 1

# Encyclopedia of Food Chemistry

Editors

Peter Varelis, Laurence Melton and Fereidoon Shahidi



# ENCYCLOPEDIA OF FOOD CHEMISTRY

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# ENCYCLOPEDIA OF FOOD CHEMISTRY

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VOLUME 1





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John teaches Food Science undergraduate and postgraduate courses specializing in food chemistry, food processing and food quality management and safety.

His research area is on physicochemical composition and properties of food components, increasing value of secondary food processing products and their contribution to consumer health and wellness. Students research on value-added oilseed products, transesterification of oils with phenolic acids (to produce antioxidants), bioactive peptides, alternative protein sources to traditional farming practices, fish oil composition, stabilization and analysis of oxidative products, biofuel production from primary wastes, nanoencapsulation of bioactives, frying oil stability and lipid derived flavour in meat. Analytical instruments: HPLC, GC, GCO, PTRMS, GC-MS, NMR, DSC, TGA and access to specialized University instruments.

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Dr Rogers operates one of only three TIM-1 simulated GI tracks in Canada and the only one where physical properties of foods can be measured directly in the system. His research area focuses on lipid digestion kinetics of processed foods, with particular interest in developing infant formula that have similar digestion kinetics to human breast milk. Dr Rogers' joined the University of Guelph after holding faculty positions at Rutgers University and University of Saskatchewan, he was also the Director of the Gastrointestinal physiology laboratory at the New Jersey of Food, Nutrition & Health. He currently holds a Tier II Canadian Research Chair in Food Nanotechnology studying how to make high fat foods healthier. For his work on lipid structure and food nanostructures he has recently been awarded The Young Scientist Research Award from the American Oil Chemists' Society and has been appointed as a Fellow of the Early Career Scientists' Section of the International Academy of Food Science and Technology. He holds two patents, 70 peer reviewed manuscripts and more than 40 technical presentations.

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## PREFACE

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This is an exciting time for all who wish to understand the nature of food and how it changes during storage, processing, cooking and digestion. This is because science has turned its focus onto soft matter and food is very largely soft matter. Earlier scientific effort was preoccupied with simple molecules in solution and solids (preferably pure crystalline proteins). When you think about it, knowing the total protein, carbohydrate, lipid and vitamin contents of a food doesn't tell you much about the nature of the food and how it will provide nutrients for us. Now we are seeing a huge effort to understand soft matter, which is directly applicable to food. Meaning we are increasing our knowledge of how food components (both macromolecules such as proteins and polysaccharides as well as smaller molecules) interact chemically with each other; leading ultimately to understanding the complex chemical structure of foods (everything from apples and ice cream to vegetable protein meat-substitutes and food for a visit to Mars).

A golden age for food research has begun, with so many new techniques available such as CRISPR, isothermal calorimetry (ITC), analytical ultracentrifugation (AUC), small angle neutron and X-ray scattering (SANS, SAXS), surface plasmon resonance, electronic tweezers, and computer modelling (for example molecular dynamics simulations for understanding the interactions of proteins and polysaccharides, enzymes and substrates, bioactives and encapsulating agents). This is in addition to the tried and tested techniques of nuclear magnetic resonance (NMR), mass spectroscopy, electron and confocal microscopy, high performance liquid chromatography (HPLC), and gas chromatography. Biosensors publications are huge and nanotechnology is a hot topic for research.

The *Encyclopedia of Food Chemistry* is for people who have a basic knowledge of food chemistry and wish to expand their understanding of a topic based on information from a reliable source. No fake news here! We welcome you to enjoy the excitement captured here by experts from across the planet.

Laurence D. Melton



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Figure 4. Formation of Selected Heterocyclic Flavor Chemicals in Beverages.

Figure 1. Interactions between polyphenols and macromolecules: effect of tannin structure.

Figure 2. Interactions between polyphenols and macromolecules: effect of tannin structure.

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Table 1. Next-generation sequencing.

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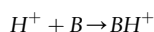
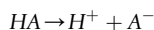
# Acids and Bases in Food

Iris J Joye, University of Guelph, Guelph, ON, Canada

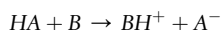
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## General Definition and Theory

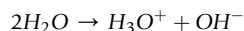
According to the theory of Brønsted-Lowry, an acid is a chemical substance that can donate protons ( $H^+$ ), *i.e.* a proton donor, while a base is a chemical substance that has a tendency to accept those protons, *i.e.* a proton acceptor:



A global acid–base reaction can, hence, be written as:



Water can both act as base and acid:



It has been shown that in pure water, the concentration of both these ions ( $H_3O^+$  and  $OH^-$ ) is  $10^{-7}$  M. In the presence of an acid, a proton donor, the concentration of protons in solution will increase and surpass the ‘neutral’ concentration of  $10^{-7}$  M, while a base, a proton acceptor, will deplete the protons in solution and the proton concentration will decrease below the ‘neutral’ concentration of  $10^{-7}$  M. A logarithmic scale has been introduced to make the concept ‘proton concentration in solutions’ more tangible, *i.e.* the pH scale:

$$pH = -\log[H_3O^+]$$

In neutral solutions, the  $H_3O^+$  (or  $H^+$ ) concentration is  $10^{-7}$  M and the pH would, hence, be 7.0. In acid solutions the pH will be lower than 7.0 while in alkaline solutions the pH will be higher. It is important to keep in mind that the pH scale is a logarithmic scale, which implies that when the pH decreases with one unit, the proton concentration actually multiplies by a factor 10.

With every acid and base a certain ‘strength’ is associated. This strength is an indication of the tendency of an acid or base to donate or accept protons, respectively. Strong acids/bases are usually completely deprotonated/protonated in water, while weak acids/bases will only be partially deprotonated/protonated in water. This is quantitatively expressed by the dissociation constants that are associated with these acids/bases. An in-depth explanation on how to use these dissociation constants can be found in most chemistry handbooks, but falls outside the scope of this chapter.

## Acid and Bases in the Food Sector

Food products are often classified according to their acidity (**Table 1**). Fruits are widely perceived as acid foods. The pH in a lime for example can be as low as pH 2.0, apples and grapes have pH values around 4.0, while bananas have a pH around 5.0. The pH of avocados is close to neutrality. Most vegetables have slightly acid pH values. Examples of neutral pH food products are milk and water. Tea, tofu and some cheeses are alkaline food products (**Table 1**) ([US. Food and Drug Administration/Center for Food Safety and Applied Nutrition, 2003](#)).

In addition to the naturally present acids and bases in food products, acids and bases are often also included in the product recipe as food additive or processing aid, or are generated during processing. Fermentation of food *e.g.* is an example of the *in situ* production of acid during processing.

Besides the targeted functionality, the selection of the acid or base which is most suitable for a specific food product depends on the flavor profile, the solubility and the hygroscopicity of the acid/base, and the food pH. Hygroscopicity, *i.e.* the tendency to absorb moisture, is especially important for those applications focusing on dry mixes or powders ([Igoe, 2011](#)). Besides the physical form of the food product, the cost, legal requirements, availability and storage and handling properties of the acids/bases are also considered when selecting the appropriate acid.

## Acids in the Food Sector

Current consumer trends towards clean label food products boost the use of so-called ‘natural’ acids (such as lime juice) or acid producing processes during food production. Biopreservation is one of the technologies that has recently gained a lot of attention and uses beneficial microbes such as lactic acid bacteria and their products (usually acids) to ensure food safety and shelf-life ([Akbar et al., 2016](#)). The most common acids for food use are organic acids such as acetic, lactic and citric acid. Phosphoric acid is an example of a mineral acid present in food. In **Fig. 1**, the structure of the different organic acids that will be discussed in this section are listed. Acids approved for use in food have been given an E-number by the European Food Safety Authority (EFSA). Their E-numbers classify most of them either in the preservative list (E200–E299) or as antioxidants and acidity regulators (E300–399). E-numbers are also increasingly found on products in North-America and largely follow the numbering of the

**Table 1** pH of some common foods or food ingredients (U.S. Food and Drug Administration/Center for Food Safety and Applied Nutrition, 2003)

<i>Food product/ingredient</i>	<i>pH value</i>
Apple juice	3.4–4.0
Apple sauce	3.1–3.6
Artichokes	5.5–6.0
Avocados	6.3–6.6
Bananas	4.5–5.2
Broccoli	6.3–6.9
Butter	6.1–6.4
Cabbage	5.2–6.8
Carrots	5.9–6.4
Cheese	4.1 (cream cheese) 7.4 (camembert cheese)
Coconut	5.5–7.8
Corn	5.9–7.5
Corn starch	4.0–7.0
Cucumber	5.1–5.8
Flour	6.0–6.3
Garlic	5.8
Grapes	2.8–3.8
Grapefruit	3.0–3.8
Honey	3.9
Fruit jam	3.5–4.5
Ketchup	3.9
Lettuce	5.7–6.1
Maple syrup	5.2
Milk	6.4–6.8
Molasses	5.0–5.5
Mushrooms	6.0–6.7
Pineapple	3.2–4.0
Potatoes	5.4–5.9
Pumpkin	4.9–5.5
Rhubarb	3.1–3.4
Rice (cooked)	6.0–6.8 (range includes brown, white and wild rice)
Spinach	5.5–6.8
Strawberries	3.0–3.9
Sugar	5.0–6.0
Tea	7.2
Tofu	7.2
Tomatoes	4.3–4.9
Vinegar	2.4–3.4

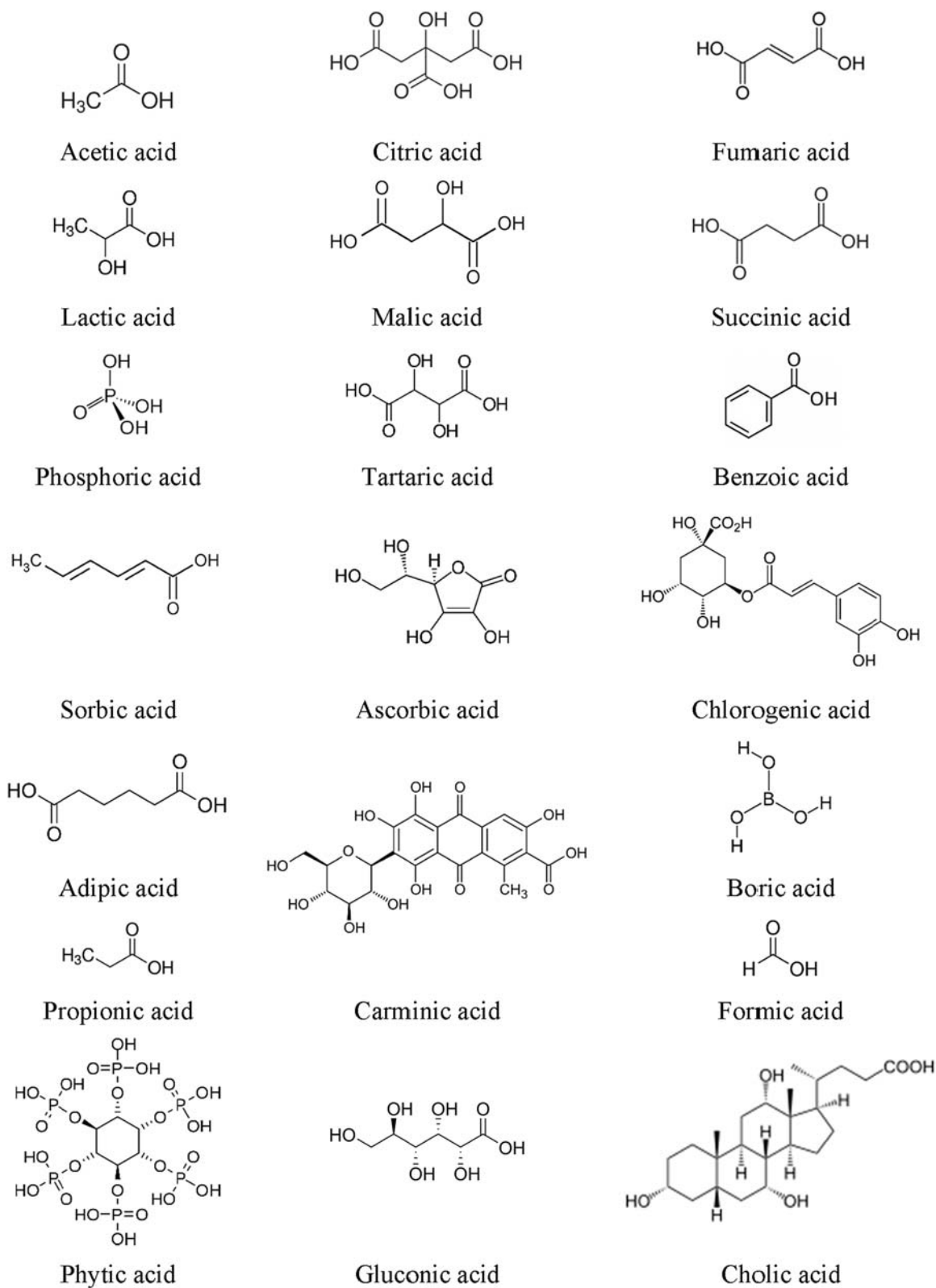
International Numbering System for Food Additives (INS) that is defined in the Codex Alimentarius (Food and Agriculture Organization of the United Nations and World Health Organization, 2017).

Functionalities that are targeted by adding acids to processed foods range from enhancing or altering flavor (*e.g.* in beverages, fruit drinks and desserts), improving food preservation (*e.g.* in mayonnaise, tomato sauce, delaying/inhibiting fruit discoloration), chelating metals (*e.g.* preventing oxidation of fatty and oily foods and delaying/inhibiting canned shrimp discoloration), buffering of food products (*e.g.* in prepared desserts), gelling (*e.g.* in desserts and jams) and/or coagulation (*e.g.* cheese and milk-based desserts) (Igoe, 2011). The perception of sour taste or flavor is a very complex phenomenon and falls outside the scope of this chapter. The interested reader is referred to the excellent review of Ramos Da Conceicao Neta *et al.* (2007).

In what follows, a few examples of acids important in the food sector are listed. It needs to be emphasized, however, that this list is not complete and other food additives exist which also have an effect on the pH of the food system.

### Acetic Acid

Acetic acid (E260 or INS 260), sometimes even called ethanoic acid (Fig. 1, Table 2), is a colorless organic acid with a very strong smell. According to the Codex Alimentarius (Food and Agriculture Organization of the United Nations and World Health Organization, 2017) it is classified as an acidity regulator and preservative. Acetic acid is the main acid in vinegar, in which it is often



**Figure 1** Chemical structure of a selection of acids important in food.

**Table 2** Overview of a selection of common acids used as additives in the food sector (Igoe, 2011; Ramos Da Conceicao Neta et al., 2007)

Acid	Appearance	pH of 1.0% solution	Sensory properties	Acid dissociation constants	Water solubility	Examples of use
Acetic acid	Liquid	2.8	Astringent, tart and sour, vinegary	4.75	High miscibility and solubility	Salad dressings, pickles, cured meat
Citric acid	Crystalline powder	2.2	Sharp, tart, delivers a 'burst' of tartness, fresh citrus flavor	3.08/4.74/5.40	Rapid and high solubility	Beverages
Fumaric acid	Crystalline powder	2.1	Sharp, tart and sour, metallic	3.03/4.44	Low solubility	Bakery applications, controlling leavening, beverages
Lactic acid	Powder or liquid	2.3	Bland or mildly acid	3.86	High solubility	Dairy foods
Malic acid	Powder	2.3	Mildly tart, green, smooth	3.40/5.11	High solubility	Sour confectionary
Phosphoric acid	Liquid	1.6	Sour, tangy	2.12/7.21/12.32	Miscible with and very soluble in water	Beverage syrups
Succinic acid	Crystalline powder	2.7	Tart and slightly bitter, salty, savory and brothy notes	4.19/5.50	Moderate solubility in water	Soup, broth, powdered drinks
Tartaric acid	Crystalline powder	2.3	Sharp, sour, very tart, slightly bitter, augments fruit flavor	2.98/4.34	Low solubility	Bakery applications, candy

present as 3% to 9% of the product volume. As a constituent of vinegar it is a very popular acid in food as e.g. a flavoring agent for salads (Table 2).

It is also used as a sanitizing agent, especially for salad vegetables which are eaten raw. The effect of acetic acid on microorganisms which are related to food spoilage has been of interest for a long time and seems to go beyond the pH effect alone. The undissociated acid is readily taken up by the cells and dissociates in the cell interior, causing a pH drop and metabolic disturbance by the acetate anion (Trcek et al., 2015). Although some bacterial food spoilage strains show a high tolerance towards acetic acid (Trcek et al., 2015), it has been proven to be very effective and toxic against *Salmonella aertrycke*, *Saccharomyces cerevisiae* and *Aspergillus niger* (Levine and Fellers, 1940). The inhibitory activity of acetic acid on *Aspergillus flavus* growth was also proven in terms of food preservation (Pelaez et al., 2012). In combination with sodium benzoate, acetic acid is used to preserve food products such as cucumbers without subjecting them to a thermal treatment (Perez-Diaz and McFeeters, 2008). Next to the above applications, acetic acid can also be used as a processing aid in the extraction of certain protein fractions from cereals. Indeed, acetic acid is a good solvent to extract the prolamin fraction of certain cereals and pseudocereals, a fact that may be of interest to food ingredient producers which are focusing on protein isolates (Taylor et al., 2005).

Acetic acid bacteria are commonly used in the food industry and *in situ* produce acetic acid in the food product or during processing (Sengun and Karabiyikli, 2011). Through the oxidation of sugars and alcohol to organic acid during food fermentation processes these bacteria have a distinct effect on the food appearance, flavor and shelf life. However, the action of these bacteria is not always wanted as they also cause wine spoilage through oxidation of ethanol into acetic acid. Next to the production of vinegar, these acetic acid producing bacteria can also be important to fermentation of cocoa beans and coffee processing (Sengun and Karabiyikli, 2011).

### Citric Acid

Citric acid (E330 or INS330) is a weak organic tricarboxylic acid (Fig. 1, Table 2) that naturally occurs in citrus fruits. Next to its role as flavoring agent, citric acid is widely used as an acidity regulator, antioxidant, color retention agent and sequestrant (Food and Agriculture Organization of the United Nations and World Health Organization, 2017). Sequestrants bind to metal ions, hence, slowing down oxidation reactions and prolonging the shelf life of food products. At the same time, it can also be considered as a nutrient enhancer. Its wide applicability and 'clean and natural' image, made it into probably the most widely used and popular acid in the food industry.

Originally, citric acid was extracted from citrus fruits. However, nowadays, it is produced through fermentation of molasses and other sugar sources. More than half of all citric acid is used in beverages. Over the years, some questions were raised on the innocence of citric acid as a food additive, especially with regard to the formation of reactive radicals (Gautier-Luneau et al., 2007).

### Fumaric Acid

Fumaric acid (E297 or INS297) is a four-carbon dicarboxylic acid (Fig. 1, Table 2) and used as acidity regulator in food (Food and Agriculture Organization of the United Nations and World Health Organization, 2017). Its low cost and non-toxicity are at the basis of its popularity as a food additive since 1946. Fumaric acid is claimed to be 1.5 times more acid than citric acid. Microbial inactivation studies have shown that fumaric acid can inactivate food-borne pathogens and extend the shelf life of products such as fresh

beef (Tango et al., 2014). It is often used as a beverage ingredient, but also finds application in bakery products, powdery dessert mixes and confectionary (Santini et al., 2012). Next to food applications, fumaric acid is used in feed as antibacterial agent and is a well-known chemical that is used in the production of polymers and as intermediate in the production of L-malic and L-aspartic acid. Fumaric acid is predominantly produced by petroleum-based chemical synthesis, but research focusing on microbial fumaric acid production (e.g. using *Rhizopus oryzae*) from starchy materials is in the process of optimizing and commercializing this 'green' technique (Xu et al., 2012; Yang et al., 2011; Alonso et al., 2014).

### Lactic Acid

Lactic acid (E270 or INS 270, Fig. 1, Table 2) is an acidity regulator and was one of the earliest food additives. It is very commonly found in sour milk products such as yoghurt and often used to regulate the acidity of processed food products. It has also been described to act as an antioxidant and preservative. Lactic acid was tested for its inhibitory effect on the growth of *A. flavus*. Its efficacy was lower than that reported for acetic acid. The combination of acetic and lactic acid, however, showed a synergistic effect (Pelaez et al., 2012).

Lactic acid is also produced by bacteria in a lot of (fermented) food products (Torino et al., 2015; de souza motta and da silva mesquita gomes, 2015, Ganzle, 2015). These bacteria are believed to have probiotic effects and, hence, caught the interest of consumers and the food industry. In the dairy industry e.g. lactic acid bacteria have been extensively researched with regard to their potential to design natural biofunctional health-promoting food (Linares et al., 2017). Through the production of products such as lactic acid, these bacteria influence the product's sensory properties and microbiological stability (De Souza Motta and Da Silva Mesquita Gomes, 2015).

### Malic Acid

Malic acid (E296 or INS 296, Fig. 1, Table 2) is a four-carbon dicarboxylic acid that is used as acidity regulator and flavor enhancer in food. It is often found in unripe fruit and is also present in wine. Malic acid levels in soft drinks, fruit juices and wine need to be strictly controlled as too low or high levels may result in product deterioration (Antonelli et al., 2008). Together with tartaric acid, malic acid makes up about 90% of the total acidity of wine. Malic acid is also used as flavoring agent in the sour confectionary sector. Similar as the other organic acids, malic acid has been found to be an effective agent for inactivating common food pathogens on fresh vegetables (Kim et al., 2016).

Malic acid is often synthesized chemically starting from fumaric acid (Chi et al., 2014). However, the increasing cost of fumaric acid production and the quest for more eco-friendly techniques has triggered more research efforts into producing malic acid from sugars using microorganisms (Chi et al., 2014).

### Succinic Acid

Succinic acid (E363, Fig. 1, Table 2) and its sodium salt are used as flavor enhancers in food products such as desserts, soups/broths and powders that can be used to prepare drinks at home (Chimirri et al., 2010). Succinic acid production through fermentation (e.g. using *Yarrowia lipolytica*) of agricultural and food waste streams is one of the strategies that is currently explored to reduce the less ecological chemical synthesis of succinic acid (Yang et al., 2017).

### Phosphoric Acid

Phosphoric or orthophosphoric acid ( $\text{H}_3\text{PO}_4$ , E338 or INS338, Fig. 1, Table 2) is used as an acidity regulator, antioxidant and sequestrant in food (Food and Agriculture Organization of the United Nations and World Health Organization, 2017). It is a widely used flavoring agent for carbonated soft drinks and lies at the basis of the tangy flavor of cola, root beers and other soft drinks. Although it is also a synthetic chemical, phosphoric acid derivatives are widely found in nature and are, hence, already naturally present in food. Phosphoric acid is the only inorganic acid that is widely used as food acid and, at the same time, is one of the least expensive food-grade acid additives. It is also, of all the acids described here, the strongest acid, which can give the lowest attainable pH values (Table 2). Next to its use in the soft drinks industry, phosphoric acid is also used in cheese manufacturing and brewing practices. It has also been shown to stimulate yeast growth (important to foods which involve fermentation by yeast) and is used in the production of gelatin.

### Tartaric Acid

Tartaric acid (E334 or INS 334) is a dicarboxylic acid (Fig. 1) that finds application as acidity regulator, antioxidant, flavor enhancer and sequestrant in the food sector. Tartaric acid is highly water soluble and has a very strong tart taste (Table 2). Tartaric acid is naturally found in grapes and bananas and has been reported to enhance the flavor of grape- and lime-flavored beverages. Together with malic acid it contributes 90% of the acidity in wine and its levels need to be tightly controlled in order to ensure product quality (Sprenger et al., 2015). Similar to malic acid, it is used as flavoring agent in sour-tasting confectionary. Its antioxidant activity stems from its function as sequestrant. Tartaric acid is commercially obtained in either a natural way (extraction from grapes or wine by-products) or by chemical synthesis starting from petroleum by-products (Serra et al., 2005).

### Benzoic Acid

Benzoic acid (E210 or INS210, Table 2), a colorless aromatic carboxylic acid (Fig. 1), is naturally present in plant (fruits, nuts, spices and vegetable), fungal and animal tissues, but it can also be produced by microorganisms during food processing and/or be added



as a food additive. Rich natural sources of benzoic acid are strawberries (up to 29 mg/kg), cayenne pepper and mustard seeds (up to 10 mg/kg), cloves, salvia, thyme and nutmeg (up to 50 mg/kg) and cinnamon (up to 335 mg/kg) (del Olmo et al., 2017). Benzoic acid levels in milk were also found to very sharply increase during fermentation, presumably due to the activity of lactic acid bacteria, *Pseudomonas* and/or *Escherichia coli*. Levels of up to 24 mg/L have been reported in fermented cow's milk. The benzoic acid levels in raw cow's milk cheese ripened for 6 months can be as high as 250 mg/kg (Del Olmo et al., 2017).

Next to its natural occurrence in food, benzoic acid and its derivatives are also commonly supplemented as antibacterial and -fungal preservatives or flavoring agents (Del Olmo et al., 2017). Benzoic acid has a low taste threshold and low volatility and wide antimicrobial spectrum (Ashurst, 1991). Although the undissociated form of benzoic acid is proven to be more effective as antimicrobial agent, the salt, due to its better water solubility, is more often used in food applications. The maximal antimicrobial activity of benzoic acid has been described between pH 2.5 and 4.5. It is clear that benzoic acid and its derivatives are, hence, most often used as preservatives in acid food products. However, when using benzoic acid as food additive, care needs to be taken that the food product's taste is not altered in such way that it becomes unacceptable. Benzoic acid is known to cause oral prickling, to increase the perception of sweetness, to reduce sourness and saltiness perception and to strongly suppress the perception of bitterness (Otero-Losada, 2003).

Notwithstanding its approval for food use and GRAS status, the toxicology of benzoic acid and its derivatives has always been controversial (Del Olmo et al., 2017). Information on maximum allowed limits of benzoic acid and its salt as food additives can be found in the Codex Alimentarius (Food and Agriculture Organization of the United Nations and World Health Organization, 2017).

### Other Acids

Examples of other non-organic acids in food are *hydrochloric acid* (HCl, E507 or INS 507), *boric acid* (E284) and *sulfuric acid* (H<sub>2</sub>SO<sub>4</sub>, E513). Hydrochloric and sulfuric acid act as acidity regulators, while boric acid can be used as a preservative. However, these acids are usually formed in the food rather than being added as a food additive. Hydrochloric acid has been described as having a faintly bitter taste (Ramos Da Conceicao Neta et al., 2007).

*Sorbic acid* (E200 or INS200, Fig. 1), analogous to benzoic acid, is used in beverages as preservative (Food and Agriculture Organization of the United Nations and World Health Organization, 2017) due to its non-volatile nature and effectiveness against yeasts (Otero-Losada, 2003). However, sorbic acid has a lower taste threshold and some consumers are very sensitive to its particular flavor in citrus flavored food products.

*Ascorbic acid* (E300 or INS300, Fig. 1) functions as acidity regulator, antioxidant, flour treatment agent and sequestrant in food applications. Ascorbic acid is highly water soluble and is often used as an antioxidant in beverages, wine and meat products (Sadecka and Polonsky, 2001).

*Chlorogenic acid* (Fig. 1) is a phenolic compound that is widely distributed in plants such as apples, artichokes, carrots, eggplants, kiwi fruits, pears, potatoes and tomatoes (Santana-Galvez et al., 2017). To this acid, a lot of health-promoting properties have been ascribed and it is, hence, often used as a food additive and nutraceutical. As a food additive, it fulfills the role of a broad spectrum antimicrobial agent effective against bacteria, yeasts, molds, viruses and amoebas. Furthermore, chlorogenic acid has also antioxidant activity and may be useful in reducing the degree of lipid oxidation or for protecting bioactive molecules (Santana-Galvez et al., 2017). Due to its properties, chlorogenic acid is certainly gaining more interest and importance for the formulation of dietary supplements or functional foods (Santana-Galvez et al., 2017). The health-improving effects ascribed to chlorogenic acid are anti-oxidant, anti-inflammatory, anti-obesity, anti-diabetic and anti-hypersensitive properties. Till date, however, the isolation of chlorogenic acid to be used as food additive is rather expensive, and, hence, not yet competitive relative to other well-known acids that are used as traditional preservatives (Santana-Galvez et al., 2017).

*Adipic acid* (E355 or INS355, Fig. 1) and its salts are used as acidity regulators, acidifiers, flavor enhancers, gelling aids and slow raising agents (Chimirri et al., 2010, Food and Agriculture Organization of the United Nations and World Health Organization, 2017). Adipic acid is used in filling and topping for bakery products, dry powdered dessert mixes, gel-like desserts, fruit-flavored desserts and beverage powders (Chimirri et al., 2010). It is also used to adjust the viscosity and melting behavior of food products such as cheese spread and as leavening acidulant in baking powder. Adipic acid's taste is sometimes described as chalky (Ramos Da Conceicao Neta et al., 2007).

*Propionic acid* (E280 or INS280, Fig. 1) is used in food as a preservative. One of its main applications is as antimicrobial additive in bread (Basler et al., 1987) (Table 1).

### Bases in the Food Sector

Next to acids, bases are also important in food. One of the most alkaline food products in the human diet is bird's nest soup, with a pH that varies between 7.2 and 7.6 (U.S. Food and Drug Administration/Center for Food Safety and Applied Nutrition, 2003). The (in the Western world) more commonly consumed Camembert cheese has a pH around 7.4 (Table 1). Acids in water will donate a proton to water and its conjugate base will be formed. Hence, for all the above acids conjugated bases also exist. For a lot of the above mentioned commonly used acids, the salt-form of the conjugated base is also an approved and commonly used food additive.

Examples of food products that require the use of bases during production are wheat noodles and pretzels (Rombouts et al., 2012b, 2014). During noodle production alkaline salts (such as sodium and potassium carbonate) are used. Pretzels, on the other

**Table 3** Overview of less common food acid additives and their properties (Food and Agriculture Organization of the United Nations and World Health Organization, 2017)

Name	E/INS-number	Type of additive	Notes	References
Carminic acid	E/INS120	Food dye	Red or purple color, obtained from an insect that lives on cacti	Muller-Maatsch and Gras (2016) (Fig. 1)
Formic acid	E236	Preservative	Produced by lactic acid bacteria, but level highly dependent on growth medium and bacterial strain	Ozcelik et al. (2016) and Ostling and Lindgren (1993) (Fig. 1)
Dehydroacetic acid	E265	Preservative	Used in <i>e.g.</i> butter, cheese and margarine	Ohtsuki et al. (2013) and Wolf (1950)
Erythorbic acid	E/INS315	Antioxidant	Stereoisomer of ascorbic acid with similar physicochemical properties, enhances the bioavailability of iron, does not have antiscorbutic activity	Fidler et al. (2004) and Kadin and Osadca (1959)
Metatartaric acid	E353	Emulsifier	Used in the wine industry to prevent the precipitation of tartrate salt	Sprenger et al. (2015)
Thiodipropionic acid	E/INS388	Antioxidant	Used in fatty foods to prevent rancidity	Stokes et al. (1975)
Phytic acid	E391	Antioxidant	Considered an antinutritional factor, tightly interacts with metals (antioxidant for lipid containing food systems and stabilization of colors) and buffers food systems	Graf (1983) (Fig. 1)
Alginic acid	E/INS400	Emulsifier, thickening and gelling agent	Used in <i>e.g.</i> soup, processed cheese, dairy spreads, pudding, mustard, jam	Usov and Zelinsky (2013)
Gluconic acid	E574	Acidity regulator	Also has raising, sequestering, hardening and flavor enhancing properties – bitter but refreshing flavor, used in fruit juices, pickled foods	Canete-Rodriguez et al. (2016) (Fig. 1)
Glutamic acid	E/INS620	Flavor enhancer	Its salt, monosodium glutamate (MSG) is an important food additive, imparts umami taste to food product	Populin et al. (2007)
Guanylic acid	E/INS626	Flavor enhancer	Enhances umami taste in food, used in soup, noodles, potato chips, savory rice, canned vegetables	
Inosinic acid	E/INS630	Flavor enhancer	Used in meat sector for intensification of meat flavor in soups, sauces and seasonings	Portela et al. (1994)
Cyclamic acid	E/INS952	Sweetener	Used in low calorie food and drinks	
Cholic acid	E1000	Emulsifier		(Fig. 1)

hand, are dipped in a hot alkaline solution (1.0 w/v NaOH at 80–90 °C) prior to baking. This causes gelatinization of starch at the dough surface, leads to dissociation of amylose–lipid complexes, promotes crosslinking of wheat gluten and induces Maillard and caramelization reactions (Rombouts et al., 2012a, 2012b). The alkaline treatment leads to the unique taste and the hard shiny surface of the pretzels (Rombouts et al., 2012a). However, in general, food consumed in a regular human diet has a more acid pH (Table 1).

In what follows, a few bases which have been approved for use as food additive in Europe and/or North America are listed. Again, this list is by no means not complete.

### Carbonates

Carbonates include sodium carbonate (E500 or INS500), calcium carbonate (E170 or INS170) potassium carbonate (E501 or INS501), ammonium carbonate (E503 or INS503), magnesium carbonate (E504 or INS504) and ferrous carbonate (E505). Besides the functionality as acidity regulators in food products, additional functionalities have been ascribed to each of these carbonates (Table 4). Carbonates are often also used as neutralizer for products which have undergone an acid treatment such as in the production of non-fermented soybean sauce (Food and Agriculture Organization of the United Nations and World Health Organization, 2017).

*Sodium bicarbonate* is commercially known under the name ‘baking soda’. Baking soda is used in the baking industry as raising agent. Upon exposure to water, sodium bicarbonate generates carbon dioxide, which leads to (chemical) leavening of bakery products.

*Calcium carbonate* is technically ground limestone or can be obtained by precipitation of calcium ions using carbonate ions. It is a white, odorless and tasteless powder that is insoluble in water and alcohol. In contact with an acid, it creates bubbles. Its traditional use was for adulteration of flour. Although it is also listed as a food coloring agent, this application is not widespread (Scotter, 2011).

### Hydroxides

*Sodium hydroxide* (E524 or INS524) and *potassium hydroxide* (E525 or INS525) are sometimes used during processing to sustain specific alkaline conditions in order to promote certain reactions in the food matrix (Rombouts et al., 2012a, 2012b).

**Table 4** Overview of food bases and their properties (Food and Agriculture Organization of the United Nations and World Health Organization, 2017)

Name	E/INS-number	Type of additive	Properties	References
Sodium carbonate	E/INS500	Acidity regulator, anticaking agent, emulsifying salt, raising agent, stabilizer, thickener	Used in cereal products, dairy products and fish products Studied as preservative in food coating, inhibiting growth of fungi	Karaca et al. (2014)
Calcium carbonate/chalk	E/INS170	Acidity regulator, anticaking agent, color, firming agent, flour treatment agent, stabilizer	Used in cereal products, dairy products, meat and fish products Use as food color is limited	Scotter (2011)
Potassium carbonate	E/INS501	Acidity regulator, stabilizer	Used in dairy products, pasta and noodles, studied as preservative in food coating, inhibiting growth of fungi	Palou et al. (2009)
Ammonium carbonate	E/INS503	Acidity regulator, raising agent	Used in frozen fish products, studied as preservative in food coating, inhibiting growth of fungi	Karaca et al. (2014)
Magnesium carbonate	E/INS504	Acidity regulator, anticaking agent, color retention agent	Used in dairy products, fish products, salt and salt substitutes	
Ferrous carbonate	E505	Acidity regulator		
Sodium hydroxide	E/INS524	Acidity regulator	Used in pretzel making, dried whey and whey products and infant formulae	Rombouts et al. (2012b), Rombouts et al. (2012a)
Potassium hydroxide	E/INS525	Acidity regulator	Used in dried whey and whey product and infant formulae	
Calcium hydroxide	E/INS526	Acidity regulator, firming agent	Improves textural properties of buckwheat noodles, used in infant formulae	Han et al. (2012)
Ammonium hydroxide	E/INS 527	Acidity regulator	Used in fermented milk	
Magnesium hydroxide	E/INS528	Acidity regulator, color retention agent	Used in fermented milk, salt substitutes, fish and coffee products	
Calcium oxide	E/INS529	Acidity regulator, flour treatment agent	Used in fermented milk	
Magnesium oxide	E/INS530	Acidity regulator, anticaking agent	Used in salt, dried whey and whey products	

Also the *hydroxides of calcium* (E526 or INS526), *ammonium* (E527 or INS527), and *magnesium* (E528 or INS528) are used in the food sector for the regulation of the acidity. Calcium hydroxide is also used as firming agent and has been proven useful for improving the texture of cereal products (Han et al., 2012).

## Conclusion

Acids and bases are omnipresent in food products. They are either naturally present in the main food ingredients, are generated during food processing and/or storage or are deliberately added to improve the quality and shelf life of food. Besides contributing to product taste and flavor, acids and bases also have roles as colorants, structure building or stabilizing agents and shelf life extenders.

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# Anthocyanins

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## Introduction

Anthocyanins are the largest group of water-soluble pigments in the Plant Kingdom, displaying a diversity of colors that range from pink-orange through red to purple and blue hues. They are present in all types of vascular plants and can be found in any plant tissue, although most commonly in flowers and fruits, being widely distributed in the human diet through plant-based foods. Their chemical nature was established at the beginning of the 20th century through the pioneer works of Richard Willstätter (Nobel Laureate in Chemistry in 1915) and Muriel Wheldale Onslow, with initial studies mostly focused in understanding their role in flower coloration (e.g. Willstätter and Everest, 1913; Wheldale, 1914). Studies on color changes dependent on pH variation were developed by Shibata et al. (1919), whereas Sir Robert Robinson (Nobel Prize in 1947) made significant contributions to anthocyanin synthesis (Robertson and Robinson, 1926) and the copigmentation process (Robinson and Robinson, 1931). Equilibria among anthocyanin structures (Brouillard and Dubois, 1977; Brouillard and Delaporte, 1977) and the copigmentation mechanisms (Goto and Kondo, 1991) were mostly elucidated in the 1970s–1980s. Over the last thirty years, favored by the advances in the analytical techniques, a large number of novel structures have been identified and new families of anthocyanin-related pigments, like pyrananthocyanins, described (Andersen, 2008). Currently the complete structures of more than 700 anthocyanins present in natural sources have been described and above 200 have been tentatively identified (Andersen and Jordheim, 2013), although the number of reported compounds is continually increasing. The interest in anthocyanin research has renovated in recent times as evidences about their potential benefits in human health have accumulated, and novel applications of anthocyanin pigments as colorants or putative bioactives to be exploited by food, pharmaceutical and cosmetic industries have arisen (He and Giusti, 2010; Pina et al., 2012).

## Structural Features

Anthocyanins belong to the family of flavonoids and their basic structure consists of an aglycone (known as anthocyanidin) derived from the 2-phenylbenzopyrylium (flavylium) skeleton diversely hydroxylated/methoxylated. Anthocyanins are generally represented as positively charged flavylium cations (Fig. 1), which are the dominant equilibrium forms in strongly acidic aqueous solutions (see below).

About 30 anthocyanidins have been identified in nature (Table 1), although only six are widespread, with more than 90% of the naturally occurring anthocyanins based on them; these are: cyanidin (around 31%), delphinidin (22%), pelargonidin (18%), petunidin, peonidin, and malvidin (21% together) (Andersen, 2008). They share the same hydroxylation pattern in 3, 5, 7 and 4' positions, and differ in the number of hydroxyl and methoxyl groups at 3' and 5' in the B-ring. Less common anthocyanidins include those additionally hydroxylated at C6 or methoxylated at 5 or 7 positions on the A-ring, and the 3-deoxyanthocyanidins that lack the hydroxyl group at position 3.

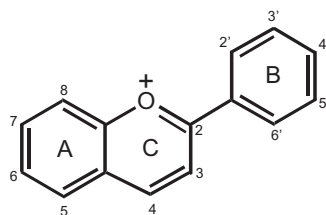
The anthocyanin aglycones are quite unstable and seldom found as such in plant tissues and food, although the natural presence of free anthocyanidins, especially cyanidin, delphinidin and pelargonidin, has been reported in dried beans (*Phaseolus* spp) (Macz-Pop et al., 2006b).

Most anthocyanins are glycosylated on the hydroxyl at position 3; additional glycosylation may exist at 5, 7, 3', or 5', and more rarely at 4' (Brouillard, 1988). Unusual anthocyanins bearing sugar functional groups only in 3' position have also been reported (Fossen and Andersen, 1999), as well as C-glycosyl anthocyanins (Saito et al., 2003). The most common substituting sugar is glucose (90%), followed by rhamnose, galactose, xylose and arabinose; glucuronic acid is the rarest monosaccharide found (Andersen and Jordheim, 2006). Several di- and trisaccharides, such as rutinose (6-O- $\alpha$ -L-rhamnosyl-D-glucose), neohesperidose (2-O- $\alpha$ -L-rhamnosyl-D-glucose), sophorose (2-O- $\beta$ -D-glucosyl-D-glucose), sambubiose (2-O- $\beta$ -D-xylosyl- $\beta$ -D-glucose), xylosylrutinose or glucosylrutinose are also found as sugar moieties (Andersen and Jordheim, 2006).

Sugars may be substituted by aliphatic, hydroxybenzoic or hydroxycinnamic acids; the most usual are malonic, acetic, *p*-coumaric, and caffeic acids, although acylation with oxalic, succinic, malic, *p*-hydroxybenzoic, gallic, vanillic, syringic, protocatechuic, ferulic and sinapic acids can also occur. Polyacylated anthocyanins that contain chains consisting of multiple glycosyl and acyl groups have been described, being particularly reported in flowers from different plant species (Goto and Kondo, 1991). It has been estimated that more than 65% of naturally occurring anthocyanins are acylated (Andersen and Jordheim, 2006), being malonic acid the most frequent acyl moiety.

Un-like usual anthocyanins, 3-deoxyanthocyanidins are commonly found as free aglycones in plants. Nevertheless, these compounds have much restricted distribution, being present in mosses and ferns, as well as in certain members of the Poaceae, Gesneriaceae, Sterculiaceae or Bignoniaceae families among higher plants (Harborne, 1966; Iacobucci and Sweeny, 1983). Sorghum is the known major edible source of these compounds, especially apigeninidin and luteolinidin (Dykes and Rooney, 2006), whereas tricetinidin is formed in black tea from the enzymatic oxidation of epigallocatechin-3-O-gallate (Coggon et al., 1973). The leaves of





**Figure 1** Structure of the flavylium cation.

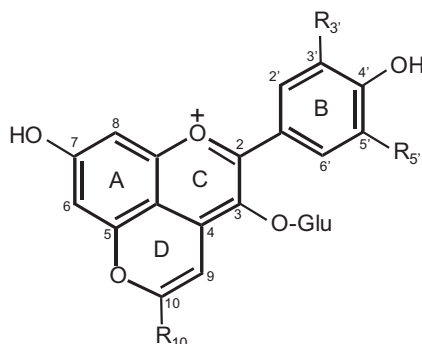
**Table 1** Naturally occurring anthocyanidins

<i>Anthocyanidins</i>	<i>Substitution pattern</i>
<b>Common anthocyanidins</b>	
Pelargonidin (Pg)	3,5,7,4'-OH
Cyanidin (Cy)	3,5,7,3',4'-OH
Delphinidin (Dp)	3,5,7,3',4',5'-OH
Peonidin (Pn)	3,5,7,4'-OH; 3'-OCH <sub>3</sub>
Petunidin (Pt)	3,5,7,4',5'-OH; 3'-OCH <sub>3</sub>
Malvidin (Mv)	3,5,7,4'-OH; 3',5'-OCH <sub>3</sub>
<b>Rare anthocyanidins</b>	
6-Hydroxypelargonidin (Aurantidin)	3,5,6,7,4'-OH
6-Hydroxycyanidin	3,5,6,7,3',4'-OH
6-Hydroxydelphinidin	3,5,6,7,3',4',5'-OH
5- <i>O</i> -Methylcyanidin (Elodenin)	3,7,3',4'-OH; 5-OCH <sub>3</sub>
7- <i>O</i> -Methylcyanidin	3,5,3',4'-OH; 7-OCH <sub>3</sub>
7- <i>O</i> -Methylpeonidin (Rosinidin)	3,5,4'-OH; 7,3'-OCH <sub>3</sub>
5- <i>O</i> -Methyldelphinidin (Pulchellidin)	3,7,3',4',5'-OH; 5-OCH <sub>3</sub>
5- <i>O</i> -Methylpetunidin (Europinidin)	3,7,4',5'-OH; 5,3'-OCH <sub>3</sub>
5- <i>O</i> -Methylmalvidin (Capensinidin)	3,7,4'-OH; 5,3',5'-OCH <sub>3</sub>
7- <i>O</i> -Methylmalvidin (Hirsutinidin)	3,5,4'-OH; 7,3',5'-OCH <sub>3</sub>
<b>3-Deoxyanthocyanidins</b>	
Apigeninidin	5,7,4'-OH
Luteolinidin	5,7,3',4'-OH
Tricetinidin	5,7,3',4',5'-OH
6-Hydroxyluteolinidin	5,6,7,3',4'-OH
8-Hydroxyluteolinidin (Columnidin)	5,7,8,3',4'-OH
5,4'- <i>O</i> -Dimethylapigeninidin (Bifloridin)	7-OH; 5,4'-OCH <sub>3</sub>
7- <i>O</i> -Methylapigeninidin	5,4'-OH; 7-OCH <sub>3</sub>
Diosmetinidin	5,7,3'-OH; 4'-OCH <sub>3</sub>
5- <i>O</i> -Methyluteolinidin	7,3',4'-OH; 5-OCH <sub>3</sub>
5- <i>O</i> -Methyl-6-hydroxyapigeninidin (Arrabidin)	6,7,4'-OH; 5-OCH <sub>3</sub>
4'- <i>O</i> -Methyl-6-hydroxyapigeninidin (Carajurone)	5,6,7-OH; 4'-OCH <sub>3</sub>
5,4'- <i>O</i> -Dimethyl-6-hydroxyapigeninidin (Carajurin)	6,7-OH; 5,4'-OCH <sub>3</sub>
5,4'- <i>O</i> -Dimethyl-6-hydroxyluteolinidin	6,7,3'-OH; 5,4'-OCH <sub>3</sub>
5- <i>O</i> -Methyl-6-hydroxyluteolinidin (3'-Hydroxyarrabidin)	6,7,3',4'-OH; 5-OCH <sub>3</sub>

*Arrabidaea chica* (syn. *Bignonia chica*), traditionally used by the indigenous populations of the Amazonia for body painting and cloth dyeing, are rich sources of carajurin, arrabidin and carajurone (Devia et al., 2002).

## Anthocyanin-Related Pigments

Pyranoanthocyanins are an important group of anthocyanin-derived pigments that possess an additional pyranic ring in the anthocyanin structure (Fig. 2). They were identified over twenty years ago in red wines (Fulcrand et al., 1996; Bakker and Timberlake, 1997), where they are formed during winemaking and ageing from the cycloaddition onto the carbon 4 and the hydroxyl at position 5 of anthocyanins of different products from the yeast metabolism (e.g., acetaldehyde, pyruvic acid, diacetyl, acetone or acetoacetic acid) or coming from the grape (e.g., flavan-3-ols, hydroxycinnamic acids). All these pigments possess the same basic structure, as presented in Fig. 2, and differ in the type of substituents linked at position C-10 on ring D. A second generation of more complex pyranoanthocyanins that are not derived from anthocyanins but from other previously formed pyranoanthocyanins was further described in Port red wines, such as the so-called portisins, oxovitisins or pyranoanthocyanin dimers (de-Freitas and Mateus,



**Figure 2** Basic structure of pyranoanthocyanins. Positions 3' and 5' may be hydroxylated or methoxylated depending on the parent anthocyanin. Substituent R10 varies as a function of the precursor and defines the different classes of pyranoanthocyanins (see **Table 2**).

2011; Oliveira et al., 2014). A summary of the different types of pyranoanthocyanins that have been described in red wines is collected in **Table 2** together with their precursors. Some pyranoanthocyanins have also been found as naturally-occurring in plants or other foodstuffs like blackcurrant seeds (Lu et al., 2002), red onion (Fossen and Andersen, 2003), strawberries (Andersen et al., 2004), or blood orange (Hillebrand et al., 2004), black carrot (Schwarz et al., 2004) and berry juices (Rein et al., 2005).

Rosacyanins are a particular type of pyranoanthocyanidins that were isolated from petals of *Rosa hybrida* (Fukui et al., 2002; Fukui et al., 2006), namely the violet pigment rosacyanin B (**Fig. 3**) and their blue analogues rosacyanins A1 and A2 that contain ellagitannin moieties linked to the anthocyanidin nuclei. These pigments are present in the plant in the form of aglycones. A non-glycosylated pyrano-3-deoxyanthocyanidin derived from apigeninidin was also identified in red *Sorghum bicolor* (Khalil et al., 2010). Similar pigments were further synthesized by Sousa et al. (2013) that give them the name of deoxyvitisins (**Fig. 3**).

Other anthocyanin-related pigments that have been found in natural sources in non-glycosylated form are sphagnorubins (**Fig. 4**), a type of red pigments described in mosses from some *Sphagnum* species (Mentlein and Vowinkel, 1983), and riccionidins (**Fig. 4**) found in liverworts (Kunz and Burkhardt, 1994) and root cultures of *Rhus javanica* (Taniguchi et al., 2000).

Oligomeric pigments where anthocyanins are covalently linked to other flavonoids have also been described in some natural sources. Anthocyanin-flavonol conjugates were identified in flowers of different plants such as orchids (Strack et al., 1989), chive (Fossen et al., 2000) or *Agapanthus* spp (Bloor and Falshaw, 2000), whilst similar anthocyanin-flavone derivatives have been isolated from *Eichhornia* flowers (Toki et al., 2004). Pigments from the direct condensation between anthocyanins and flavan-3-ols (**Fig. 5A**) have been long indicated to be formed in red wines during winemaking and ageing (Jurd, 1969) and their presence further confirmed

**Table 2** Classes of pyranoanthocyanins identified in red wines

Type	Structure	Precursors
Non-substituted pyranoanthocyanins (B-type vitisins)		Anthocyanins + ethanal
Carboxypyrananthocyanins (A-type vitisins)		Anthocyanins + pyruvic acid
Methylpyrananthocyanins		Anthocyanins + acetone or acetoacetic acid

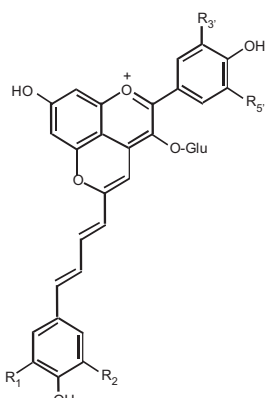
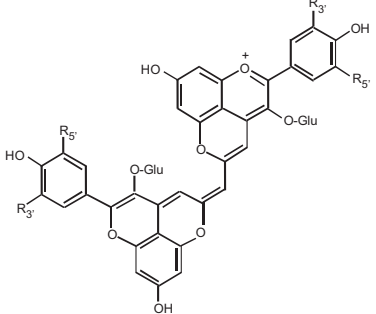
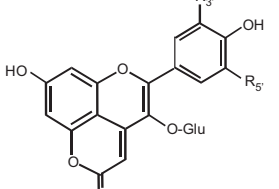


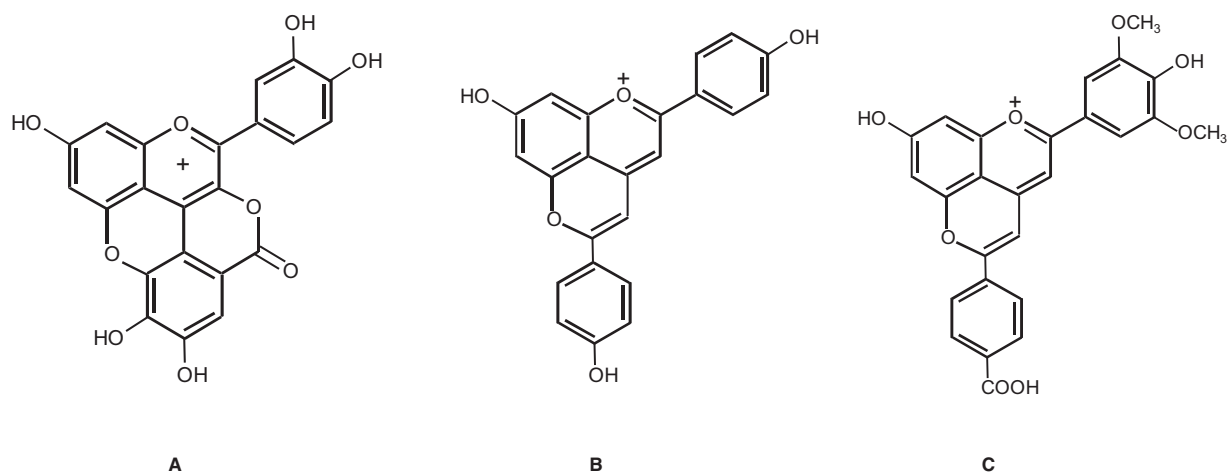
**Table 2** Classes of pyranoanthocyanins identified in red wines—cont'd

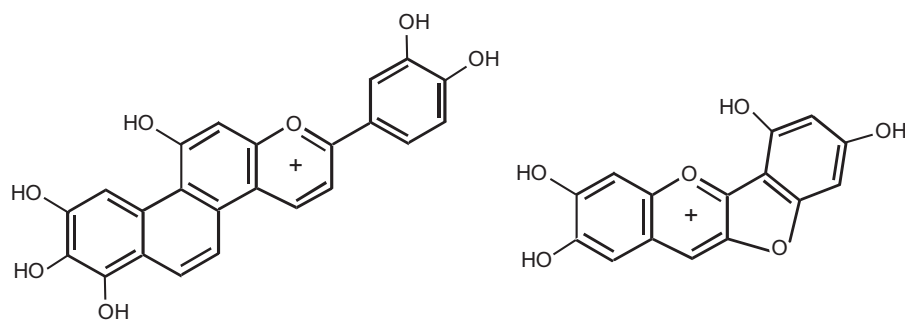
Type	Structure	Precursors
Acetylpyranoanthocyanins		Anthocyanins + diacetyl
Phenyl-pyranoanthocyanins (pinotins)		Anthocyanins + hydroxycinnamic acids or vinylphenols
Flavanol-pyranoanthocyanins		Anthocyanins + vinylflavanols or flavan-3-ols and acetaldehyde
Vinylflavanol-pyranoanthocyanins (A-type portisins)		Carboxypyrananthocyanins + vinylflavanols or flavan-3-ols and acetaldehyde
Vinylphenyl-pyranoanthocyanins (B-type portisins)		Carboxypyrananthocyanins + hydroxycinnamic acids or vinylphenols

(Continued)

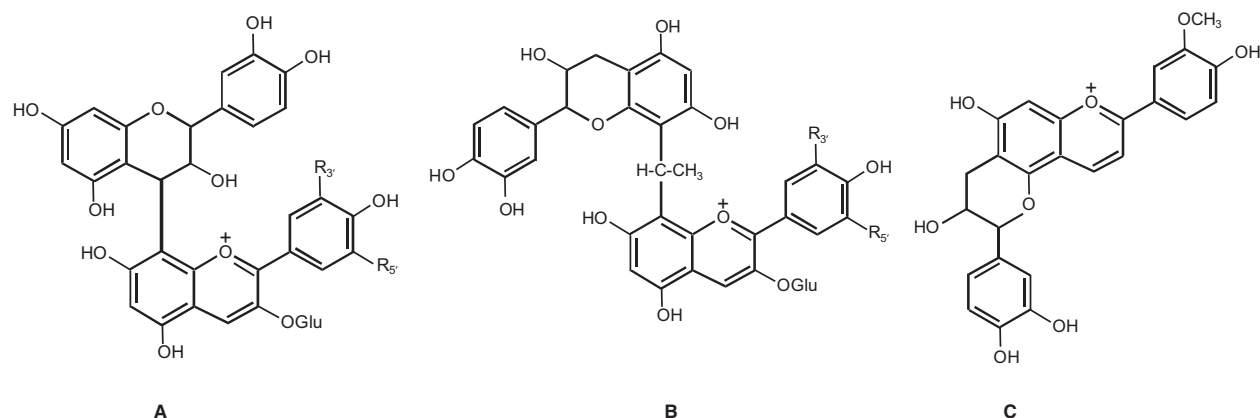
**Table 2** Classes of pyranoanthocyanins identified in red wines—cont'd

Type	Structure	Precursors
Phenyl-butadienylidene-pyranoanthocyanins		Methylpyranoanthocyanins + hydroxycinnamaldehydes
Pyranoanthocyanin dimers		Carboxypyrananthocyanins + methylpyranoanthocyanins
Pyranone-anthocyanins (oxovitisins)		Carboxypyrananthocyanins + water

**Figure 3** Structures of particular non-glycosylated pyranoanthocyanidins. (A) Rosacyanin B (Fukui et al., 2002), (B) pyrano-apigeninidin 4-vinylphenol (Khalil et al., 2010), and (C) malvidin-derived type-A deoxyvitisin (Sousa et al., 2013).



**Figure 4** Structures of sphagnorubins (left) and riccionidins (right).



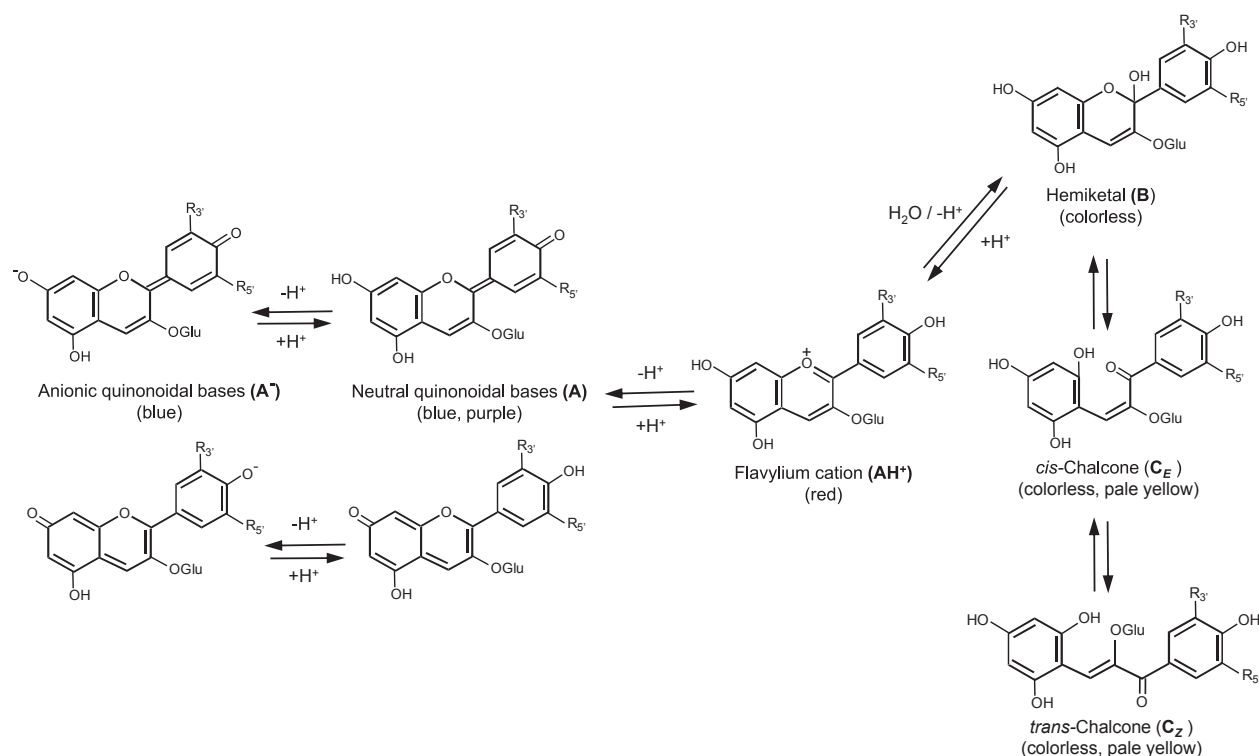
**Figure 5** Structures of different anthocyanin-related pigments. (A) Flavanol-anthocyanin condensed pigments; (B) pigments from the anthocyanin-flavanol condensation mediated by acetaldehyde, and (C) an oaklin (guaiacylcatechinpyrylium).

(Remy et al., 2000; Salas et al., 2004). Similar pigments have been found in extracts of different fruits and grains, such as strawberries (Fossen et al., 2004), black currant concentrates (McDougall et al., 2005), purple corn and red grape skin (González-Paramás et al., 2006), fig (*Ficus carica*) (Duenas et al., 2007) or dried beans (Macz-Pop et al., 2006a). Nevertheless, it is not totally clear whether these pigments actually occur in the plant or they are artefacts produced during extracts preparation or storage. Pigments from the condensation between anthocyanins and flavan-3-ols mediated by acetaldehyde (Fig. 5B) are another type of oligomeric anthocyanins that have also been described in red wines and assigned a crucial role in wine color evolution (Timberlake and Bridle, 1976). The presence of anthocyanin dimers has also been reported in grapes and red wines (Vidal et al., 2004; Alcalde-Eon et al., 2007), as well as that of oaklins (Fig. 5C), a type of anthocyanin-like brick-red pigments that are formed in red wines aged in oak barrels from the reaction between catechins and wood cinnamic aldehydes, such as sinapaldehyde or coniferaldehyde (Sousa et al., 2005).

## Anthocyanins Equilibria

In aqueous solutions anthocyanins exist in various equilibrium forms following different reactions (proton transfer, hydration, tautomerization) of the flavylium cation determined by the pH value. The basic mechanisms of these transformations were established by Brouillard and coworkers in the 1970s (Brouillard and Dubois, 1977; Brouillard and Delaporte, 1977; Brouillard et al., 1978) and are summarized in Fig. 6.

The flavylium cation ( $AH^+$ ), which shows a red color in common anthocyanins, is the predominant form in strongly acidic solutions ( $pH < 2$ ). The positive charge is delocalized through all the pyrylium moiety, although carbons 2 and 4 are the more positively charged atoms (Brouillard et al., 2010). In slightly acidic to neutral solutions, as the pH increases, competition occurs between two reactions: (a) deprotonation of the acidic hydroxyl groups to yield purple/blue quinonoidal bases (A), and (b) hydration of the flavylium cation to give colorless hemiketal forms also referred to as carbinol pseudo-bases (B). Although both positions 2 and 4 of the flavylium cation are charge-defective and susceptible to nucleophilic attack, the hydration reaction mainly takes place at C-2, since the formation of the 4-hydroxy adduct is thermodynamically less favored. The hemiketal form can eventually undergo a ring opening with the formation of the also almost colorless *cis*-chalcone ( $C_E$ ) by a tautomeric process, followed by further isomerization to the *trans*-chalcone ( $C_Z$ ) form (Pina et al., 2012; Brouillard et al., 2010). From the flavylium



**Figure 6** Scheme of the pH-dependent equilibria between main anthocyanin forms.

cation forward, the reactions in anthocyanins equilibria are endothermic, so that chalcone formation is favored by the increase in the temperature. Acidification reverts the equilibria to the flavylium cation; nevertheless, while there is a quick reversion from the hemiketal, the reconversion of the chalcone to  $AH^+$  is very slow and more than 1 h may be required to re-establish equilibrium (Iacobucci and Sweeny, 1983).

By their part, at pH values close to neutrality, the quinonoidal bases undergo a second deprotonation leading to more bluish resonance-stabilized quinonoidal anions ( $A^-$ ). The proton transfer is faster than the hydration reaction, lasting from microseconds to several seconds, respectively (Brouillard and Dubois, 1977). However, the hemiketal ( $B$ ) and chalcone species ( $C_E$ ,  $C_Z$ ) are more stable than the quinonoidal forms ( $A$ ), so that while these latter are rapidly formed as a kinetic product, later they totally or partially disappear in favor of the colorless species as the thermodynamic equilibrium is reached (Pina et al., 2012). However, in planta, the colorless forms ( $B$ ,  $C_E$ ,  $C_Z$ ) are rare, indicating the existence of vacuolar mechanisms that stabilize the colored species, such as copigmentation and metal complexation, which are described below.

In contrast to the 3-substituted flavylium derivatives, quinonoidal bases of 3-deoxyanthocyanins are quite stable and predominate over the colorless hemiketal (Devia, 2003). Actually, carajurin, the first isolated deoxyanthocyanin, was obtained in the form of the quinonoidal base (Chapman et al., 1927).

## Anthocyanin Color and Stability

The stability of the anthocyanins is affected by several factors like their structure, pH, temperature, concentration, oxygen, light and presence of other coexisting components, such as nucleophiles, copigments, metal ions or antioxidants, factors that also have a strong impact on their color. For the six most common anthocyanidins, the stability is influenced by the pattern of substitution in the B-ring, decreasing with the number of hydroxyls and increasing with that of methoxyls. Pelargonidin would be the most stable anthocyanidin, followed by malvidin, peonidin, petunidin, cyanidin and delphinidin. B-ring substitution also affects the color increasing the blue tint with the number of substituents. Nevertheless, anthocyanidins are quite unstable and suffer easy degradation in slightly acidic to neutral mediums as those existing in foods and beverages. Both glycosylation and acylation are stabilizing factors, so that anthocyanins are more stable than their parent aglycones (Iacobucci and Sweeny, 1983).

As above indicated, anthocyanins are usually glycosylated at position 3; further glycosylation at position 5 improves the stability and, thus, 3,5-*O*-diglycosides are more stable than corresponding 3-*O*-glycosides (Hrazdina et al., 1970). Acylated derivatives are generally more resistant to hydration than non-acylated counterparts, showing higher color stability in weakly acidic or neutral solutions. Thus, acylated anthocyanins, such as those present in black carrot, red radish or red cabbage, can provide acceptable color at slightly acidic pH values (Giusti and Wrolstad, 2003). These compounds are not only more stable to pH changes but also against

light and heat. Both aromatic and aliphatic acylation improve stability, but in the first case a blue shift in anthocyanin color is produced that is not observed for aliphatic acylation (Giusti and Wrolstad, 2003).

Bisulfite ions ( $\text{HSO}_3^-$ ), present in food as a result of the addition of sulfite preservatives, act as nucleophiles and attack the flavylium, producing colorless sulfonate adducts, which lead to anthocyanin discoloration. The reaction mostly takes place on C-4 position, which is less sterically hindered than C-2 and it is reversible in a variable extent upon acidification, depending on the anthocyanin and the pH value (Berke et al., 1998). When the position 4 of the pyrylium ring is occupied, the anthocyanins are less susceptible to the nucleophilic attack and subsequently they are totally or partially resistant to bleaching effect of water or bisulfite. This is one of the reasons for the greater color stability of the pyranoanthocyanins and other anthocyanin-derived pigments, as those formed in red wines (de Freitas and Mateus, 2011). Depending on their class, pyranoanthocyanins present different colors that range from yellow to turquoise blue color, through orange, red or purple, which are quite stable within the range of pH existing in most foods and beverages (Oliveira et al., 2014).

The lack of the hydroxyl group in position 3 greatly improves the stability of the anthocyanidins, so that 3-deoxyanthocyanins are much more stable to pH changes than common anthocyanins, showing greater resistance to color bleaching by water or sulfur dioxide, as well as increased stability against ascorbic acid and thermal degradation (Awika et al., 2004; Ojwang and Awika, 2008; Yang et al., 2014). As above indicated, these compounds can be commonly found in nature as free aglycones, and show yellow to orange colors in acidic media (Awika et al., 2004), in contrast with common anthocyanins that possess red to purple hues.

Phenolic compounds with catechol groups are known to possess antioxidant properties, related to their ability to scavenge free radicals by donation of phenolic hydrogen atoms to form relatively stable phenoxyl radicals, and metal chelating capacity (Heim et al., 2002). This activity is expected for cyanidin, delphinidin and petunidin, which would also be the anthocyanins most susceptible to oxidation. The antioxidant capacity of some fruits and vegetables, such as berries, has been related to their anthocyanin content, as well as their protective effects against degenerative and chronic diseases (Heinonen, 2007).

Ascorbic acid, either naturally present or incorporated to food as an additive, induces the degradation of anthocyanins in the presence of oxygen, leading to their bleaching through a mechanism that has been related to ascorbic acid auto-oxidation and may involve the oxidative cleavage of the pyrylium ring (Iacobucci and Sweeny, 1983). Light exposure also speeds anthocyanin degradation by a photo-oxidative mechanism regardless of the pH of the medium, whereas the increase in the temperature leads to the degradation of the anthocyanins by favoring heterocycle ring opening and further cleavage (Furtado et al., 1993).

## Copigmentation and Metal Complexation

In intact plant tissues stabilizing mechanisms exist that contribute to protect anthocyanin color from its degradation and pH-dependent changes. Among them, the most widespread are copigmentation and metal complexation and combinations of both (Brouillard et al., 2010).

Copigmentation consists of a hydrophobic  $\pi$ - $\pi$  molecular non-covalent interaction between the planar polarizable nuclei of the colored forms of the anthocyanins with other organic molecules (i.e., copigments). Copigmentation complexes adopt a vertical stacking (sandwich configuration) that protects the flavylium chromophore from the attack of water and other nucleophilic species like sulfur dioxide, thus preventing at least in part the formation of the colorless adducts. This results in an enhancement and generally a modification in the original color of the pigment containing solution. Thus, at the same time color stabilization and variation can be obtained (Brouillard and Dangles, 1993). The copigmentation effect is evident in weakly acidic to neutral conditions where anthocyanins should mostly exist under colorless forms, and it is well known to occur in plant tissues offering an explanation for the large color variations in flowers and fruits (Goto and Kondo, 1991). However, its contribution to the color in processed foods and beverages is more debatable, due to their lower anthocyanin and copigment concentrations compared to those existing in plant vacuoles. Despite it and the dissociating effects of ethanol on the formation of copigmentation complexes, this process is believed to play a relevant role on color definition in young red wines (Santos-Buelga and de Freitas, 2009).

Copigments are generally colorless and can have different chemical nature, including most polyphenols, but also alkaloids, amino acids, nucleotides, organic acids and some carbohydrates, their efficiency depending on their chemical structures (Escribano-Bailón and Santos-Buelga, 2012). Copigmentation may also occur among anthocyanins themselves, in whose case is also referred to as self-association. This is observed at increasing the concentration of anthocyanins in the medium, which results in a positive deviation from Beer's law, and it is accepted to contribute to the color expression in flowers (Hoshino, 1991).

Another-type of copigmentation is intramolecular copigmentation, where the anthocyanin chromophore interacts with other residues of its own molecule. Such residues are generally hydroxycinnamic acids attached to the anthocyanidin chromophore through one or more sugar units that would act as "spacers", allowing the molecule to fold in such a way that the  $\pi$ -orbitals of the aromatic acyl group(s) can interact with the benzopyrylium nucleus and protect it from hydration (Dangles et al., 1993). Intramolecular copigmentation has the entropic advantage of the copigment being directly attached to the chromophore and consequently the non-requirement of bringing together two molecules initially separated in solution. Those structures are found in many plants and give rise to pigments that are colored through a wide range of pH values (Brouillard et al., 2010).

A process that also influences the color of the anthocyanins is metal complexation. Anthocyanins possessing an o-dihydroxyl substitution (catechol group) in their ring B (e.g., derivatives of cyanidin, delphinidin and petunidin) are able to form complexes with di- or trivalent metals leading to changes in color. Metals most commonly involved in the formation of metalloanthocyanins are iron (III), magnesium (II) and aluminium (III). Metals selectively link with the quinonoidal forms of the anthocyanins,

especially with the anionic quinonoidal bases that result from the deprotonation of the hydroxyls on 4' and 7, which show high acidity at weakly acidic pH values (Brouillard et al., 2010). Metal complexation and hydration are competitive processes, so that as a result of the association with the metal cation, anthocyanin equilibria are displaced to the formation of quinonoidal bases, provoking a bathochromic shift of the absorption spectrum and a change of the color to more violet hues (Dangles et al., 1994). Ternary complexes anthocyanin:metal:copigment can also be formed where the metal links to both pigment and copigment, strengthening the copigmentation effect (Takeda et al., 1990). This type of associations has been indicated to contribute to the blue color in some flowers (Goto and Kondo, 1991; Goto et al., 1986).

## Occurrence in Food and Dietary Intake

As above indicated, most of the anthocyanins occurring in foods are derived from six anthocyanidins: cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin, being cyanidin glycosides the most commonly found in fruits and vegetables.

The most commonly eaten anthocyanin sources belong to the fruits in family Rosaceae (blackberries, raspberries, strawberry, cherries, plums, apples), whose composition is based on cyanidin derivatives as major anthocyanins (Andersen and Jordheim, 2013). The total anthocyanin content reported to occur in fruits varied from a few milligrams to more than 1000 mg per 100 g (fw), with higher levels present in some commonly consumed berries, such as blackcurrants, blackberry, blueberries or chokeberry (Andersen and Jordheim, 2013; Clifford, 2000). Anthocyanins are also abundant in certain cereals and leafy and root vegetables, such as pigmented potatoes, eggplant, cabbage, beans, or red onion, with higher values found in purple corn and purple sweet potato (*Ipomoea batatas*) reaching contents as high as 1400 mg/100 g (Andersen and Jordheim, 2013; Clifford, 2000).

Anthocyanins in the vegetables are considerably more complex than those of the fruits. The proportion of anthocyanins without acyl groups and just one or two monosaccharide units is 74% in fruits, whereas above 77% of the anthocyanins in the vegetables have one or more acyl groups, generally aromatic acyl groups. The families Brassicaceae (red cabbage, radish), Apiaceae (purple carrot), Solanaceae (purple potato), and Convolvulaceae (purple sweet potato) are especially rich in anthocyanins with aromatic acylation. Among fruits, only 23% of the anthocyanins contain acyl groups and 11% have just one aromatic acyl group (Andersen and Jordheim, 2013).

The qualitative and quantitative anthocyanin content in processed products have been much less studied than the anthocyanin content of intact plant sources, except for red wine. The anthocyanin composition in processed products usually reflects that of the original fruit or vegetable, although there are huge variations in their contents depending on the methods of processing and storage conditions. In red wines the intact anthocyanins (mainly malvidin-3-O-glucoside and its derivatives) are present in considerable amounts in young wines (accounting for around 200 mg/L to more than 500 mg/L). Anthocyanin levels notably decrease during wine ageing and storage, until their almost completely disappearance, at the same time that they are replaced by other more stable anthocyanin-derived pigments (de Freitas and Mateus, 2011; Fulcrand et al., 2006). Although less studied, similar reactions may also take place in other processed products like jams or juices during processing and storage (Fukui et al., 2002).

There are large variations in the anthocyanin intake depending on the country, season and dietary habits, especially related to the consumption of fruits, berries and red wine. A first estimation of the average daily intake of anthocyanins was made by Kühnau (1976) that situated it in the range of 180–215 mg, although further reports offer much lower daily consumptions. Dietary intakes of 3.1 mg/day (Chun et al., 2007) and 12.5 mg were estimated for US adults (Wu et al., 2006), whereas mean values of 47 mg/day (Ovaskainen et al., 2008) and 82.5 mg/day (Heinonen, 2007) were calculated in Finland and explained by the high berry consumption. An estimate of 18.9 mg/day was made by Zamora-Ros et al. (2010) for a Spanish adult population, with a relevant contribution of red wine, which accounted for up to 46% of the anthocyanin intake. In general, Andersen and Jordheim (2013) estimated that the average adult intake of anthocyanins in Western countries could be on the scale of 10 mg/day. More studies are, however, required regarding anthocyanin consumption according to the country and the consumers' profiles, taking into account that not only total intake is important but also qualitative composition should have an influence on their putative health promoting effects. Moreover, attention will also have to be paid to the increasing popularity of food supplements and the developing market for functional foods, as they may contribute substantially to a rise in the dietary intake levels (Santos-Buelga et al., 2014).

Actually, a serious limitation to investigate the relation between anthocyanin consumption and health promotion is the difficulty to make adequate estimations of their dietary intake. Data on anthocyanins and other phytochemicals contents in selected foods are already collected in some databases, such as the US flavonoids database (<http://www.ars.usda.gov/Services/docs.htm?docid=6231>), the Canadian FoodDB (<http://foodb.ca/>), or the European eBASIS (Bioactive Substances in Food Information Systems; <http://ebasis.eurofir.org/Default.asp>), Phenol-Explorer (<http://phenol-explorer.eu/>) and PhytoHub ([www.phytohub.eu](http://www.phytohub.eu)). Also, the EU PlantLIBRA initiative (PLANT food supplements: Levels of Intake, Benefit and Risk Assessment; [www.plantlibra.eu](http://www.plantlibra.eu)) was fostered with the aim of gathering knowledge on plant food supplement bioactive compounds. However, accurate data on the qualitative and quantitative anthocyanin composition in foods and beverages are not always available and they are also difficult to obtain, owing to their structural diversity, distribution across a wide range of products, and the strong influence of agronomic and environmental factors on their contents and profiles (Andersen and Jordheim, 2013; Santos-Buelga et al., 2014). Besides, further changes may take place during food processing, storage and culinary preparation, owing to their instability and the formation of anthocyanin-derived products, whose significance and putative biological activities are still to be established. In these circumstances, the description of adequate biomarkers could represent a promising alternative to traditional dietary assessments in order to estimate the exposure to different phytochemicals, including anthocyanins and other polyphenols (Zamora-Ros et al., 2014).

## Anthocyanins as Food Additives

Anthocyanins are approved as natural food colorant additives in both the European Union and the United States. They are usually employed in the form of extracts or concentrated juices obtained from different edible plants, so that they may also contain other components of the original plant material, such as organic acids, tannins, sugars or minerals. In the EU, the anthocyanins receive the common code of E-163 regardless of their origin ([Commission Directive, December 2008](#); [Regulation \(EC\) No 1333/2008 of the European Parliament and of the Council of 16, December 2008](#); [Commission Regulation, November 2011](#)), indicating that, at least from a regulatory point of view, they are looked upon as one homogeneous group of harmless compounds ([Andersen and Jordheim, 2013](#)). Several sources are authorized for their extraction, including grape skins, black currants and other berries, purple corn or red cabbage. In the United States, FD&C numbers are only given for synthetic food dyes approved for their use in foods, drugs, and cosmetics, whereas natural colorants are exempt from batch certification. Grape color and grape skin extracts (enocianina) and fruit and vegetable juices are included in this latter category ([US Food and Drug Administration, 2015](#)).

The interest in anthocyanins and other natural pigments as food colorants to replace synthetic dyes has increased significantly over the last years due to safety issues and consumers' concerns. However, the applications of anthocyanins as food colorants are seriously limited due to their problems of stability. They are generally unsuitable for foodstuffs that do not have a sufficiently acidic pH or that contain sulfites, because of the color changes that they suffer in those conditions. Also, they are susceptible to heat degradation, so that they are not well suited for products submitted to high processing temperatures, such as extruded foods. Typical products for anthocyanin addition are soft drinks, acid dairy products like yogurts, or sugar candies and jams, as well as low water activity products like some snack foods. Acylated anthocyanins, as those present in some vegetables like colored potatoes, black carrot, red radish or red cabbage, usually possess greater heat and light stability, so that they are better candidates for their use as colorants ([Giusti and Wrolstad, 2003](#)). The recent description of several classes of anthocyanin-derived pigments, such as pyranoanthocyanins, with greater stability against pH changes and showing a diversity of colors from yellow to bright blue, has opened promising expectations regarding their use as food colorants ([Oliveira et al., 2014](#)). Moreover, owing to their putative health-promoting effects, anthocyanins have also been proposed for their use as nutraceuticals in the formulation of functional foods or dietary supplements, making them multifunctional food additives ([He and Giusti, 2010](#)).

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## Further Readings

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# Aromas

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## Overview

Flavor plays an important role in food selection and consequently overall nutrition. Flavor is the integrated response to the simultaneous perception (total sensation) of the taste, odor and chemical feeling factors (somatosensory perceived) characteristics of a substance, and is often influenced by the color, shape, texture and overall appearance or esthetic quality of the product. Although the peripheral sensory organs for detection of taste and smell stimuli are quite distinct, their signals are integrated in the orbitofrontal and other areas of the cerebral cortex of the brain to generate the perception of “flavor” and to mediate food recognition (Chaudhari and Roper, 2010). It is generally accepted by most researchers that olfaction (aroma) plays the dominant role in the flavor of food (Spence, 2015).

This chapter provides an overview of the chemicals responsible for aroma or odor perception and to a lesser degree on the physiology and mechanisms involved in odor perception. The discussion is mainly focused on some important and well-defined aroma compounds and in some cases chemical pathways for the formation of specific aroma compounds are provided. Throughout this chapter it is recognized that more detailed information for certain subjects is covered in much greater detail by other publications. Especially useful resources are provided in the *Further Reading* and *Relevant Websites* sections provided at the end of this chapter.

## Olfaction and Odor Perception

The olfactory system of humans is capable of detecting and discriminating a multitude of odorants with diverse chemical structures and widely varying concentrations (Zarzo, 2007; DeMaria and Ngai, 2010). Odorants are transported, by either orthonasal or retronasal pathways, to the olfactory mucosa, which contains millions of bipolar olfactory neurons (Hutchins, 2017). Each neuron contains a single dendrite with multiple (10–20) cilia that are embedded in the surface mucosa and each cilium contains numerous receptor membrane proteins which can interact with different odorant molecules (Menini et al., 2004). Unbound hydrophilic odor molecules diffuse across the layer of mucus, whereas hydrophobic odors must become bound to a specific odorant binding protein to be transported to each cilium for interaction with specific receptors. All of these receptors have the same general structure, seven hydrophobic transmembrane regions, but the amino acid sequence within the cylinders spanning the membrane are extremely diverse which permits the discrimination of a large number of odorants.

Odorant molecules bind reversibly to the odorant receptors which are composed of a diverse group of G-protein coupled receptors. The binding of an odorant activates adenylyl cyclase leading to the formation of cAMP, which triggers a cascade of events that ultimately lead to a nerve signal (Firestein, 2001). Individual odorant molecules stimulate multiple receptors and neurons across the olfactory mucosa to produce spatial patterns of activity that code for its odor character and intensity (Malnic et al., 1999).

## Odor Quality

The way in which people perceive and classify the odor quality of an aroma substance is as much a matter of perceptual learning and memory as it is sensitivity to the odorant (Stevenson, 2001). In general, people can discriminate among individual odors, but are generally poor at naming them (Jöhnson et al., 2005; Stevenson et al., 2007; Frank et al., 2011; Kaeppler and Mueller, 2013). The perceived odor quality of a single odorant is mainly related to its chemical structure. However, food aromas are not composed of single odorants but are instead complex odorant mixtures. The recognition and categorization of perceived smells emanating from odorant mixtures is complex (Thomas-Danguin et al., 2014). Studies have shown that people can identify only about three odorants in mixtures containing up to eight odorants (Laing and Jinks, 2001). But people do not generally perceive and process odor mixtures in this way, but instead as a global mixture percept, referred to as odor object perception (Rescorola et al., 1985). This means that complex olfactory information is coded and stored in the brain as perceptual odor objects which are later recalled. Odor objects or odor quality attributes are believed to be concentration invariant and mainly a function of the defined ratios of the perceived odorants in a mixture (Cleland et al., 2007). For example, a strawberry or a mixture of strawberry aroma components in the correct ratios would be perceived, stored and later recalled as a strawberry odor object. In some instances, people may respond to only a single odorant or just a few odorants in a food containing a complex mixture of odorants. These are often the character-impact odorants of that food, or if these odorants are considered to be unusual or foreign to that food they might be perceived as off-odors or taints.

## Aroma Substances

Not all volatile compounds have perceivable odors in foods. In many food products it is possible to identify thousands of volatile compounds; however, only a few (estimated to be ~3%) of these are actually odor-active and contribute substantially to the overall aroma or flavor of the product (Dunkel et al., 2014). Often a volatile compound has a detectable odor at high concentrations, but

cannot be detected at the low levels at which it is typically found in a food product. Therefore, when we speak of aroma substances, we generally refer to only those volatile compounds that have perceivable odors at relatively low concentrations and which exceed their odor detection thresholds.

### Odor Threshold and Odor Activity (Value)

The human nose is an extremely sensitive and specific chemical detector. One measure of the potency of an aroma substance is its odor detection threshold. The odor detection threshold of a substance is the lowest concentration required to elicit an odor response. Some odorants can be detected at sub parts-per-trillion (ppt, ng/L) levels, while others must be present at parts-per-billion (ppb, µg/L), parts-per-million (ppm, mg/L) or even higher concentrations in order to elicit odors. **Table 1** illustrates the enormous odor threshold differences that exist among aroma compounds with the same aliphatic nine-carbon backbone structure but which differ in their functional groups. In addition to general structural features the stereochemistry and absolute configuration (chirality) of a molecule is often also important to its perceived odor quality and odor detection threshold (Brenna et al., 2003). For example, linalool, which has a single chiral center (at carbon 3), can exist in two chiral forms (3S and 3R). It is known that the S and R enantiomers can evoke different neural responses in humans and therefore each possesses a distinct odor (Sugawara et al., 2000) and odor detection threshold, e.g., 0.8 ppb for the 3R-enantiomer versus 7.4 ppm for the 3S-enantiomer (Padrayuttawat et al., 1997).

Thresholds can vary widely both within and across individuals. In addition, some people lack sensitivity to specific odor-active compounds. That is, they are *anosmic* to these odor stimuli. To further complicate matters, detection thresholds for specific aroma compounds depend on their vapor pressures, which are influenced by temperature and the food matrix (or medium) in which they exist. Also, some aroma compounds may undergo odor quality (attribute) changes as a function of concentration (Gross-Isseroff and Lancet, 1988).

One way of gauging or estimating the impact of a compound on a food's aroma is based on its odor-activity value (OAV), sometimes referred to as aroma value or odor unit. An OAV is a dimensionless number and is defined as the ratio of the concentration of an odorant in a food to its odor detection threshold value of the odorant in a suitable matrix (i.e., which mimics that of the food). Based on this concept, compounds having high OAVs should make the greatest contribution to the overall aroma of the food under investigation. However, this assumes that the aroma compounds are below levels that saturate the olfactory receptors. In fact, the olfactory sense can only accommodate a relatively narrow range of response differential for most aroma compounds. The effect of concentration (dose) on the perceived odor intensity can vary greatly among different aroma compounds. This relationship is governed by the Stevens' Power Law (Stevens, 1971). For this reason OAVs do not necessarily allow for accurate ranking of aroma compounds by intensity (or odor importance) in a food. Nevertheless, OAVs are still a common way of providing a preliminary ranking of the relative importance of aroma compounds in foods. As an example, the concentrations and OAVs for the main aroma components of a lemon-lime carbonated beverage are compared in **Table 2**.

**Table 1** Odor detection thresholds for some aliphatic nine-carbon aroma compounds

Compound	Odor Quality	Threshold (µg·L <sup>-1</sup> ) <sup>a</sup>
Nonanoic acid	Fatty, soapy	3000
Nonan-1-ol	Melon	50
Nonanal	Pungent, fatty	1
(E)-2-nonenal	Fatty, hay	0.08

<sup>a</sup>Odor detection threshold in water (Buttery et al., 1988).

**Table 2** Concentrations, odor thresholds and odor-activity values (OAV) for selected potent aroma components of a lemon-lime flavored carbonated beverage

Compound	Cncn (µg·kg <sup>-1</sup> )	Threshold (µg·L <sup>-1</sup> )	OAV
Octanal	585	0.7	836
limonene	3220	10	322
Decanal	341	2	171
Linalool	483	6	80.4
Nonanal	61.5	1	61.5
Geranyl acetate	248	9	27.6
Geranial	874	32	27.3
1,8-Cineole	19.0	1.3	14.6
Geraniol	321	40	8

Data from Hausch, B.H., Lorjaroenphon, Y., Cadwallader, K.R., 2015. Flavor chemistry of lemon-lime carbonated beverages. J. Agric. Food Chem. 63, 112–119.

### Aroma Analysis by Combined Instrumental-Sensory Methods

Being able to distinguish the predominant and characterizing aroma components of a food system, so called character-impact components, from the other odorless or less important aroma components is a difficult task, and sometimes this is not feasible if a large number of compounds make a seemingly equal contribution to the aroma. Individual aroma-significant volatile components are generally present at low parts-per-million ( $\mu\text{g}\cdot\text{kg}^{-1}$ ), parts-per-billion ( $\text{ng}\cdot\text{kg}^{-1}$ ), and sometimes part-per-trillion ( $\text{pg}\cdot\text{kg}^{-1}$ ) levels, making the detection and identification of these compounds difficult. Nonetheless, due to modern advances in analytical chemistry, especially application of instrumental-sensory techniques, like gas chromatography-olfactometry (GC-O) combined with GC-mass spectrometry and sensory analysis of aroma models, significant progress has been made toward our understanding of the aroma chemistry of foods (Grosch, 2001; Marsili, 2007; Dunkel et al., 2014). Such efforts have eventually led to the identification of character-impact components in various foods. Some examples of character-impact compounds are given in Table 3.

### Formation and Occurrence of Aromas in Foods

There are numerous ways in which flavor systems can be classified. In foods there exists a great diversity of classes (alcohols, aldehydes, ketones, acids, esters, amines, etc.) of aroma compounds. The diversity of chemical structures indicates the involvement of numerous chemical reactions in the formation of food aromas. Even in aroma systems as seemingly different as vegetables and cooked meats there are numerous common reactions that produce similar or overlapping aroma profiles. In this discussion, the chemistry of aroma components will focus on a few general categories, with the understanding that inevitably there will be some overlap in the chemical pathways involved in aroma formation.

### Aroma Biogenesis in Plants

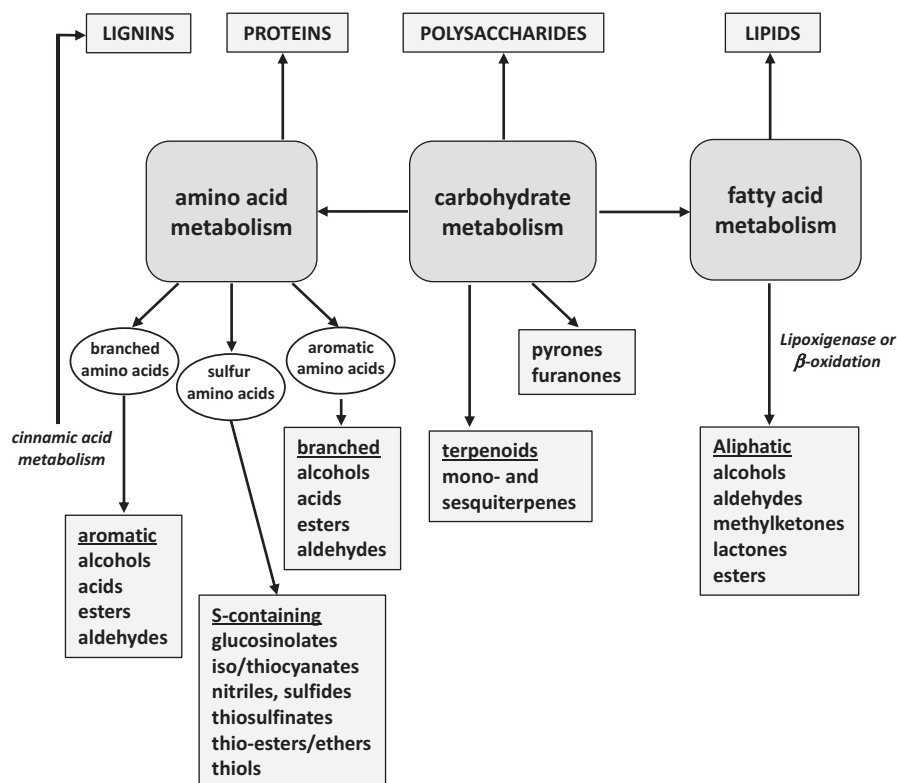
The volatile profiles of fruits and vegetables are composed of a large number of different compound classes, and depending on species, variety, cultivar and/or other factors, one or more these classes may predominate (Cadwallader, 2005; Schwab et al., 2008a). Among the thousands of volatile compounds identified in plants, only a relative few actually contribute to the distinctive aromas of specific fruits and vegetables. The great diversity of the chemical structures indicates the involvement of various biosynthesis pathways and chemical reactions in the formation of plant-derived aroma compounds. In fruits and vegetables there are some common reactions that produce similar or overlapping aroma profiles. The biogenesis and occurrence of aroma compounds in plants has been previously reviewed (Cadwallader, 2005; Schreier, 1986; Leahy and Roderick, 1999; Schwab et al., 2008a). This discussion presents a brief overview of some pathways involved in the biogenesis of aroma compounds in fruits and vegetables. The references cited provide additional information for each pathway mentioned.

The major biosynthetic pathways involved in the formation of aroma compounds in plants are shown in Fig. 1 (Schwab et al., 2008b). Monoterpene ( $\text{C}_{10}$ ) and sesquiterpene ( $\text{C}_{15}$ ) hydrocarbons and their oxygenated derivatives (terpenoids) are formed by enzymes known as terpene synthases (Degenhardt et al., 2009). Acyl pathways play a central role in the metabolism of fatty acids, terpenes, amino acids, and carbohydrates (Tressl and Albrecht, 1986; Leahy and Roderick, 1999). Lipoxygenase and associated enzymes (hydroperoxide lyases, alcohol dehydrogenases and isomerases) are responsible for the breakdown of fatty acids, in particular linoleic and linolenic acids, into  $\text{C}_6$ ,  $\text{C}_8$  and  $\text{C}_9$  volatile aldehydes and alcohols important in the aromas of green plants (Hatahaka, 1996). Fatty acid metabolism, e.g.,  $\beta$ -oxidation of linolenyl-CoA, results in the formation of volatile esters, ketones and alcohols. Hydroxylated fatty acids ( $\text{C}_8$ – $\text{C}_{12}$ ) are also formed *via*  $\beta$ -oxidation and may cyclize (spontaneously) to form  $\gamma$ - and  $\delta$ -lactones (Engel et al., 1989). Branched-chain and aromatic esters are formed *via* amino acid metabolism to give typical fruity aromas (Wyllie et al., 1996). In strawberries, the character-impact compound 2,5-dimethyl-4-hydroxy-3(2H)-furanone is formed *via* metabolism of carbohydrates (Schwab et al., 2008b). Metabolism of carotenoids leads to the formation of aroma significant  $\text{C}_{10}$  and  $\text{C}_{13}$  norisoprenoids (Winterhalter and Rouseff, 2002). Volatile sulfur-containing compounds are of particular importance in the aromas of some plants such as members of the Cruciferae family and *Alliums* and are formed by the action of specific enzymes released after tissue disruption (Cadwallader, 2005).

**Table 3** Examples of some character-impact aroma compounds in foods

Compound	Aroma Quality	Product	Threshold ( $\mu\text{g}\cdot\text{L}^{-1}$ ) <sup>a</sup>
(E,Z)-2,6-nonadienal	Fatty, melon	Fresh-cut cucumber	0.001
2-Methyl-3-furanthiol	Meaty, sulfurous	Cooked meat	0.0004
1-Octen-3-one	Metallic, mushroom	Mushroom	0.005
2-Acetyl-1-pyrroline	Roasted, nutty	Aromatic rice	0.1
Methional	Potato, sulfurous	Potato products	0.2
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	Burnt sugar, caramel	Strawberry, pineapple	0.6
1-(p-Hydroxyphenyl)-3-butanone	Floral, raspberry	Raspberry	10
Vanillin	Vanilla-like	Vanilla	20

<sup>a</sup>Odor detection threshold in water (Rychlik et al., 1998).



**Figure 1** Main pathways and precursors involved in the formation of aroma compounds in plants. Adapted from Tressl, R., Albrecht, W., 1986. Biogenesis of aroma compounds through acyl pathways. In: Parliament, T.H., Croteau, R. (Eds.), *Biogenesis of Aromas*. ACS Symposium Series 317 American Chemical Society, Washington, DC, pp. 114–133 and Schwab et al. (2008).

### Thermal Generation of Aromas

The *Maillard* reaction between amino acids (or other amines) and sugars (or other carbonyls) plays a predominant role in the formation of aroma in thermally treated food (Motttram, 2007). This complex reaction leads to the formation of hundreds of volatile compounds that can be grouped into many chemical classes (Fig. 2). The *Strecker* degradation is an important reaction associated with the *Maillard* reaction which involves the oxidative deamination and decarboxylation of an  $\alpha$ -amino acid in the presence of a dicarbonyl compound to produce a volatile aldehyde, so called *Strecker* aldehyde (Fig. 2). Table 4 contains a list of some of the common odor-important *Strecker* aldehydes, their amino acid precursors and odor detection thresholds. The *Strecker* degradation of cysteine is of particular importance since it leads to the formation of the highly reactive intermediate compounds hydrogen sulfide and ammonia. Hydrogen sulfide can react to form various potent heterocyclic sulfur compounds, e.g., 2-methyl-3-furanthiol, a potent character-impact aroma component of cooked meat (Motttram, 1998a,b).

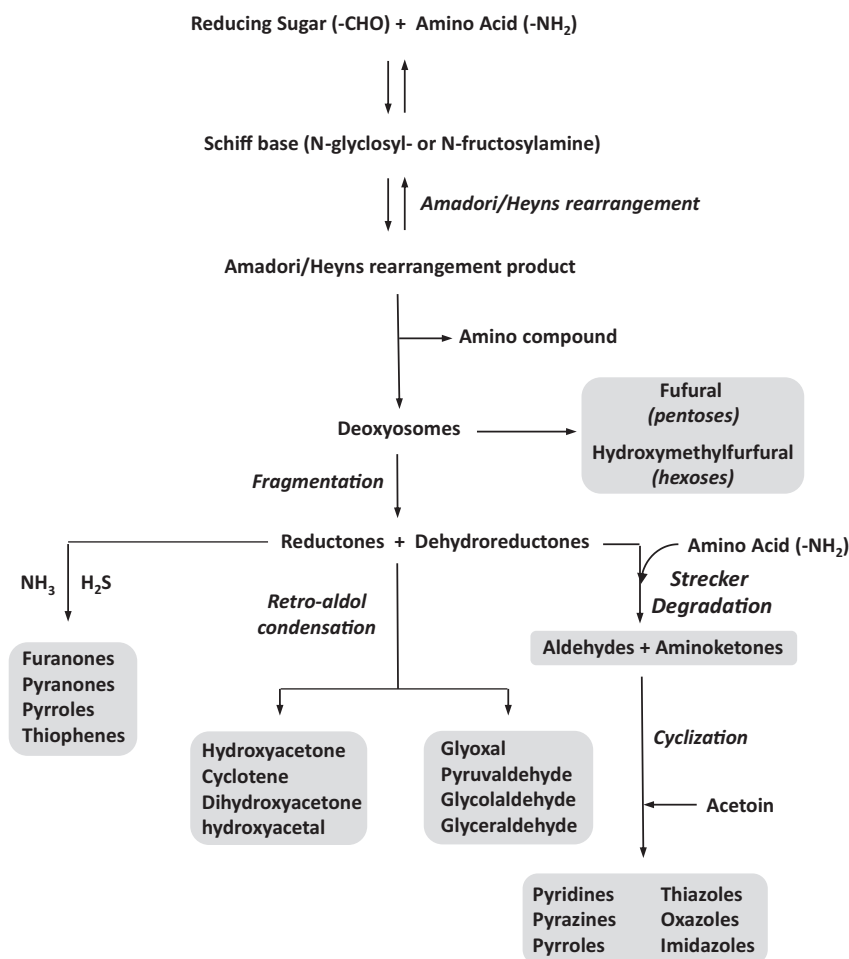
### Lipid Degradation as a Source of Aromas

Degradation of lipids by hydrolytic and oxidative processes leads to the formation of numerous volatile aroma compounds in foods (Table 5). Hydrolytic rancidity is caused by the hydrolysis (lipolysis) of fats or oils (triglycerides) which leads to the release of pungent, rancid or sweaty smelling short-chain free fatty acids such as butyric acid. Lactones, with fruity or coconut-like aroma characteristics, also may be produced from hydroxy fatty acids released by lipid hydrolysis (e.g., in dairy products). In general, oxidation of fats and oils, especially polyunsaturated lipids, is of greater importance than lipolysis, since it is a source of volatile aldehydes and ketones with low odor detection thresholds (Pegg and Shahidi, 2007). These compounds are generally unstable and may be transformed to other volatile compounds through further chemical reactions. For example, the retro-aldol condensation of unsaturated aldehydes, e.g., (*E,E*)-2,4-decadienal to form (*E*)-2-octenal (Table 5), is an important reaction that can further impact the aroma of lipid-containing foods (Josephson and Lindsay, 1987). It is important to note that in addition to forming aroma-active compounds, the oxidative and thermal degradation of lipids gives rise to many volatile compounds (e.g., carbonyls) that also can take part in the *Maillard* reaction (Zhang et al., 1994).

### Fermentation-Derived Aromas

Fermentations have been used for millennia to preserve foods and for modification of food flavors. This section will briefly focus on flavors derived by yeast and lactic acid bacteria fermentations. The aroma components of fermented products can be derived





**Figure 2** Formation of aroma compounds by the Maillard reaction. Adapted from Ho, C.-T., 1996. Thermal generation of Maillard aromas. In: Ikan, R. (Ed.), The Maillard Reaction: Consequences for the Chemical and Life Sciences. John Wiley and Son Ltd. pp 27–53.

**Table 4** Aroma-active Strecker aldehydes found in foods

Compound	Odor Quality	Amino Acid Precursor	Threshold ( $\mu\text{g} \cdot \text{L}^{-1}$ ) <sup>a</sup>
Acetaldehyde	Pungent, yoghurt	Alanine	25
Methylpropanal	Malty, green	Valine	1
2-Methylbutanal	Malty, bug	Isoleucine	2
3-Methylbutanal	Malty	Leucine	0.2
Phenylacetaldehyde	Floral, honey	Phenylalanine	4
Methional	Potato, sulfurous	Methionine	0.2

<sup>a</sup>Odor detection threshold in water (Rychlik et al., 1998).

through primary metabolism or by residual enzymes produced by the organism. The biosynthetic pathways and reactions involved formation of fermentation aromas are often analogous to those previously discussed for aroma biogenesis in plants. Extensive reviews have been published on flavor chemistry of fermented dairy products (Singh et al., 2007), fermented meat products (Toldrá, 2008) and alcoholic beverages (Piggot, 2012). Of particular interest in recent years has been the application of fermentation technology (biotechnology) for the sustainable production of natural aroma compounds (Berger, 2007; Gupta et al., 2015).

### Off-Odors and Taints

Off-odors (off-flavors) and taints can cause severe problems in foods, leading to economic losses due to product recalls, reduced consumer confidence and to potentially a tarnished brand image. Owing to its importance to the food industry numerous



**Table 5** Some important lipid-derived aroma compounds

Compound	Odor Quality	Precursor	Mode of Formation
Butyric acid	Cheesy, fecal	Tributyryl	Hydrolysis (lipolysis)
Hexanal	Green, cut-grass	Linoleic acid	Oxidation
(E)-2-hexenal	Green, cut-leaf	Linolenic acid	Oxidation/isomerization
via (Z)-3-hexenal			
Nonanal	Pungent, fatty	Oleic acid	Oxidation
(E)-2-nonenal	Fatty, hay	Linoleic acid	Oxidation/isomerization
via (Z)-3-nonenal			
(E,Z)-2,6-nonadienal	Fresh-cut cucumber	Linolenic acid	Oxidation/isomerization
via (Z,Z)-3,6-nonadienal			
(E,E)-2,4-decadienal	Fatty, fried	Linoleic acid	Oxidation
(E,E,Z)-2,4,7-decadienal	Fatty, fishy	Linolenic acid	Oxidation
(Z)-4-heptenal	Rancid, fishy	(E,Z)-2,6-nonadienal	Retro-aldol condensation
(E)-2-octenal	Fatty, nutty	(E,E)-2,4-decadienal	Retro-aldol condensation

comprehensive reviews have been published on this subject (Goldenburg and Matheson, 1975; Saxby, 1982; Whitfield, 1986; Mottram, 1998a,b; Jelén, 2006; McGorin, 2007; Pegg and Shahidi, 2007; Reineccius, 1991). For the sake of this discussion off-odors are considered to be the occurrence of any atypical odors resulting from compounds formed by internal deterioration of the food, including chemical reactions and microbial spoilage; whereas, taints result from contamination of the food by foreign chemicals derived from the environment or other external sources (Whitfield, 1986).

Off-odors in foods may result from oxidation, light-catalyzed reactions, nonenzymatic browning, chemical reactions and interactions among food constituents, enzymatic reactions and microbial spoilage. Many of the reactions involved in the formation of off-odors are the same or analogous to those already discussed in previous sections. Oftentimes, compounds that impart a positive aroma character in one instance may cause an off-odor when present in the wrong context or at elevated levels. For example, lipid degradation caused by autoxidation and/or lipolysis is a common source of either desirable aromas or off-odors in foods (Pegg and Shahidi, 2007).

In addition to chemical reactions, certain aspects of plant and animal metabolism can also lead to formation of off-odors. The so called “boar taint” off-odor associated with meat derived from intact male pigs has been attributed to the steroid 5- $\alpha$ -androsterone or “androst-16-en-3-one” which has an ammoniacal and urine-like odor. This is sometimes accompanied by the fecal-smelling tryptophan metabolite 3-methylindole (skatole) (Bonneau, 1982).

As mentioned above, taints as opposed to off-odors, originate from external sources. Taints may be introduced into food materials as a result of exposure to environments or packaging materials contaminated by either synthetic chemicals or chemicals produced or transformed by microbial action. Some common taints of chemical and microbial origins include halogenated phenolic compounds (e.g., trichloroanisole) which cause cork taint in wine (Simpson and Sefton, 2007), as well as the musty/earthy smelling geosmin and 2-methyl isoborneol in aquaculture products (Whitfield, 1988, 1998; Rimando and Schrader, 2003).

## Conclusions

Much has been learned over the past 50 years with respect to our understanding of olfaction and odor perception and on the identities and origins of volatile foodborne odorants involved food aromas. This above discussion is just a small sampling of what is known and available in the vast literature on the subject. However, despite our already great understanding of the subject, the identification and characterization of food aromas will continue to be of interest to food chemists concerned with food quality.

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[www.leffingwell.com](http://www.leffingwell.com) – Leffingwell.

<https://www.acs.org/content/acs/en/education/whatischemistry/landmarks/usda-flavor-chemistry.html> – What Is Chemistry.

## Artificial Sweeteners

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### Glossary

ADI Acceptable Daily Intake

EDI estimated daily intake

GRAS generally recognized as safe

FDA Food and Drug Administration.

### Introduction

While no single factor is responsible for the recent, dramatic increases in overweight and obesity, a scientific consensus has emerged suggesting that consumption of sugar-sweetened products, especially beverages, is casually linked to increases in risk of chronic, debilitating diseases including type 2 diabetes, cardiovascular disease, hypertension and stroke (Swithers, 2015). Sweeteners have been used extensively in food and beverages for decades, yet there continues to be controversy about their net effects on energy balance. There has been renewed interest in this discussion in recent years in the wake of a number of prospective cohort studies in which it was observed that the risk of weight gain was increased in a dose responsive manner among participants consuming diet beverages compared to those who did not consume them (Fowler et al., 2008, 2015). Recognizing our desire for sweet flavours, the food industry has developed and supplied sugar free alternatives designed to satisfy our cravings, referred to as sugar substitutes, artificial sweeteners or non-nutritive sweeteners. Nowadays they occupy a large portion of commercial space on the supermarket shelves worldwide.

Sweeteners are functional food additives that impart sweetness in food (DuBois and Prakash, 2012). Some of the low-calorie sweeteners currently approved by different international authorities as direct food additives include acesulfame, aspartame, cyclamate, saccharin and sucralose. On the other hand, there is a wide range of natural, unrefined sweeteners which aside from providing sweetness to the product, contain various bioactive compounds, such as vitamins, minerals or polyphenols that are known to exhibit positive health effects and contribute to the concept of functional food. Natural sweetener can be further divided into saccharide and non-saccharide sweeteners (Surana et al., 2006). There are many other natural alternatives to sugar that are available, though not widely used, despite the fact that natural non-refined sugar alternatives potentially contain beneficial bioactive compounds, especially polyphenolic compounds, known for their antioxidant properties. Some of these natural sugar alternatives include plant saps/syrups (eg, maple syrup, agave nectar), syrups made from raw sugar and grains (eg, molasses, barley malt, and brown rice syrup), honey, fruit or vegetable sugars (eg, date sugar, carrot). Among the natural alternatives to sucrose that can be used as sweeteners with low glycemic index are lucuma (*Pouteria obovata*) and yacon (*Smallanthus sonchifolius*), which - undergo less refining process and may therefore provide a substantial content of other beneficial nutrients and bioactives. Likewise, the use of Stevia and liquorice (*Glycyrrhiza glabra*) are also well known in confectionery industry, but they still do not have a wider application due to the aftertaste that often occurs in the products. Black locust (*Robinia pseudoacacia*) is a tree native and widely spread in the southeast European region, appreciated for its medicinal properties (prepared and consumed as tea) and culinary uses (the flower nectar is used for production of honey, flowers are fried, added to dishes or used for preparation of beverages), mostly due to its specific sweet taste and mild, flowery aroma (Belšcak-Cvitanovic et al., 2015). Sweetener can be broadly divided into two categories, natural and artificial or synthetic sweetener.

### Artificial Sweeteners: An Alternative to Limiting Table Sugar in the Diet

Sensory properties of food are highly influenced by the sensational properties like taste, smell texture and appearance (Sorensen et al., 2003). The selection and consumption of food plays a crucial role in the regulation of human appetite and nutrient intake. A sweetener is a food additive, which mimics the effect of sugar on taste. Therefore, they are called sugar substitutes. Consumers often select foods that are composed of low calorie sweetener because they want the taste of sweetness without added calories.

Artificial sweeteners are being used as sugar substitutes in considerable and increasing amounts in food and beverages, especially by those who are diabetic and/or obese (Benton, 2005). They have also been used in other personal care and pharmaceutical products such as toothpastes (Zygler et al., 2009). Although, from the beginning of their use, there has been a controversy over their risk as potential carcinogens (Weihrauch and Diehl, 2004), these sweetener compounds are generally considered to be safe for use in food stuffs (Kroger et al., 2006; Ahmed and Thomas, 1992; Cohen et al., 2008). Artificial sweeteners are becoming increasingly used

to sweeten beverages, such as soda, juice, coffee and tea. In an era where obesity has become a serious health problem with large populations affected, sweeteners are a way to replace sucrose consumption. These products have been considered as safe in a recently published position article from the Academy of Nutrition and Dietetics (Caballero, 2007; Fitch and Keim, 2012). Acesulfame-k, aspartame, cyclamate, saccharin and sucralose are currently approved as artificial sweeteners as direct food additives. Others are continually being developed and are gradually used in more foodstuffs, especially because they contribute to longer shelf-life. In terms of environmental degradation, among the five most commonly used artificial sweeteners named above, only aspartame decomposes under normal usage conditions, and safety clearance was given to the intake of even its breakdown derivatives (US FDA, 2006; EU, 2003; US FDA, 1983). The number of times that a sweetener is sweeter than sucrose is called sweetener potency. The potency of a sweetener is compared with sucrose mainly in the threshold levels of the sweetener and sucrose (Yasuura et al., 2014). Sugars and sugar alcohols, such as sucrose and xylitol, are low-potency sweeteners, whose sweetener potencies are about 1 and under. On the other hand, sweeteners which have a sweetener potency exceeding 10 are called high-potency sweeteners, such as saccharin and aspartame. Interestingly, low-potency sweeteners, such as sucrose, exhibit higher sweetness intensity than high-potency sweeteners at very high concentrations. That is why low-potency sweeteners are also called high-intensity sweeteners (Hayes, 2008; Cardello et al., 1999; Farkas and Hid, 2011).

### Acesulfame—k

Acesulfame—k, a high intensity sweetener, is a potassium salt of 6-methyl-123-oxathiazine-4 (3H)-one 2, 2-dioxide with molecular formula  $C_4H_4KNO_4S$  and molecular weight of 201.24 was developed as sweetener by Hoechst. Synthesis of Acesulfame-K involves the treatment of acetoacetamide with at least two equivalents of sulfur trioxide. This results in formation of N-sulfoacetoacetamide, which is then dehydrated by sulfur trioxide to form oxathiazinone dioxide. Neutralization with potassium hydroxide gives Acesulfame-K (Clauss et al., 1993). Acesulfame—k is a white crystalline, water soluble powder, which is roughly 120 times sweeter than sucrose. It is heat stable so it can be used in cooking and baking. Acesulfame-K or Ace-k is often blended with other sweeteners (usually sucralose or aspartame) whereby each sweetener masks the other's after taste and exhibit a synergistic effect by which the blend is sweeter than its components. The European Commission's Scientific Committee on food reevaluated this sweetener and supported its safety but recommended an ADI from  $9 \text{ mg kg}^{-1}$  to  $15 \text{ mg kg}^{-1}$  bw per day. The amount of acesulfame-K added to food products is very small because of its intense sweetening power and because it is often used in combination with other sweeteners. The EDI is estimated at 20% of the ADI because of its intense sweetening power.

### Alitame

L-aspartic acid, D-alanine, and a novel C-terminal amide moiety are the main component of Alitame. It is 2000 times sweeter than sucrose without the bitter or metallic qualities of high-intensity sweetener (Auerbach et al., 2001). This sweetener blends with other high-intensity sweeteners to maximize the quality of sweetness. From an oral load of alitame, 7% to 22% is unchanged and excreted in the feces. The remaining amount (78% to 93%) is hydrolyzed to aspartic acid and alanine amide. An ADI of  $0\text{--}1 \text{ mg kg}^{-1}$  body weight was allocated on the basis of the no-observed-adverse-effect-level (NOAEL) of  $100 \text{ mg kg}^{-1}$  body weight/day to an 18 month study in dogs. Joint FAO/WHO Expert Committee on Food Additives (JEFCA) reviewed safety data on alitame in 2002 and postponed making ADI or other toxicology recommendations about alitame until findings of a 90-day tolerance study were made available. In the FDA petition, the estimated daily intake as a sole sweetener in all products is  $0.34 \text{ mg kg}^{-1}$  bw per day. The level at which no observed adverse effects occurred in animals was  $100 \text{ mg kg}^{-1}$  (Auerbach et al., 2001). Alitame has already been approved in Mexico, Colombia and China as well as Australia and New Zealand.

### Aspartame

Aspartame was discovered in 1965 by James Schlatter. It is an artificial, non-saccharide sweetener, L-aspartyl-L phenylalanine methyl ester that is a methyl ester of the dipeptide of the amino acids aspartic acid and phenylalanine. Under strongly acidic or alkaline conditions, aspartame may generate methanol by hydrolysis. Under more severe conditions, the peptide bonds are also hydrolyzed, resulting in the free amino acids. It is slightly soluble in water. The solubility increases with higher or lower pH as well as with increased temperature (Mazur et al., 1970; Mazur and Ripper, 1979). Intestinal esterases hydrolyze aspartame to aspartic acid, methanol, and phenylalanine. These components are found in much greater amounts in the normal diet in fruits, vegetables, meat, and milk. For example, a serving of nonfat milk provides about six times more phenylalanine and 13 times more aspartic acid, whereas a serving of tomato juice has about six times more methanol than an equal volume beverage sweetened 100% with aspartame. In 1981, the FDA approved aspartame as a sweetener for a number of dry uses (eg, tabletop sweetener, cold breakfast cereal, gelatins and puddings) and in chewing gum. This approval was expanded in 1983 to include carbonated beverages. The first safety assessment of aspartame carried out in Europe was published by the Scientific Committee on Food (SCF) in 1984 and ADI for aspartame of  $40 \text{ mg kg}^{-1}$  bw per day was established. The Council on Scientific Affairs of The American Medical Association in 1985 concluded that "Available evidence suggests that consumption of aspartame by normal humans is safe and is not associated with serious adverse health effects". In European Food Safety Authority's (EFSA's) first ever full risk assessment of the food additive aspartame, the experts concluded that aspartame and its breakdown products are safe for human consumption at current levels of exposure. Aspartame is used in low-calorie soft drinks, foods, and sweeteners because aspartame is approximately 200 times sweeter than sugar. Aspartame is a low calorie option for people who should or need to limit their sugar intake (Ishii, 1981).

## Neotame

Neotame is one of the newest artificial sweetener, a derivative of aspartame. Another similar compound, alitame, is pending approval before the FDA. Neotame is 7000 to 13,000 times sweeter than sugar and has no calories. Synthesis of neotame involve the hydrogenation of L- $\alpha$ -aspartyl-L-phenylalanine I methyl ester and 3-3 dimethylbutyraldehyde produced in-situ by the hydrolysis or cleavage of a 3-3-dimethylbutyraldehyde precursor. Neotame is an odorless white to gray-white powder with a strong sweetness and is readily soluble in alcohols and slightly soluble in water (Prakash et al., 2002; Prakash, 2007). Globally, neotame is approved for use in multiple countries in North America, South American, Europe, Africa, Asia, and Australia. In June 2003, the JECFA confirmed the safety of neotame and granted an ADI of 2 mg kg<sup>-1</sup> bw per day.

## Saccharin

Saccharin, the first artificial sweetener, was discovered serendipitously, as were most artificial sweeteners. Constantine Fahlberg was researching on the oxidation mechanisms of toluenesulfonamide, during research, a substance accidentally splashed on his finger; he later licked his finger and noticed the substance had a sweet taste, which he traced back to saccharin (Arnold, 1983). Since that time, a number of compounds have been discovered and used as food additives for their sweetener properties. Saccharin has been in use since 1900 and obtained FDA approval in 1970. Saccharin has no calories and is 300 times sweeter than sugar (Food and Drug Administration, 2006). Synthesis involves diazotization of methyl anthranilate and then treatment of the diazonium salt with sulfur dioxide and chloride gas to give the sulfonyl chloride which is then treated with ammonia to give saccharin (Tarbell and Tarbell, 1978). The label must state saccharin in the ingredient declaration, the amount of saccharin listed per fluid ounce for beverages, milligrams in the dispensing unit for cooking or tabletop use, and milligrams per serving for processed goods. The FDA lists the ADI for saccharin at 5 mg kg<sup>-1</sup>.

## Sucralose

Sucralose was accidentally discovered in 1976 when Tate & Lyle, a British sugar company, was looking for ways to use sucrose as a chemical intermediate. This non-nutritive sweetener is made from sucrose by a process that substitutes 3 chloride atoms for 3 hydroxyl groups on the sucrose molecule (Food and Drug Administration, 2006). Sucralose is 600 times sweeter than sugar and contains no calories. Sucralose was approved by the FDA in 1998 for use in 15 food categories, including a tabletop sweetener under the brand name Splenda. In 1999, sucralose was approved as a general-purpose sweetener. The FDA concluded from a review of more than 110 studies in human beings and animals that this sweetener did not pose carcinogenic, reproductive, or neurologic risk. According to European Food Safety Authority's (EFSA's) ADI of sucralose is 40 mg kg<sup>-1</sup> bw per day.

## Artificial Sweetener Use and Health Effects

The nutritive sweeteners include the monosaccharide polyols (e.g., sorbitol, mannitol, and xylitol) and the disaccharide polyols (e.g., maltitol and lactitol). They are approximately equivalent to sucrose in sweetness (Dills, 1989). The non-nutritive sweeteners, better known as artificial sweeteners, include substances from several different chemical classes that interact with taste receptors and typically exceed the sweetness of sucrose by a factor of 30 to 13,000 times. Nutritive sweeteners (eg, sucrose, fructose) are generally recognized as safe by FDA, yet concern exists about increasing sweetener intakes relative to optimal nutrition and health. In the United States, estimated intakes of nutritive sweeteners fall below this, although one in four children (ages 9 to 18 years) can surpass this level. In addition to their sensory qualities, nutritive sweeteners add functional properties to foods through their effects on physical (eg, crystallization, viscosity), microbial (eg, preservation, fermentation), and chemical (eg, caramelization, antioxidation) characteristics (Davis, 1995). Nutritive sweeteners are easily digestible except in the cases of rare genetic abnormalities of carbohydrate metabolism (eg, galactosemia, inherited fructose intolerance) (Rumessen, 1992). Over the years, the effects of artificial sweetener use on health have been a concern among health professionals as well as the public for a variety of reasons (Jones and Elam, 2003). One area involves the safety of sweeteners for use by children, when sweetener intakes are high relative to body weight, and pregnant women, when the goal of the diet is to support maternal and fetal health (Kaiser and Allen, 2002). Concern about sweetener intakes has shifted from diabetes in the 1960s, to hyperactivity and behavior issues in children in the 1990s, and to the etiology of obesity in the 2000s. A key recommendation related to the use of non-nutritive sweeteners is to control total energy intake and increase physical activity to manage body weight (US Departments of Agriculture and Health and Human Services, 2010). Eating patterns that are low in energy density improve weight loss and weight maintenance, and may be associated with a lower risk of type 2 diabetes in adults. Substituting non-nutritive sweeteners for higher-energy foods and beverages can decrease energy intake, but evidence of their effectiveness for weight management is limited.

Dental caries are the localized destruction of dental hard tissue by acidic material from bacterial fermentation of dietary carbohydrate (American Academy of Pediatric Dentistry, 2011). Factors that control the development of dental caries include microbiological shifts in the biofilm, salivary flow, buffering capacity of saliva, frequency and kind of dietary sugars consumed,



length of time oral bacteria have to ferment the fermentable carbohydrate and create organic acids, tooth susceptibility, preventive behaviors such as cleaning of teeth (Selwitz et al., 2007), and exposure to fluoride (National Institutes of Health, 2001). Use of polyol-based gum can reduce the risk of dental caries in children, with the greatest benefit in xylitol-based gums, but non-nutritive sweeteners do not promote dental caries (Makinen et al., 1995). A child who consumes more than three nutritive sweetener-containing snacks or beverages between the meals per day is considered at increased risk for dental caries. Xylitol is considered cariostatic and anticariogenic and aids in the prevention of dental caries. It is very soluble in water and sparingly soluble in ethanol. Human tolerance studies indicate that consumption exceeding 50 g per day leads to diarrhoea. The estimated intakes of non-nutritive sweeteners in children are below the established acceptable daily intakes for all approved sweeteners. As a percentage of ADI, they are as low as 10.4% for aspartame to as high as 60% for acesulfame-K. It has been suggested that caregivers may want to limit intake of saccharin by young children because of the limited amount of data available for its use in children (Council on Scientific Affairs, 1985). The wide range of nutritive and non-nutritive sweeteners available in the food supply, as well as blending these sweeteners in food and beverage systems, should continue to keep estimated intakes of non-nutritive sweeteners in children well below the acceptable daily intakes.

Obesity is another complex problem, and its cause cannot simply be attributed to any one component of the food supply such as sweeteners. The causes of obesity are multifactorial, and the focus on any single factor no doubt oversimplifies the issue. Nevertheless, with regard to recent and rapid increases in the prevalence of obesity, scientific evidence has implicated a number of dietary factors as likely contributors. Most recently, special attention has been focused on the extremely high levels of consumption of sugars in general and sugar-sweetened beverages in particular. For example, in the US overall consumption of sugar-sweetened soft drinks in 2001 was roughly 37 gallons per capita (USDA, 2008). The prevalence of obesity has increased substantially at the same time as the consumption of non-nutritive sweeteners has increased (Sylvetsky et al., 2012). The question is, do these sweeteners maintain a highly sweet food environment to increase risk of obesity through appetite, intake, and energy regulation mechanisms? Some evidence primarily from studies with animals suggests that high intakes of sweets (nutritive sweeteners alone or in mixtures with fat) promotes weight gain through changes in neuropeptide control of appetite, intake, and energy expenditure (Levine et al., 2003). In 2012 over 70% of adults reported that they consumed sugar-sweetened beverages (SSB; soft drinks or fruit drinks with added sugar; Kumar et al., 2014), with over 25% reporting daily intake. A recent meta-analysis also showed strong links between SSB consumption and increased body weight (Malik et al., 2013). Thus, the rise in prevalence clearly relates to all factors that cause an energy imbalance.

Pregnancy is a time of special concern because the focus is on maternal and fetal health. All FDA-approved nutritive sweeteners and non-nutritive sweeteners are approved for use by the general public, which includes pregnant and lactating women. The position of the Academy is that use of nutritive sweeteners is acceptable during pregnancy (Position of the American Dietetic Association, 2008). The safety of acesulfame-K, aspartame, sucralose, and neotame in pregnancy has been determined with rat studies; the scientific community accepts rats and some other animals as appropriate models for reproductive toxicology testing that are applicable to human beings. At high doses, there was no change observed in fertility, size of litter, body weight, growth, or mortality for acesulfame-K, sucralose, or neotame (Food and Drug Administration, 1998, 1998, 2002).

## Conclusions

Artificial sweeteners add to the pleasure of eating. Consumers can enjoy a wide range of sweeteners in a wide variety of foods and beverages. Artificial sweeteners are safe for use within the approved regulations. Artificial sweeteners are those that sweeten with minimal or no carbohydrate or energy. They can increase the palatability of fruits, vegetables, and whole-grain breads/cereals and thus have the potential to increase the nutrient density of the diet while promoting lower energy intakes. They are regulated by the Food and Drug Administration as food additives or generally recognized as safe. The Food and Drug Administration approval process includes determination of probable intake, cumulative effect from all uses, and toxicology studies in animals. Five non-nutritive sweeteners are approved by FDA, those are acesulfame-K, aspartame, neotame, saccharin, sucralose. They have different functional properties that may affect perceived taste or use in different food applications. There is a chance that, continuous exposure to artificial sweeteners could persistently alter sweet preferences, leading to enhanced intake of sugars throughout life and it could interfere with learning of basic relations between sweet tastes and the delivery of calories, which in turn could negatively affect regulation of metabolic processes. Artificial sweeteners could alter the composition of the gut microbiota, which in turn can contribute to metabolic dysregulation. Thus, consumers must be aware of science-based information about particular artificial sweetener and supportive research on the use of that to promote eating enjoyment, optimal nutrition, and health.

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## Betalains

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### Introduction

Betalains are water-soluble nitrogen-containing vacuolar pigments, consisting of the red to red-violet betacyanins and the yellow-orange betaxanthins (Fig. 1). They are immonium conjugates of betalamic acid with *cyclo*-Dopa (cyclo-3,4-dihydroxyphenylalanine) and amino compounds (amino acids, amines, or derivatives), respectively. Examples are betanin (betanidin-5-O- $\beta$ -glucoside) of red beet and indicaxanthin (betalamic acid linked to proline) of cactus pear, the first structurally characterized betalains.

Glycosylation and acylation result in a diversity of betacyanin structures. The betanidin aglycone is usually linked with glucose, occasionally with glucuronic acid, sophorose, rhamnose, and apiose, at the C-5 or C-6 position. Further modification occurs by aliphatic or aromatic acid esterification of the sugar moiety. Malonic, 3-hydroxy-3-methyl-glutaric, caffeic, *p*-coumaric, cinnamic, and ferulic acids are typical acid substituents (Strack et al., 2003). To date, about 78 plant betalains have been identified and characterized (Slimen et al., 2017).

The biosynthesis of betalains is discussed in detail in review articles (Esatbeyoglu et al., 2015; Khan and Giridhar, 2015; Strack et al., 2003). Betalains are found in numerous sources (flowers, fruits, roots, leaves, stalks, seeds, grains) in the plant kingdom. In food, however, their occurrence is limited, the red beet being regarded for a long time as practically the sole source of betacyanin. More recently, other edible sources have been investigated, such as *Ullucus tuberosus*, an important root crop in the Andean region of South America (Cejudo-Bastante et al., 2014); *Basella rubra*, known as Malabar spinach, a leafy vegetable that accumulates pigments in its fruits (Kumar et al., 2015, 2016); prickly (cactus) pear (*Opuntia ficus-indica* and *Opuntia stricta*) (Castellar et al., 2003; Melgar et al., 2017; Moßhammer et al., 2005a,b; 2006; Stintzing et al., 2002b, 2003); red-purple pitaya (dragon fruit) *Hylocereus polyrhizus* (Herbach et al., 2006a, 2007; Moßhammer et al., 2005b; Stintzing et al., 2002a; Wybraniec et al., 2001) and *Amaranthus* leaf and grain (Stintzing et al., 2004; Cai and Corke, 2000; Cai et al., 2005).

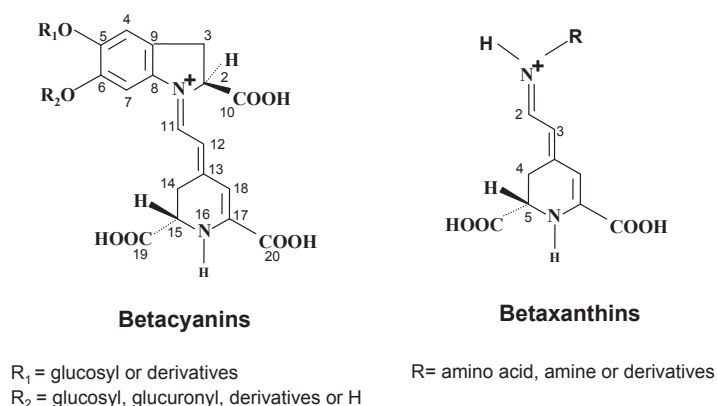
Being minor pigments in red beet, betaxanthins have received much less attention. Food sources of betaxanthins are yellow varieties of Swiss chard, beet, and cactus fruits (Kugler et al., 2004; Stintzing et al., 2002b; Strack et al., 2003).

### Physical and Chemical Properties

Betacyanins and betaxanthins exhibit absorption maxima at 532–550 nm and 457–485 nm, respectively (Khan, 2016). The conjugated double bond system of the betalamic acid moiety constitutes the chromophore responsible for these absorptions and consequently, the vivid color. The bathochromic shift of 50 to 70 nm of betacyanins compared to betaxanthins is due to conjugation of the aromatic ring's double bonds of *cyclo*-Dopa, extending the electronic resonance system. Glycosylation of betanidin causes a hypsochromic shift (Stintzing et al., 2004) while esterification with aliphatic acids has little impact on the maximum absorption of betacyanins (Stintzing et al., 2002a; Wybraniec et al., 2001). Acylation with aromatic acids results in a bathochromic shift (Heuer et al., 1992).

As with other natural pigments, stability is a major concern with betalains. It is enhanced by high betalain content, high degree of glycosylation and acylation, low  $a_w$ , pH 3 to 7, antioxidants, chelating agents, low temperature, protection from light, and nitrogen atmosphere (Herbach et al., 2006a,b). Conversely, degrading enzymes (peroxidase, polyphenol oxidase, glucosidase), low degree of glycosylation and acylation, high  $a_w$ , metal cations, pH < 3 or > 7, high temperature, exposure to light, O<sub>2</sub>, and H<sub>2</sub>O<sub>2</sub> lowers stability.

The lower oxidation–reduction potential of betanidin as compared to betanin coincides with its greater susceptibility to destruction by molecular oxygen (von Elbe and Attoe, 1985). Glycosylation increased the half-life of betanidin and isobetanidin by about 17 times in O<sub>2</sub>-saturated solutions. Betacyanin stability may be increased by substitution with aromatic acids, the



**Figure 1** Structures of betacyanins and betaxanthins.

6-O— being more effective than the 5-O-substitution, explained by intramolecular stacking that may protect the aldimine bond from hydrolytic attack (Schliemann and Strack, 1998).

It is widely known that betalains are stable over a broad pH range, from 3 to 7, which makes them well suited for application in low-acid and neutral foods. Betanin is most stable at pH 4–5 (Huang and von Elbe, 1987).

Betanin undergoes water-dependent hydrolysis;  $a_w$  is a crucial factor for betanin susceptibility to aldimine bond cleavage. Lower  $a_w$  improved betanin stability, most effectively at below 0.63 (Kearsley and Katsaboxakis, 1980). In encapsulated beetroot pigments, betanin degradation was greatest at  $a_w = 0.64$  (Serris and Biliaderis, 2001). Greater betanin stability was ascribed to decreasing mobility of the reactants at lower  $a_w$  and by dilution effects at higher  $a_w$ .

Metal cations ( $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Sn}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Cu}^{2+}$ ) accelerate betanin degradation (Attoe and von Elbe, 1984; Czapski, 1990; Sobkowska et al., 1991). EDTA can prevent metal-catalyzed betanin degradation by pigment stabilization and complex formation with metal ions (Attoe and von Elbe, 1984).

Ascorbic acid showed a protective effect in purple pitaya juice and pigment preparations (Herbach et al., 2006a,b) and on Amaranthus pigments (Cai and Corke, 1999). Added to the juice prior to storage, it prevented the detrimental effects of light exposure (Herbach et al., 2007). Addition of 0.25% ascorbic acid, pH 4.0, and pasteurization at 65 °C for 30 min were selected as the best processing conditions to retain betacyanin content in red-fleshed pitaya juice (Wong and Siow, 2015). Ascorbic acid and Se synergistically protected and regenerated betalains efficiently after thermal degradation in *Rivina humilis* L. berry juice (Khan and Giridhar, 2014).

### Alterations During Processing and Storage of Food

Decomposition of betalains may be catalyzed by degrading enzymes. Several polyphenol oxidases were isolated from red beet (Escribano et al., 2002). A betalain oxidase catalyzed betanin degradation to *cyclo*-Dopa-5-O- $\beta$ -glucoside, betalamic acid, and 2-hydroxy-2-hydro-betalamic acid (Zakharova et al., 1987).

Degradation of betalain during thermal processing has been reported in many papers (e.g. Herbach et al., 2004a,b, 2006a; 2007). The color shift of red beet juice to orange on thermal treatment was attributed to the formation of yellow and orange-red degradation products (Herbach et al., 2004b).

Betacyanin undergoes isomerization, deglycosylation, dehydrogenation, hydrolysis, and decarboxylation (Fig. 2). The predominating reaction differs with different betacyanin (Stintzing and Carle, 2007). Betanin was mainly hydrolyzed into betalamic acid and *cyclo*-Dopa-5-O-glucoside. Decarboxylation and combined decarboxylation/dehydrogenation predominated with hylocerenin (3-hydroxy-3-methylglutaryl-betanin). Phyllocactin (malonyl-betanin) yielded betanin and various yellow and red dehydrogenated and decarboxylated derivatives.

Betacyanins are generally accompanied by their respective isobetacyanins, but isomerization can be induced by acidic or alkaline conditions (Schwartz and von Elbe, 1983; von Elbe et al., 1981). It has also been observed during thermal treatment of red beet juice (Herbach et al., 2004b; von Elbe et al., 1981).

The glucose moiety of betanin may be cleaved in the presence of  $\beta$ -glucosidase, under strongly acidic conditions, or at high temperature (Herbach et al., 2006b). This deglycosylation results in increased susceptibility towards oxidation (Stintzing and Carle, 2004).

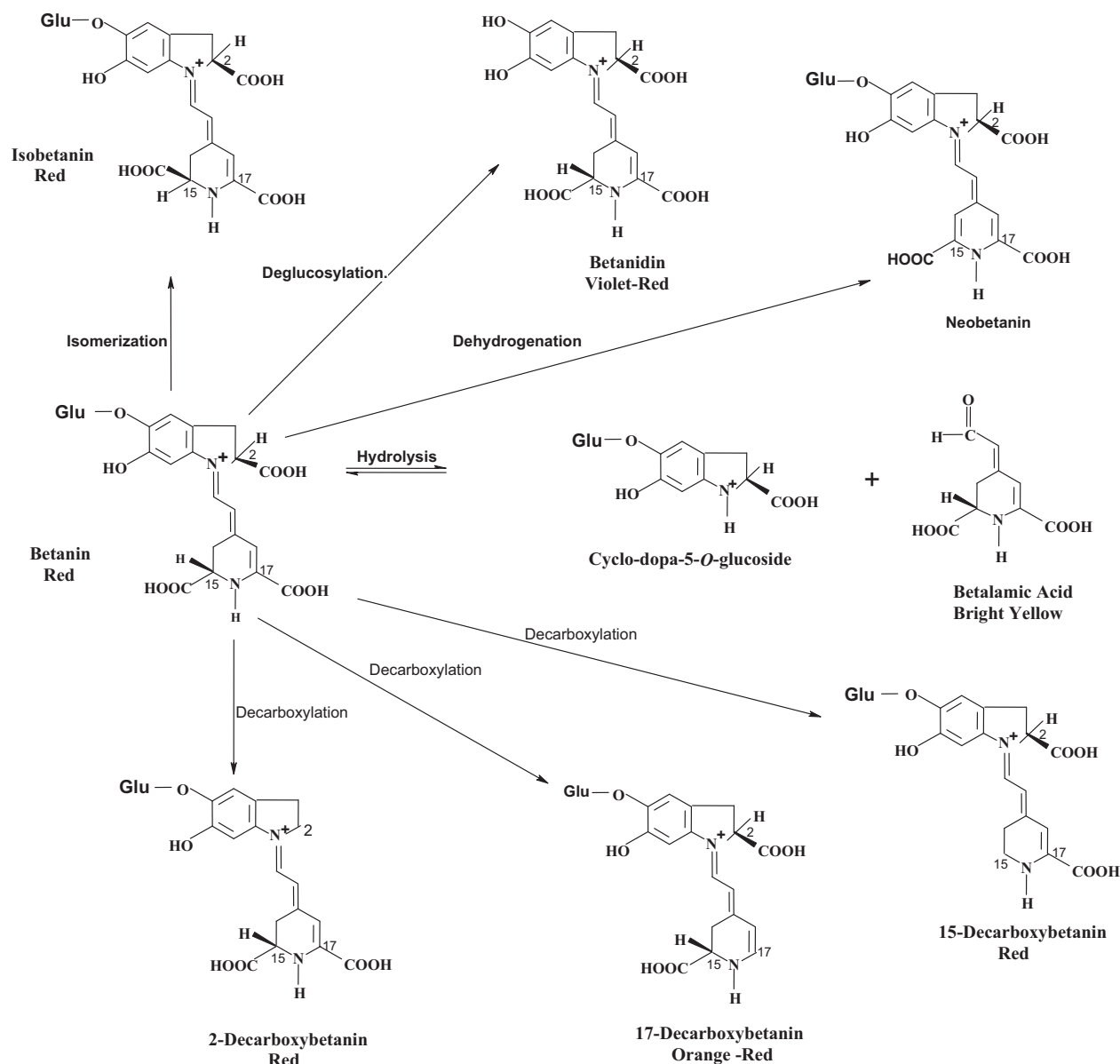
Yellow neobetainin (14,15-dehydrobetainin) is an endogenous pigment, as in red beet (Alard et al., 1985; Kujala et al., 2001) and prickly pear (Strack et al., 1987). It can also be generated on heat exposure (Herbach et al., 2004b). Dehydrogenation of phyllocactin and hylocerenin on thermal treatment of purple pitaya juice resulted in the formation of the corresponding yellow neo-derivatives (Herbach et al., 2004a).

At pH above 6 or during thermal processing, betanin is cleaved to the bright yellow betalamic acid and the colorless *cyclo*-Dopa-5-O- $\beta$ -glucoside (Schwartz and von Elbe, 1983). The aldimine bond may be protected from cleavage by acylation with aromatic or aliphatic acids (Schliemann and Strack, 1998; Herbach et al., 2005). Partial regeneration of betanin occurs after short-term heating (Czapski, 1985; Huang and von Elbe, 1985; von Elbe et al., 1981). Ascorbic, isoascorbic, metaphosphoric, and gluconic acids improved the regeneration of red beet juice pigments after heating (Han et al., 1998).

Betanin may be decarboxylated at the C-2, C-15, or C-17 position (Herbach et al., 2005). The monodecarboxylated betanins are subsequently transformed to di- and tridecarboxylated betanins (Wybraniec, 2005). Decarboxylation at either C-2 or C-15 do not alter the betanidin chromophore, thereby maintaining the color of the original betacyanin. On the other hand, a carboxyl substituent in conjugation with the conjugated double bond system is eliminated in 17-decarboxybetanin, which thus exhibit an orange hue (Herbach et al., 2006b). Monocarboxylated betanin, phyllocactin, and hylocerenin were shown to be considerably more stable towards degradation than nondecarboxylated betacyanins (Huang and von Elbe, 1987; Herbach et al., 2006c). Isobetainin and neobetainin are also decarboxylated in a manner similar to that of betanin (not shown in Fig. 2) (Wybraniec, 2005).

During heating of *Basella alba* L. fruit juice, the principal decarboxylation products of major gomphrenins (simple and acylated betanidin-6-O- $\beta$ -glucoside) were 2-, 17-, and 2,17-decarboxy-gomphrenins (Kumorkiewicz and Wybraniec, 2017). Their diastereomers, as well as minor levels of their dehydrogenated derivatives were also found.

On prolonged thermal treatment of red beet, a diversity of betacyanin degradation products may be formed by multiple decarboxylation or by combined decarboxylation and dehydrogenation of betanin (Herbach et al., 2006b). Mixtures of mono-, di-, and tridecarboxylated betacyanins, together with their corresponding neobetacyanins were identified in purified extracts of red beet and purple pitaya (Wybraniec, 2005; Wybraniec and Mizrahi, 2005). Main products were 17-decarboxy-betacyanin,



**Figure 2** Alterations of betanin during processing and storage of food. Based on Herbach, K.M., Stintzing F.C., Carle, R., 2004. Impact of thermal treatment on color and pigment pattern of red beet (*Beta vulgaris* L.) preparations. J. Food Sci. 69, C491–C498; Herbach, K.M., Stintzing, F.C., Carle, R., 2006. Betalain stability and degradation – structural and chromatic aspects. J. Food Sci. 71, R41–R50.

17-decarboxy-isobetanin, 2-decarboxy-betanin, 2,17-didecarboxybetanin, 2,17-didecarboxyisobetanin, and 14,15-dehydrogenated-neobetanin. Heating of betanin, phyllocactin, and hylocerenin resulted in decarboxylated neo-derivatives together with the corresponding decarboxylated betacyanins (Herbach et al., 2005).

## Betalains as Food Colorants

Concern about food safety and strong demand for more natural products have stimulated efforts for the replacement of artificial food dyes by natural pigments, strengthened in more recent years by the potential health benefits of the latter. Natural colorants, however, are usually less stable, more costly, and not as easily utilized as synthetic dyes, besides having weaker tinctorial strength, interaction with food components, and limited range of hues (Sigurdson et al., 2017; Wrolstad and Culver, 2012).

Betanin extracted from beetroot has been commercialized as a natural food colorant, approved by the European Union and the U.S. Food and Drug Administration. It is used mainly to color such foods as dairy products, confectionery, ice cream, desserts, beverages and sausages (Obón et al., 2009). Its use is, however, restricted by its high nitrate content and its earthy flavor caused by geosmin and pyrazine derivatives (Lu et al., 2003; Stintzing and Carle, 2004). Alternative sources such as purple and yellow cactus pear and purple pitaya are therefore being explored.

Along with continued search for new and economically viable sources, appropriate pretreatment and extraction methods (Ngamwonglumlert et al., 2017), advances in processing technology and stabilization (Celli and Brooks, 2017), availability of wide spectrum of color, better quality of pigments devoid of off-flavor, and possibility of developing betalain-fortified formulations containing certain other bioactive and nutritional components could be expected (Khan and Giridhar, 2015).

Microencapsulation of betalains can be a successful strategy to improve stability, make handling easier during processing, and ensure bioavailability of microcapsules to be used as food colorants (Otálora et al., 2015). Microencapsulation of beetroot juice (Janiszewska, 2014; Pitalua et al., 2010) or extract/pigment (Azeredo et al., 2007; Serris and Biliaderis, 2001), pulp and cactus pear extract (Vergara et al., 2014), and Amaranthus pigments (Cai and Corke, 2000) has been tested.

## Health Benefits

Various health-promoting biological activities have been attributed to betanin and betalain-rich foods such as: scavenging of free radicals/reactive oxygen species, inhibition of lipid peroxidation and LDL oxidation, prevention of DNA-damage, induction of antioxidant (e.g. paraoxonase 1, glutathione peroxidase, heme oxygenase 1) and phase II detoxifying enzymes (e.g. glutathione S-transferase, NAD(P)H dehydrogenase [quinone] 1), gene regulatory activity (e.g. Nrf2-dependent signaling pathway); anti-inflammatory (e.g. inhibition of cyclooxygenase-2), anti-proliferative and anti-microbial activities (Esatbeyoglu et al., 2015; Gandía-Herrero et al., 2016; Gengatharan et al., 2015; Khan, 2016; Ninfali et al., 2017). Most of the studies have, however, been conducted in vitro with cells and animal models; human studies are lacking.

The antioxidant, anti-inflammatory, antiangiogenic and GST-inducing activities of betalains from pitaya fruit peels were enhanced by encapsulation in maltodextrin–gum Arabic and maltodextrin–pectin matrices (Rodriguez et al., 2016).

## Concluding Remarks

The coming years will witness continued intense research on betalains. Research topics will include: new and economically viable sources of both betacyanins and betaxanthins, optimization of processing to control alterations of betalains, efficient and green extraction and stabilization methods, production of high quality betalain colorants with wide color spectrum and free from off-flavor, and human studies to evaluate betalain's health benefits.

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## Carotenoids

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### Glossary

**Antioxidants** Compounds that inhibit or reduce the ability of oxidizing compounds (e.g., singlet oxygen and free radicals).

**Isomerization** Process that changes arrangement of atoms in a molecule without adding or removing anything from the original molecule.

**Oxidation** Chemical reaction that involves combination of oxygen with another substance or process that involves loss of electron.

**Oxidative stress** Presence of excessive level of oxidizing compounds known as free radicals in a cell; lack of antioxidant compounds that can eliminate free radicals.

**Pro-vitamin A** Substances in food that can be converted into vitamin A in organism.

### Introduction

Carotenoids are among the most widely distributed pigments and naturally exhibit red, orange and yellow colors. Carotenoids are lipid-soluble pigments, which can be found in many kinds of fruit, vegetables, fungi, flowers and some kinds of animals (Ötles and Cagindi, 2008). Photosynthetic bacteria, algae, fungi and plants can produce carotenoids through biosynthesis, whereas carotenoids found in humans as well as animal cells (e.g., lutein and zeaxanthin in human eyes and astaxanthin in salmon) are only from diets (Ellison, 2016; Kaczor et al., 2016). More than 750 different structures of carotenoids have so far been isolated from natural sources; about 500 structures have been fully characterized (Rodriguez-Amaya, 2016). Based on their structures, carotenes and xanthophylls are two main subclasses of carotenoids (Ngamwonglumlert et al., 2017). Different carotenoids structures naturally possess different physical, chemical and functional properties as well as stabilities.

Carotenoids exert several beneficial functions. The compounds can help, for example, attract insect pollinators in flowers, indicate maturity in fruit, absorb visible light in photosynthesis and defend light-induced damage of cells in the cases of photosynthetic bacteria, algae and green plants (Lerfall, 2016). Carotenoids are also essential for human health. For instance, lutein and zeaxanthin in human eyes are responsible for the filtering of the high-energy wavelengths of blue light, resulting in the reduction of oxidative stresses on the retina (Roberts et al., 2009). Some carotenoids provide additional health benefits since they play essential roles as pro-vitamin A and antioxidants. Based on their various benefits, carotenoids have long been utilized by nutraceutical and pharmaceutical industries (Ötles and Cagindi, 2008; Alcaïno et al., 2016). In addition, various carotenoids are used in many food-related industries as food colorants in lieu of the synthetic ones.

This chapter aims to provide basic information on carotenoids in terms of their occurrences, sources, structures, properties and stabilities. Relationships between the carotenoids structure and some selected properties as well as stability are outlined. The chapter ends with some sample applications of carotenoids as food colorants.

### Occurrences, Structures and Sources

The origin name of carotenoids is from carrot (*Daucus carota*) as they are the major pigments in a carrot root (Kaczor et al., 2016). Carotenoids, which are polyisoprenoid compounds consisting of eight isoprene units, are essential for all photosynthetic organisms due to their important photoprotective, antioxidant and visible-light absorbing roles. Plants, algae, bacteria and some fungi can naturally produce carotenoids through biosynthesis (Ötles and Cagindi, 2008; Lohr, 2009; Lerfall, 2016).

Carotenoids biosynthesis occurs in plastids, which are chloroplasts in the case of green tissues or chromoplasts in the case of yellow or red tissues. Carotenoids are synthesized from a C5 compound (isopentenyl pyrophosphate, IPP), which is obtained from the cytosolic mevalonic acid (MVA) as well as plastid methylerythritol 4-phosphate (MEP) pathways. Three IPP units and one unit of its allylic isomer (dimethylallyl diphosphate, DMAPP) are merged to form a C20 compound (geranylgeranyl pyrophosphate, GGPP), which is a precursor of the carotenoids biosynthesis, with GGPP synthase as the catalyst. Two molecules of GGPP are condensed together by the activation of phytoene synthase (PSY) enzyme, leading to the formation of 15-*cis*-phytoene, which is a C40 carotenoid skeleton. Phytoene later undergoes a series of dehydrogenation (desaturation reaction) and isomerization, with the activation of phytoene desaturase (PDS) and  $\zeta$ -carotene desaturase (ZDS) enzymes, resulting in the transformation of the colorless phytoene into red lycopene, which is the origin compound that can be transformed into a large variety of carotenoids structures (Ötles and Cagindi, 2008; Rosas-Saavedra and Stange, 2016). Other structures of carotenoids can thus be considered as lycopene derivatives.



Structures of carotenoids have been classified into two main subclasses, which are carotenes and xanthophylls. Carotenes consist of pure hydrocarbons, while xanthophylls consist of hydrogen, carbon and one or more oxygen-containing functional groups (Ötles and Cagindi, 2008; Alcaïno et al., 2016; Ngamwonglumlert et al., 2017). In the xanthophylls structure, various forms of oxygen groups (hydroxy, methoxy, carboxy, keto and epoxy groups) can be found. The presence of oxygen in the structure leads to many structural derivatives and the higher polarity of xanthophylls in comparison to that of carotenes. Common carotenes found in green plants and algae are  $\beta$ -carotene, while common xanthophylls found in plants are lutein, antheraxanthin, neoxanthin, violaxanthin and zeaxanthin (Nobel, 2009).

Carotenoids are naturally found in the form of all-*trans* isomer; an exception is noted in the case of neoxanthin, which is exclusively present in the form of 9'-*cis*-isomer (Lohr, 2009). However, all-*trans*-isomer can easily be isomerized to *cis*-isomer when the former is being exposed to heat and light (Provesi and Amante, 2015). Although the transformation of carotenoids structure from *trans*- to *cis*-isomer is noted to slightly reduce the color saturation of the pigments (Ngamwonglumlert et al., 2017), change in the carotenoids color is indeed more affected by the change in the number of conjugated double bonds in their structure. The characteristic color of carotenoids changes from colorless (phytoene) to yellow, red and pink when the number of conjugated double bonds increases as listed in Table 1. In addition, the color of carotenoids can also change to green, purple or blue when they form complexes with proteins (carotenoproteins). Crustacyanin, for example, is a dark blue pigment found in lobster shell (Lerfall, 2016; Alcaïno et al., 2016).

Carotenoids play essential roles in nutrition, vision, enhancement of immune function and prevention of cellular differentiation (Arscott, 2013; Rosas-Saavedra and Stange, 2016). Unfortunately, humans and animals cannot synthesize carotenoids. Carotenoids found in human and animal cells are thus acquired from dietary intake. Among many carotenoids,  $\beta$ -carotene, lutein, zeaxanthin and lycopene (Fig. 1) are the major carotenoids found in food sources (Boon et al., 2010; Arscott, 2013) and are well known to exert many health benefits. Food and plant sources of the four major carotenoids and their carotenoid contents are listed in Table 2.

## Functional Properties

Consumption of carotenoids-rich foods as well as carotenoids supplements has become more popular as a number of researchers have reported that carotenoids can help reduce the risks of several degenerative disorders, including cancer, cardiovascular and ophthalmological diseases due to their antioxidant properties (Stahl and Sies, 2003). In addition, diets containing carotenoids are important for human health since some carotenoids are pro-vitamin A or can be converted into vitamin A in human body. Antioxidant and conversion mechanisms of carotenoids into vitamin A are of focus here.

### Antioxidant Property

In human body, reactive oxygen species (ROS), which are singlet oxygen ( $^1\text{O}_2$ ) and free radicals, are naturally generated during respiration and metabolism. The generated singlet oxygen and free radicals normally have a beneficial role of killing pathogenic organisms that attack the body. However, ROS generation is an uncontrolled process; this, in combination with the fact that humans also typically obtain additional free radicals from such exogenous sources as air pollution, tobacco smoke and pesticides, may result in exceeding ROS level in the body. When the ROS level is excessive, oxidative stresses occur. ROS would react with fatty acids in cell membranes, enzymes, nucleic acids and endothelial cells, leading to cell damage and cell deterioration, mutation and/or inflammation, which are related to aging and occurrence of degenerative disorders, chronic diseases and cancer (Stahl and Sies, 2003; Lerfall, 2016).

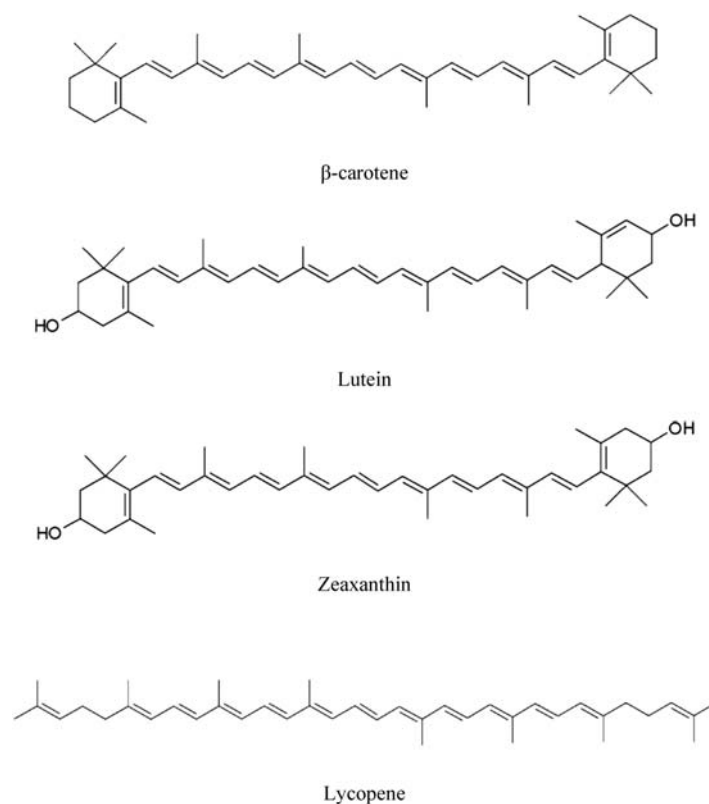
Consumption of carotenoids-rich sources has been reported to reduce the risks of several disorders and diseases that are caused by oxidative damage as carotenoids are a potent natural antioxidant. The antioxidant property of carotenoids is due to physical scavenging of ROS.

### Scavenging of Singlet Oxygen

The role of singlet oxygen quencher is based on the conversion of excess energy of such oxygen into heat. The process of singlet oxygen quenching consists of two main steps: (1) energy transfer between singlet oxygen and carotenoids molecules and (2) return to the ground state of carotenoids as shown in Eqs. (1) and (2), respectively. Carotenoids would first interact with singlet

**Table 1** Characteristic colors of carotenoids pigments

Pigment	Number of conjugated double bonds	Color
Phytoene	3	Colorless
$\zeta$ -Carotene	7	Light yellow
4,4'-Diaponeurosporene	9	Yellow
Capsanthin	10	Red
$\beta$ -Carotene	11	Orange
Bacterioruberin	13	Pink



**Figure 1** Structures of major carotenoids found in foods. Reprinted with permission from Rao and Rao (2007) and Zhang et al. (2018).

oxygen ( $^1\text{O}_2$ ) through the absorption of the energy of the oxygen, resulting in the formation of triplet ground state oxygen ( $^3\text{O}_2$ ) and triplet excited carotenoids. The triplet excited carotenoids would then return to the ground state by converting the excess energy to heat. In this process, the damage effect that is caused by the excited carotenoids is mostly neglected since the excited carotenoids possess low energy and short lifetime. After the excited carotenoids have returned to the ground state, they can be reused to quench another singlet oxygen (Stahl and Sies, 2003; Edge and Truscott, 2010; Fiedor and Burda, 2014; Lerfall, 2016).



The influence of various carotenoids on the singlet oxygen quenching ability has been demonstrated; most of the works were carried out in organic solvents (e.g., benzene, methanol and toluene). The results show that four major carotenoids found in foods, which are  $\beta$ -carotene, lutein, zeaxanthin and lycopene, possess high ability to quench singlet oxygen. This is probably because the triple energy levels of those carotenoids are closer to that of the singlet oxygen and hence the facilitated energy transfer (Stahl and Sies, 2003; Edge and Truscott, 2010). Many researchers also investigated the relationship between the quenching efficiency of carotenoids and the number of conjugated double bonds in the carotenoids structure and reported that the number of conjugated double bonds is related to the triple energy level. Carotenoids containing the larger number of conjugated double bonds exhibit a closer triple energy level to that of singlet oxygen and hence the enhanced quenching efficiency (Edge et al., 1997; Conn et al., 1991; Edge and Truscott, 2010).

The ability to quench singlet oxygen of carotenoids not only depends on the number of conjugated double bonds but also on other factors, including stereo-structure and functional groups in the carotenoids molecule. For example, Mascio et al. (1991) reported that lycopene (11 conjugated double bonds) possesses a higher quenching rate than astaxanthin (13 conjugated double bonds) although it has the smaller number of conjugated double bonds. All-*trans*- $\beta$ -carotene has also noted to exhibit a higher quenching rate than 15-*cis*- and 9-*cis*- $\beta$ -carotene although they all have similar numbers of conjugated double bonds (Conn et al., 1991; Edge and Truscott, 2010). The rate constants of singlet oxygen quenching of some selected carotenoids are listed in Table 3.

**Table 2**  $\beta$ -Carotene, lutein, zeaxanthin and lycopene contents in some selected food and plant sources

Source	Carotenoids content (mg/100 g)		
	$\beta$ -Carotene	Lutein + zeaxanthin	Lycopene
Apricot	6.640	ND	0.065
Apple	0.031–0.072	–	0.209
Asparagus	0.493	–	–
Avocado	0.053	–	–
Broccoli	0.779	2.445	ND
Brussels sprout	0.140	–	ND
Cabbage	0.010–3.020	–	ND
Carrot	6.500–21.000	–	–
Cauliflower	0.130	–	–
Cantaloupe	1.595	0.040	ND
Celery	0.770	–	ND
Chinese kale	4.090	–	ND
Chinese mustard leaves	2.930	–	ND
Chive	0.830	–	ND
Collard	3.323	–	–
Corn	0.030	0.884	–
Curry leaves	7.100	–	–
French bean	0.240	–	ND
Garlic leaves	5.000	–	–
Green bean	0.377	0.640	ND
Green pea	0.485	–	–
Guava	0.001	–	0.114
Ivy gourd	3.200–4.100	–	–
Jackfruit	0.026–0.036	–	0.037
Kale	9.226	39.550	ND
Long bean	0.410–0.570	–	ND
Mango	0.445	–	–
Okra	0.432	–	–
Onion leaves	4.900	–	–
Orange	0.040–0.059	–	–
Peach	0.097	0.057	ND
Pineapple	0.056	–	ND
Pink and red grapefruit	0.240–2.343	0.000–2.000	0.160–3.362
Pink guava	–	–	5.400
Pumpkin	0.060–14.850	–	ND
Spinach	5.597	11.938	–
Spring onion leaves	1.280	–	–
Sweet green pepper	0.198	–	–
Sweet red pepper	2.379	–	–
Tomato (ripe)	0.393	0.130	3.025
Watermelon	0.287–0.310	0.017	4.868

ND = Not detected; – = Data not available.

Source: Khoo et al. (2011), Arscott (2013).

### Scavenging of Free Radicals

Other antioxidative role of carotenoids is free radical scavenging. Radical adduct formation, electron transfer and allylic hydrogen abstraction (or hydrogen atom transfer) are the three accepted possible interactions to quench free radicals by carotenoids (Krinsky and Yeum, 2003; Fiedor and Burda, 2014); these are shown in Eqs. (3)–(6).

Radical adduct formation:



Electron transfer:



or

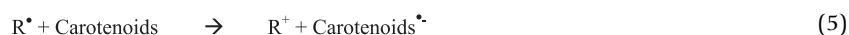
**Table 3** Singlet oxygen quenching rate constants of carotenoids in benzene and toluene

Carotenoid	N	$k_q (\times 10^9 M^{-1} s^{-1})$	
		Benzene <sup>a</sup>	Toluene <sup>b</sup>
Dodecapreno- $\beta$ -carotene	19	23.0	29.0
Tetradecahydrolycopene	15	10.7	–
Astaxanthin	11 (+2, C=O)	14.0	–
Canthaxanthin	11 (+2, C=O)	12.0	13.0
Lycopene	11	17.0	18.0
All- <i>trans</i> - $\beta$ -carotene	11	13.0	14.0
15- <i>cis</i> - $\beta$ -carotene	11	11.0	12.0
9- <i>cis</i> - $\beta$ -carotene	11	11.0	9.0
Zeaxanthin	11	12.0	–
$\alpha$ -carotene	10	12.0	8.2
Lutein	10	6.64	–

N = Number of conjugated double bonds.

<sup>a</sup>Source: Edge and Truscott (2010).

<sup>b</sup>Source: Conn et al. (1991).



Allylic hydrogen abstraction:



Carotenoids-free radicals interaction mechanism depends on the species of free radicals (e.g., sulfur-containing, nitrogen dioxide and peroxy radicals) and solvent that are used to test the scavenging ability. For example, carotenoids would interact with thiyl radical ( $RS^\bullet$ ) through the process of radical adduct formation, while they would interact with thiyl sulfonyl radical ( $RSO_2^\bullet$ ) through radical adduct formation and electron transfer (Everett et al., 1996). In a non-polar solvent, carotenoids would react with acylperoxy radicals via radical adduct formation but would interact with those radicals via both radical adduct formation and electron transfer in a polar solvent (Edge and Truscott, 2010). Other interactions between carotenoids and various species of free radicals are listed in Table 4. However, relationships between the carotenoids structure and the carotenoids-free radicals interaction mechanism have never been established.

There are three types of products from the carotenoids-free radicals interactions: (I) adduct radical ( $R$ -Carotenoid $^\bullet$ ) from radical adduct formation, (II) carotenoids radical cation ( $\text{Carotenoids}^{\bullet+}$ ) or anion ( $\text{Carotenoids}^{\bullet-}$ ) from electron transfer and (III) neutral carotenoid radical ( $\text{Carotenoids}^\bullet$ ) from allylic hydrogen abstraction. Among the various types of the products,  $\text{carotenoids}^{\bullet+}$  are mostly studied as they possess strong oxidizing activity and long lifetime. This radical can play a role as pro-oxidant since it is well known to be able to interact with oxygen, other carotenoids and other biological substrates such as water-soluble antioxidants and amino acids (Edge and Truscott, 2010). El-Agamey et al. (2004) noted that  $\text{carotenoids}^{\bullet+}$  (i.e.,  $\beta$ -carotene, zeaxanthin, astaxanthin,  $\beta$ -cryptoxanthin and lycopene radical cations) could oxidize amino acids (i.e., tryptophan, cysteine and tyrosine), resulting therefore in protein damage. Removing and/or repairing  $\text{carotenoids}^{\bullet+}$  is thus necessary; this can be achieved through the oxidation of  $\text{carotenoids}^{\bullet+}$  by other antioxidants such as another carotenoids and ascorbic acid. Transferring the oxidizing radicals from lipid phase to aqueous phase through an interaction between  $\text{carotenoids}^{\bullet+}$  and water-soluble antioxidants such as ascorbic acid is one of the interesting ways to reduce the risk of additional lipid oxidation (El-Agamey et al., 2004).

### Pro-vitamin A Ability

Besides the antioxidant activity, ability to serve as pro-vitamin A is another most important property of carotenoids. However, only carotenoids that contain at least one unsubstituted  $\beta$ -ionone ring at the terminal end possess the ability to be pro-vitamin A (Chandrika, 2009; Lerfall, 2016). For this reason,  $\beta$ -carotene and  $\alpha$ -carotene are noted to be pro-vitamin A, whereas lycopene, lutein and zeaxanthin are not. Among the many types of carotenoids, all-*trans*- $\beta$ -carotene is well recognized as the most important precursor of vitamin A. Transformation of  $\beta$ -carotene into vitamin A (or retinol) in humans and mammals has long been investigated and the process predominantly occurs in intestine (Tang, 2010). Two different pathways for the cleavage of  $\beta$ -carotene as well as other carotenoids into retinol, which are central cleavage pathway and eccentric cleavage pathway, have been proposed (Rodriguez-Amaya, 2016).

Central cleavage or symmetric cleavage is the pathway that involves the activity of 15,15'-dioxygenase (BCO1) to break the central 15,15'-double bond of  $\beta$ -carotene to yield two molecules of retinal. Retinal can then be reversibly converted into retinol

**Table 4** Interactions of carotenoids with various free radical species and their products in different solvents

Carotenoid	Free radical	Solvent	Result
All- <i>trans</i> - $\beta$ -carotene <sup>a</sup>	$\text{NO}_2^\bullet$	$\text{N}_2$ -saturated <i>tert</i> -butanol/water mixture (50:50, v/v)	Interaction: Electron transfer Product: $\beta$ -carotene $^{\bullet+}$
	$\text{GS}^\bullet$ (glutathione thiyl radical)		Interaction: Radical adduct formation Product: $\text{GS}-\beta$ -carotene $^\bullet$
	$\text{C}_2\text{H}_5\text{SO}_2^\bullet$		Interaction: Electron transfer, radical adduct formation Product: $\beta$ -carotene $^{\bullet+}$ , $\text{C}_2\text{H}_5\text{SO}-\beta$ -carotene $^\bullet$
All- <i>trans</i> - $\beta$ -carotene <sup>b</sup>	$\text{CCl}_3\text{O}_2^\bullet$	Triton X-100 micelles (aqueous 2% v/v Trion X-100)	Interaction: Electron transfer, radical adduct formation Product: $\beta$ -carotene $^{\bullet+}$ , $\text{CCl}_3\text{O}_2-\beta$ -carotene $^\bullet$
All- <i>trans</i> - $\beta$ -carotene <sup>c</sup>	$\text{C}_6\text{H}_5\text{O}^\bullet$ (phenoxy radical)	Di- <i>tert</i> -butyl peroxide/benzene (70:30, v/v)	Interaction: Electron transfer, radical adduct formation Product: $\beta$ -carotene $^{\bullet+}$ , $\text{C}_6\text{H}_5\text{O}-\beta$ -carotene $^\bullet$
All- <i>trans</i> - $\beta$ -carotene <sup>d</sup>	$\text{CH}_3\text{C}(\text{O})\text{OO}^\bullet$	Benzene	Interaction: Radical adduct formation Product: $\text{CH}_3\text{C}(\text{O})\text{OO}-\beta$ -carotene $^\bullet$
7,7'-dihydro- $\beta$ -carotene (77DH) <sup>e</sup>	$\text{C}_6\text{H}_5\text{CH}_2\text{C}(\text{O})\text{OO}^\bullet$ (phenylacetylperoxyl radical, PAP)	Hexane	Interaction: Radical adduct formation Product: $\text{PAP}-77\text{DH}^\bullet$
		Methanol	Interaction: Electron transfer, radical adduct formation Product: $77\text{DH}^{\bullet+}$ , $\text{PAP}-77\text{DH}^\bullet$
Astaxanthin <sup>b</sup>	$\text{CCl}_3\text{O}_2^\bullet$	Triton X-100 micelles (aqueous 2% v/v Trion X-100)	Interaction: Electron transfer, radical adduct formation Product: Astaxanthin $^{\bullet+}$ , $\text{CCl}_3\text{O}_2$ -astaxanthin $^\bullet$
Astaxanthin <sup>f</sup>	$\text{NO}_2^\bullet$	$\text{N}_2$ -saturated <i>tert</i> -butanol/water mixture (60:40, v/v)	Interaction: Electron transfer Product: Astaxanthin $^{\bullet+}$
	$\text{GS}^\bullet$ (glutathione thiyl radical)		Interaction: Radical adduct formation Product: $\text{GS}-\text{astaxanthin}^\bullet$
	$\text{HO}(\text{CH}_2)_2\text{S}^\bullet$		Interaction: Radical adduct formation Product: $\text{HO}(\text{CH}_2)_2\text{S}-\text{astaxanthin}^\bullet$
	$\text{CH}_3\text{SO}_2^\bullet$		Interaction: Electron transfer, radical adduct formation Product: Astaxanthin $^{\bullet+}$ , $\text{CH}_3\text{SO}_2$ -astaxanthin $^\bullet$
Canthaxanthin <sup>b</sup>	$\text{CCl}_3\text{O}_2^\bullet$	Triton X-100 micelles (aqueous 2% v/v Trion X-100)	Interaction: Electron transfer, radical adduct formation Product: Canthaxanthin $^{\bullet+}$ , $\text{CCl}_3\text{O}_2$ -canthaxanthin $^\bullet$

(Continued)

**Table 4** Interactions of carotenoids with various free radical species and their products in different solvents—cont'd

<i>Carotenoid</i>	<i>Free radical</i>	<i>Solvent</i>	<i>Result</i>
Canthaxanthin <sup>f</sup>	NO <sub>2</sub> •	N <sub>2</sub> -saturated <i>tert</i> -butanol/water mixture (60:40, v/v)	Interaction: Electron transfer Product: Canthaxanthin• <sup>+</sup>
	GS• (glutathione thiyl radical)		Interaction: Radical adduct formation Product: GS–canthaxanthin•
	HO(CH <sub>2</sub> ) <sub>2</sub> S•		Interaction: Radical adduct formation Product: HO(CH <sub>2</sub> ) <sub>2</sub> S–canthaxanthin•
	CH <sub>3</sub> SO <sub>2</sub> •		Interaction: Electron transfer, radical adduct formation Product: Canthaxanthin• <sup>+</sup> , CH <sub>3</sub> SO <sub>2</sub> –canthaxanthin•
Zeaxanthin <sup>b</sup>	CCl <sub>3</sub> O <sub>2</sub> •	Triton X-100 micelles (aqueous 2% v/v Triton X-100)	Interaction: Electron transfer, radical adduct formation Product: Zeaxanthin• <sup>+</sup> , CCl <sub>3</sub> O <sub>2</sub> –zeaxanthin•
Zeaxanthin <sup>c</sup>	C <sub>6</sub> H <sub>5</sub> O• (phenoxyl radical)	Di- <i>tert</i> -butyl peroxide/benzene (70:30, v/v)	Interaction: Electron transfer, radical adduct formation Product: Zeaxanthin• <sup>+</sup> , C <sub>6</sub> H <sub>5</sub> O–zeaxanthin•
Zeaxanthin <sup>f</sup>	NO <sub>2</sub> •	N <sub>2</sub> -saturated <i>tert</i> -butanol/water mixture (60:40, v/v)	Interaction: Electron transfer Product: Zeaxanthin• <sup>+</sup>
	GS• (glutathione thiyl radical)		Interaction: Radical adduct formation Product: GS–zeaxanthin•
	HO(CH <sub>2</sub> ) <sub>2</sub> S•		Interaction: Radical adduct formation Product: HO(CH <sub>2</sub> ) <sub>2</sub> S–zeaxanthin•
	CH <sub>3</sub> SO <sub>2</sub> •		Interaction: Electron transfer, radical adduct formation Product: Zeaxanthin• <sup>+</sup> , CH <sub>3</sub> SO <sub>2</sub> –zeaxanthin•
Lutein <sup>b</sup>	CCl <sub>3</sub> O <sub>2</sub> •	Triton X-100 micelles (aqueous 2% v/v Triton X-100)	Interaction: Electron transfer, radical adduct formation Product: Lutein• <sup>+</sup> , CCl <sub>3</sub> O <sub>2</sub> –lutein•
Lutein <sup>c</sup>	C <sub>6</sub> H <sub>5</sub> O• (phenoxyl radical)	Di- <i>tert</i> -butyl peroxide/benzene (70:30, v/v)	Interaction: Electron transfer, radical adduct formation Product: Lutein• <sup>+</sup> , C <sub>6</sub> H <sub>5</sub> O–lutein•
Lutein <sup>f</sup>	NO <sub>2</sub> •	N <sub>2</sub> -saturated <i>tert</i> -butanol/water mixture (60:40, v/v)	Interaction: Electron transfer Product: Lutein• <sup>+</sup>
	GS• (glutathione thiyl radical)		Interaction: Radical adduct formation Product: GS–lutein•
	HO(CH <sub>2</sub> ) <sub>2</sub> S•		Interaction: Radical adduct formation Product: HO(CH <sub>2</sub> ) <sub>2</sub> S–lutein•
	CH <sub>3</sub> SO <sub>2</sub> •		Interaction: Electron transfer, radical adduct formation Product: Lutein• <sup>+</sup> , CH <sub>3</sub> SO <sub>2</sub> –lutein•

**Table 4** Interactions of carotenoids with various free radical species and their products in different solvents—cont'd

Carotenoid	Free radical	Solvent	Result
Lycopene <sup>c</sup>	C <sub>6</sub> H <sub>5</sub> O• (phenoxy radical)	Di- <i>tert</i> -butyl peroxide/benzene (70:30, v/v)	Interaction: Electron transfer, radical adduct formation Product: Lycopene <sup>•+</sup> , C <sub>6</sub> H <sub>5</sub> O–lycopene <sup>•</sup>
Lycopene <sup>f</sup>	NO <sub>2</sub> •	N <sub>2</sub> -saturated <i>tert</i> -butanol/water mixture (80:20, v/v)	Interaction: Electron transfer Product: Lycopene <sup>•+</sup>
	GS• (glutathione thiyl radical)		Interaction: Radical adduct formation Product: GS–lycopene <sup>•</sup>
	HO(CH <sub>2</sub> ) <sub>2</sub> S•		Interaction: Radical adduct formation Product: HO(CH <sub>2</sub> ) <sub>2</sub> S–lycopene <sup>•</sup>
	CH <sub>3</sub> SO <sub>2</sub> •		Interaction: Electron transfer, radical adduct formation Product: Lycopene <sup>•+</sup> , CH <sub>3</sub> SO <sub>2</sub> –lycopene <sup>•</sup>

<sup>a</sup>Source: Everett et al. (1996).<sup>b</sup>Source: Hill et al. (1995).<sup>c</sup>Source: Mortensen and Skibsted (1997).<sup>d</sup>Source: Mortensen (2001).<sup>e</sup>Source: El-Agamey and McGravey (2002).<sup>f</sup>Source: Mortensen et al. (1997).

via the reaction of retinal reductase; retinal can otherwise be irreversibly transformed into retinoic acid via the reaction of retinal dehydrogenase (Rodriguez-Amaya, 2016). The conversion of  $\beta$ -carotene into retinol through the central cleavage pathway is shown in Fig. 2.

The other alternative route, which is the eccentric cleavage or asymmetric cleavage pathway, involves the activation of dioxygenase 2 (BCO2). BCO2 activates the cleavage of double bond in the polyene chain beside the central 15,15'-double bond, yielding  $\beta$ -ionone and several  $\beta$ -apo-carotenals, which can subsequently be transformed into retinal and then retinol as shown in Fig. 3.

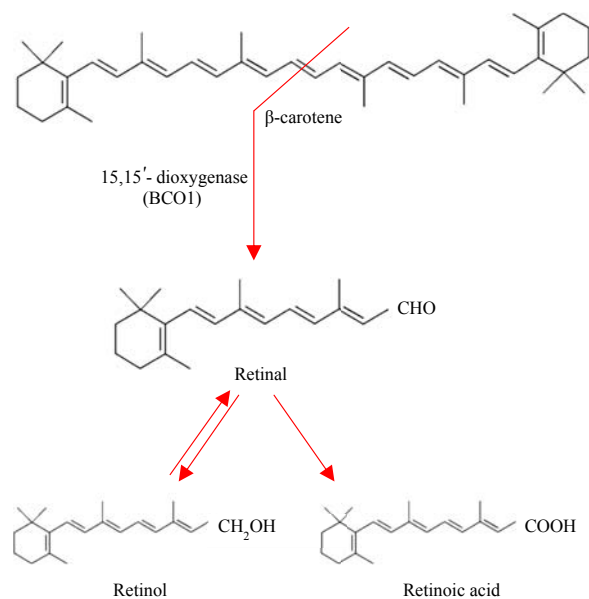
Conversion efficiency of carotenoids into vitamin A is related to the absorption and bioconversion abilities and also depends on the status of vitamin A and pro-vitamin A in the body. The efficiency increases when vitamin A status becomes low, but decreases when a high dose of pro-vitamin A is consumed (Rodriguez-Amaya, 2016). This occurs to prevent the toxicity that may occur due to excess vitamin A level. Excess consumption of pro-vitamin A (e.g.,  $\beta$ -carotene and  $\alpha$ -carotene) have nevertheless never been reported to be related to toxicity, but can simply cause deep yellow or orange coloration of skin (Francis, 1996; Lerfall, 2016). On the other hand, vitamin A deficiency (VAD) is known to lead to several disorders. VAD has been reported to be directly linked to night blindness and reduction of immune function, which causes an increase in the risks of morbidity and mortality, especially in infant and preschool-age child due to bacterial or viral infection (Haskell, 2013).

## Carotenoids Stability

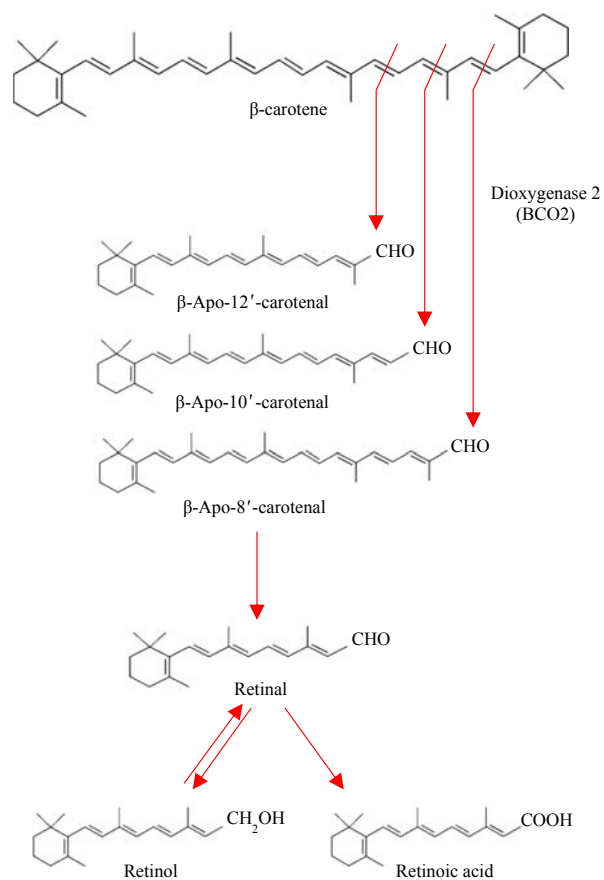
Change in the molecular structure as well as degradation of carotenoids normally begin even during the process of material preparation. Most preparation processes (e.g., chopping and grinding) cause tissue disruption and hence increased surface area exposing to oxygen, which leads to the oxidation of carotenoids (Yahia and de Jesús Ornelas-Paz, 2010). Heat, light and enzymes are the important factors stimulating the oxidation reaction, which are the main cause of the losses in both the activity and color of carotenoids (Ngamwonglumlert et al., 2017). Carotenoids can be oxidized into the primary oxidation products, which are apo-carotenoids and epoxides, which subsequently degrade into secondary degradation products, which are low molecular weight or volatile compounds (Borsarelli and Mercadante, 2010).

To prevent the oxidation of carotenoids due to the activity of such enzymes as lipoxygenase, short-time thermal treatment (i.e., blanching) can be conducted (von Elbe and Schwartz, 1996; Bahçeci et al., 2005). However, although blanching can help inactivate the oxidative enzymes, a number of researchers have reported that blanching as well as other thermal processes may lead to change in carotenoids structure through isomerization. Heat is a major factor inducing carotenoids isomerization from *trans*- to *cis*- isomers. Although transformation from *trans*- to *cis*-isomers only slightly leads to a decrease in the color





**Figure 2** Central cleavage pathway of  $\beta$ -carotene. Adapted from [Harrison et al. \(2012\)](#).



**Figure 3** Eccentric cleavage pathway of  $\beta$ -carotene. Adapted from [Harrison et al. \(2012\)](#).

saturation without a significant change in the hue (Ngamwonglumlert et al., 2017), such a change may affect the antioxidant activity of carotenoids. Bohm et al. (2002), for example, noted that all-*trans*- $\beta$ -carotene has higher antioxidant activity than 13-*cis*- $\beta$ -carotene.

Food processing generally leads to degradation of carotenoids through both oxidation and isomerization. Increasing temperature and processing time as well as large amount of oxygen concentration lead to extensive degradation of carotenoids. Fratianni et al. (2010), for example, investigated the effect of temperature on the degradation of carotenoids in orange juice (*Citrus sinensis* Osbeck) during microwave pasteurization. Increasing pasteurization temperature resulted in the more extensive loss of carotenoids. Total carotenoids,  $\alpha$ -carotene,  $\beta$ -carotene, lutein and zeaxanthin contents of the juice pasteurized at 70 °C for 60 s did not change, while those of the sample pasteurized at 85 °C for 60 s decreased by about 50%, 57%, 66%, 54% and 58%, respectively. Chen et al. (1995) earlier compared the change in the carotenoids content of carrot juice subjected to pasteurization and sterilization. Carrot juice undergone pasteurization (110 °C for 30 s) suffered less extensive loss of  $\alpha$ -carotene than the juice undergone sterilization (121 °C for 30 min) due to the use of the lower heating temperature and shorter heating time of the former. Isomerization of  $\alpha$ -carotene into 9-*cis*-, 13-*cis*- and 15-*cis*- $\alpha$ -carotenes was noted in both the pasteurized and sterilized products; sterilized product nevertheless possessed lower contents of 13-*cis*- and 15-*cis*- $\alpha$ -carotenes than the pasteurized one due to the compound degradation at extended heating time.

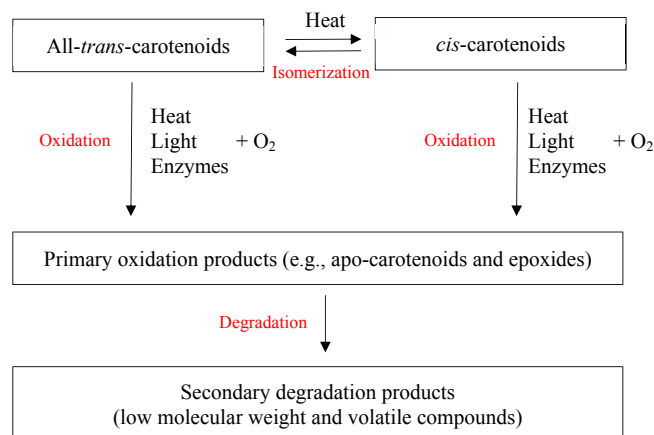
To retain food quality as well as to prevent degradation of carotenoids due to heat, non-thermal techniques such as high-pressure processing (HPP) and pulsed electric field (PEF) treatment have been proposed. Knockaert et al. (2012), for example, compared the quality of tomato puree (in terms of its lycopene content) obtained from thermal and non-thermal preservation techniques. Lycopene content of the puree obtained from mild pasteurization (60 °C for 1 min) and from HPP at a milder condition (450 MPa at 20 °C for 15 min) were not significantly different. However, pasteurization at a more intense condition (90 °C for 10 min) resulted in the loss of lycopene content by about 14%, while the content of the product treated by HPP at a more intense condition (600 MPa at 20 °C for 15 min) was not altered. Tomato puree sterilized at 120 °C for 1.5 min suffered more extensive loss of lycopene than the puree treated by HPP at a sterilization condition (600 MPa at 117 °C for 1.5 min). Lycopene content decreased by about 38% and 5% after sterilization and HPP at the sterilization condition, respectively.

Cortés et al. (2006) investigated the effect of high-intensity PEF on carotenoids in orange juice; the results were also compared with those of the sample undergone pasteurization. The juice treated by PEF (at 30 kV/cm for 100  $\mu$ s) suffered less extensive losses of total carotenoids,  $\alpha$ -carotene,  $\beta$ -carotene, lutein and zeaxanthin than the pasteurized juice (treated at 90 °C for 20 s). Total carotenoids,  $\alpha$ -carotene,  $\beta$ -carotene, lutein and zeaxanthin were lost by about 7%, 7%, 4%, 7% and 16% after PEF and 13%, 34%, 10%, 12% and 17% after pasteurization, respectively.

Structural changes and degradation of carotenoids through isomerization, oxidation and degradation to low molecular weight or volatile compounds are shown in Fig. 4.

## Applications as Food Additives

Carotenoids have long been used as a colorant to yield yellow-orange or red color in foods. Carotenoids can be directly added into foodstuffs or indirectly added by mixing into animal feeds such as in chicken feed to give a color to egg-yolk. Oil-based extract, emulsion and dried powder are the most popular forms of commercially available carotenoids. Carrot, annatto, marigold, paprika, saffron and tomato are usually used as sources of carotenoids. Some carotenoids are permitted for use in foods, while some are restricted to such specific uses as animal feeds. Such allowance depends on the regulation of each country.



**Figure 4** Structural changes and degradation of carotenoids. Adapted from Borsarelli and Mercadante (2010).

### Astaxanthin

Astaxanthin, which is a keto-carotenoid derivative of canthaxanthin, is an orange-red pigment found in green algae, red algae and bacteria. The United States permits the use of astaxanthin from *Haematococcus* sp. as a pigment source in animal and fish feeds, while the use in foods is still not permitted. Limitation of the use is less than 80 mg/kg of finish product. Suspension, water- or oil-dispersion and freeze-dried products can be found in the market (Delgado-Vargas and Paredes-López, 2003; Solymosi et al., 2015).

### β-Carotene

β-carotene, the most widely distributed carotenoids, is usually used as a colorant to yield yellow-orange color in various foods (Francis, 1996). β-carotene obtained from halo-tolerant algae (*Dunaliella salina*) or fungi (*Blakeslea trispora*) is permitted for use as food additive in Europe, while β-carotene extracted from vegetables such as carrot and grass alfalfa is only permitted for feed additive (Delgado-Vargas and Paredes-López, 2003; Kendrick and Limited, 2012). Although there is no evidence indicating that oral consumption of β-carotene is related to toxicity or harm to humans, hypercarotenemia, which results in orange skin coloration, may be observed when consuming food containing β-carotene at a higher concentration for an extended period of time (Francis, 1996).

### Bixin

Bixin, which is an oil-soluble red-brown pigment, can be extracted from the resinous coating on the seed of annatto (*Bixa orellana*). Water-soluble annatto extract can be prepared by saponification; the extract has been used as a colorant for foods as well as cosmetic and textile products (Delgado-Vargas and Paredes-López, 2003; Britton, 1996). Oil-soluble annatto is, on the other hand, more suitable for use in oily foods such as dairy spreads, salad dressings and extruded snacks (Delgado-Vargas and Paredes-López, 2003). Dried powder of annatto extract has also been applied in sausage to replace the use of nitrite (Zarringhalami et al., 2009). Maximum use level, which has been established by JECFA, of annatto extract depends on the type of foods. For example, limitation for the use in cakes, cookies, and pies is 15 mg/kg of a final product, while limitation for the use in processed meat and poultry is less than 50 mg/kg of a final product (Delgado-Vargas and Paredes-López, 2003).

### Canthaxanthin

Canthaxanthin is a red keto-carotenoid, which is naturally found in bacteria, algae and some fungi (Esatbeyoglu and Rimbach, 2017). However, most commercially available canthaxanthin is chemically synthesized. This is because natural canthaxanthin is generally found at lower concentrations (Sanchez et al., 2013). Canthaxanthin is used as poultry feed additive to yield red color in skin and yolks (Esatbeyoglu and Rimbach, 2017). The European Union permits the use of canthaxanthin in feedstuff at a maximum content of 25 mg/kg of final feedstuff (Oehlenschläger and Ostermeyer, 2016), while the United States allows the use of this pigment in broiler chicken and salmonid fish feeds at a concentration below 30 mg/0.45 kg of solid or semi-solid feed or below 30 mg/0.74 L of liquid food (Harp and Barrows, 2015). Canthaxanthin is also permitted for use as food additive. ADI of canthaxanthin when used as a food colorant has been defined by JECFA as 0–3 mg/kg of body weight (Esatbeyoglu and Rimbach, 2017).

### Capsanthin and Capsorubin

Capsanthin and capsorubin are orange-red pigments generally obtained from paprika (*Capsicum annum*). Paprika powder and oleoresin, which is an oil-based mixture containing oil, fat, wax and pigment, are the major forms that are widely used in foods, especially in spice mixes, sauces and emulsified processed meats (Delgado-Vargas and Paredes-López, 2003; Kendrick and Limited, 2012). Besides enhancing the color of food, the other aim of adding paprika is to add flavor. Both paprika and paprika oleoresin are allowed in general foods in the United States (Harp and Barrows, 2015). However, the limitation for use of paprika as food colorant is its hot and spicy flavor, which may not be desirable in some products.

### Crocetin and Crocin

Crocetin and crocin are yellow pigments that are normally obtained from saffron (*Crocus sativus*). Saffron powder as well as extract are used as both colorant and spice (Delgado-Vargas and Paredes-López, 2003; Britton, 1996). However, the use of saffron powder and extract as food colorant is limited due to its high cost and is restricted only in the United States. The European Union has not approved saffron for its use as colorant but allows its use as spice (Solymosi et al., 2015; Harp and Barrows, 2015). In Japan and China, crocin extracted from fruit of gardenia yellow (*Gardenia jasminoides* and *Gardenia augusta*) has been approved for use in foods. On the other hand, the use of crocin obtained from the fruit has not been permitted in the United States and European Union (Solymosi et al., 2015).

### Lycopene

Lycopene extracted from tomato (*Lycopersicon esculentum*) is permitted for use as colorant in the United States and European Union to yield red color in food (Delgado-Vargas and Paredes-López, 2003; Solymosi et al., 2015; Harp and Barrows, 2015). Besides

tomato, lycopene extract from fungus *B. trispora* has been approved for use in the European Union (Harp and Barrows, 2015). Lycopene extracted from tomato suffers one important limitation, however; the intense flavor of tomato may not be favored by some consumers (Britton, 1996).

### Lutein and Zeaxanthin

Lutein and zeaxanthin, which are pigments that give yellow color, are usually obtained from petals of marigold flower (Kendrick and Limited, 2012). In the European Union, dried petals and oleoresin of lutein and zeaxanthin are permitted for use in poultry and fishery feeds, while the purified oleoresin is permitted for use as additives in various kinds of foods such as pastas, vegetable oils, margarine and baked products (Delgado-Vargas and Paredes-López, 2003). On the other hand, the United States allows the use of the dried petals and extracts only in chicken feed (Harp and Barrows, 2015).

To enhance the color stability of carotenoids when being used as colorants, some antioxidants such as ascorbic acid are added into food to prevent the oxidation of carotenoids. Addition of ascorbic acid at a maximum level (400 mg/kg) has been noted to help increase the color stability of carotenoids (Kendrick and Limited, 2012). Products containing carotenoids should also be kept in a low-oxygen environment and in dark.

### Conclusions

Occurrences, sources, structures, selected properties, stabilities and applications in food of carotenoids are here outlined. Properties, including color, antioxidant and pro-vitamin A activities, as well as stability of carotenoids are related to their structures. *Trans*-carotenoids normally possess higher antioxidant activity than *cis*-ones. Only carotenoids containing at least one unsubstituted  $\beta$ -ionone ring at the terminal end (i.e.,  $\beta$ -carotene and  $\alpha$ -carotene) exhibit pro-vitamin A activity. Heat induces isomerization of carotenoids structure from *trans*- to *cis*-isomers, while completed degradation of carotenoids is generally due to oxidation.

Foods processed under high oxygen-concentration environment (e.g., hot air drying) and high temperature (e.g., sterilization) suffer more extensive losses of carotenoids. Processes involving low oxygen concentration (e.g., vacuum microwave drying and low-pressure superheated steam drying) and non-thermal processes (e.g., high pressure processing and high-intensity pulsed electric field) have therefore been proposed to alleviate such losses.

For utilization of carotenoids as a coloring agent, regulations and limitations, which depend on the type of carotenoids, must be considered. Some carotenoids are permitted for use in foods as colorant, additive or spice, while some are restricted only for animal feeds.

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# Clarifying Agents

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## Introduction

In broader terms, “clarification” is the crucial step, in either juice processing or wine/beer making, which removes suspended solids and clarity compromising colloids, to achieve the required degree of clarity and sensory attributes. Clarification is a process by which the semistable emulsion of colloidal plant carbohydrates that support the insoluble cloud material of a freshly pressed juice or wine is “broken” such that the viscosity is dropped and the opacity of the cloudy juice is changed to an open splotchy appearance. Manufacturers and processors can clarify the juice or any other beverage by either using mechanical separations (centrifugation or filtration) alone or a combination of clarifying/fining agents and mechanical separations.

Kilara and Van Buren (1989) classified ways for attaining clarification as enzymatic and nonenzymatic where nonenzymatic clarification involves breaking the emulsion by other means, the most common of which is heat. Other techniques include addition of gelatin, casein, and tannic acid–protein combinations (Kilara and Van Buren, 1989). Additionally, the uses of honey and combined honey–pectinase treatments have been found to be effective clarification agents. It is believed that the proteinaceous component of honey is responsible for a synergistic effect when honey and pectinase are used in combination (McLellan et al., 1985).

## Need for Clarification

Fruit juices, beer, wine, and many beverages face a major problem of haziness. The interaction between natural phenolic substances and pectic and proteinaceous substances causes the formation of clarity compromising colloids. The consumers’ expectations for clarity and sensory quality attributes have prompted many manufacturers of juice and beverage makers to incorporate clarifying/fining agents during their processing.

## Clarifying Agents

Proteins and polyphenols are the most common substances that require clarification in wines and fruit juices. There is one other contributing cause of suspended haze that we often overlook, for example, entrained carbon dioxide. Moving the wine into a warmer area from a cold cellar can frequently reduce this contributor. Clarifying agents can be classified according to their composition as shown in Fig. 1.

Generally, conventional clarifying procedures can be achieved by centrifugation, enzymatic treatment, applying clarifying agents such as gelatin, bentonite, silica sol, polyvinylpyrrolidone (PVPP), or a combination of these compounds (Sin et al., 2006). A number of agents, including gelatin, bentonite, activated carbon, casein, ion-exchange waxes, and PVPP, have been studied for the removal of polyphenols from fruit juices (Balik, 2003; Fang et al., 2006; Stankovic et al., 2004; Turkyölmaz et al., 2012). Zoecklein et al. (1999) compared desirable effects and potential problems for different clarifying agents in their decreasing order of activity or effectiveness (Table 1). The strong negative charge of the bentonite surface exerts an important action on positively charged proteins of the juice that enables its flocculation. Effective use of clarification agents requires optimization of their methods of preparation and determination of the suitable concentrations needed to achieve the desired clarification.

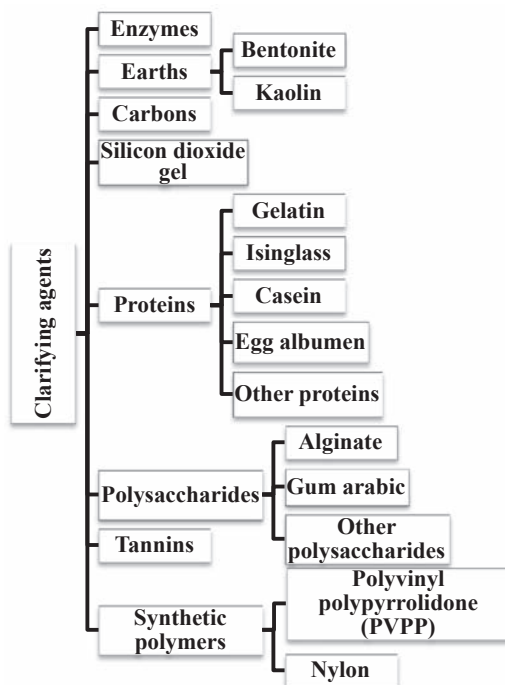
## Enzyme as a Clarifying Agent

Enzymes are effective protein catalysts for biochemical reactions. Enzymes are classified into six groups according to the reaction catalyzed and denoted by an EC (Enzyme Commission) number, viz., EC1: Oxidoreductases, EC2: Transferases, EC3: Hydrolases, EC4: Lyases, EC5: Isomerases, and EC6: Ligases. Systematic nomenclature is the addition of the suffix *-ase* to the enzyme-catalyzed reaction with the name of the substrate (Bayindirli, 2010).

Fruit juice contains pectin and other polysaccharides, so it may lead to fouling during membrane filtration. Enzymatic treatments lead to the degradation of pectin. Enzymatically clarified juice resulted in viscosity reduction and cluster formation, which facilitates separation through centrifugation or filtration. As a result, the juice presents higher clarity, as well as more concentrated flavor and color (Abdullah et al., 2007).

Enzymatic treatments lead to an increase in the clarity of juice. Juice clarity can be determined in terms of absorbance and transmittance at 660 nm using ultraviolet–visible spectrophotometer. An increase in enzymatic concentration increases the rate of clarification by exposing part of the positively charged protein, thus reducing electrostatic repulsion between cloud particles which caused these particles to aggregate into larger particles and eventually settle out (Sin et al., 2006). Clarity showed the lowest absorbance value at highest enzyme concentration, where lower absorbance indicates a clearer juice, is being produced. It was also observed that the absorbance values decreased with increasing incubation time at fixed temperatures (Table 2).





**Figure 1** Classification of clarifying agents.

**Table 1** Comparison of various clarifying agents in terms of desirable effects and potential problems in their decreasing order of activity or effectiveness

<i>Desirable effects and potential problems for various fining agents</i>					
<i>Clarity and stability</i>	<i>Tannin reduction</i>	<i>Color reduction</i>	<i>Volume of lees formed</i>	<i>Potential for overfining</i>	<i>Quality impairment</i>
Chitosan	Gelatin	Carbon	Bentonite	Gelatin	Carbon
Bentonite	Albumen	Gelatin	Gelatin	Albumen	Bentonite
Ferrocyanide	Isinglass	Casein	Casein	Isinglass	Casein
Carbon	Casein	Albumen	Albumen	Casein	Gelatin
Isinglass	Bentonite	Isinglass	Isinglass	Ferrocyanide	Albumen
Casein	Carbon	Bentonite	Ferrocyanide		Isinglass
Gelatin	Ferrocyanide	Ferrocyanide	Carbon		Ferrocyanide
Albumen					Chitosan

Modified from Zoecklein, B.W., Fugelsang, K.C., Gump, B.H., Nury, F.S., 1999. Fining and fining agents. In: Wine Analysis and Production. Springer, Boston, MA, pp. 242–271.

In general, the time required to obtain a clear juice is inversely proportional to the concentration of enzyme used at a constant temperature (Kilara, 1982). At the lowest temperature, the clarity of banana juice was found to increase rapidly at the beginning of the reaction but slowed as the reaction progressed. The temperature increases the rate of enzymatic reactions, hence the rate of clarification, as long as the temperature is below the denaturation temperature of the enzyme. A similar behavior for the clarity was observed for the changes in incubation time in case of banana juice (Lee et al., 2006). The clarity of centrifuged litchi juice increased with an increase in enzyme concentration. Among the different concentrations used for the optimization of pectinase, the litchi pulp added with 500 ppm of pectinase resulted in maximum transmittance of 80% at 660 nm. The clarity of mosambi juice decreases with time up to 90 min and increases thereafter. Similarly at constant time and temperature, the clarity decreases with enzyme concentration and remains constant and increases thereafter. From both the observations, it is evident that there exists an optimum enzyme concentration and time for the juice clarity (Rai et al., 2003).

#### **Effect of the Enzymes on Viscosity of Juice During Clarification Process**

The use of enzymes leads to the drop of fruit juice viscosity and disintegrating the jelly structure and making it easier to obtain the fruit juices (Singh et al., 2012). The effect of different enzymes on viscosity of different juices is presented in Table 3. The high concentration of pectin leads to colloid formation, which constitutes one of the main problems during the processing of clear fruit juices (Sulaiman et al., 1998). The depectinisation of fruit juices through the use of pectinases has been presented as an efficient



**Table 2** Effect of pectinase enzyme on clarity of different fruit juices

Fruit	Incubation time <sup>a</sup>	Incubation temperature <sup>b</sup>	Enzyme concentration <sup>c</sup>	Clarity <sup>d</sup>	References
Banana ( <i>Musa sapientum</i> cv. Berangan)	80	43.2	0.084%	0.009 Abs	Lee et al. (2006)
Carambola ( <i>Averrhoa carambola</i> L.)	20	30	0.10%	0.019 Abs	Abdullah et al. (2007)
White grape ( <i>Vitis vinifera</i> )	30	27–30	0.048%	0.031 Abs	Sreenath and Santhanam (1992)
Sapodilla ( <i>Achras sapota</i> )	120	40	0.1%	0.023 Abs	Sin et al. (2006)
Mosambi ( <i>Citrus sinensis</i> (L.) Osbeck)	99.27	41.89	0.0004 w/v%	83.97% T	Rai et al. (2003)
Lichi ( <i>Litchi chinensis</i> L.)	120	40	500 ppm	80% T	Vijayanand et al. (2010)

<sup>a</sup>Incubation time in minutes.<sup>b</sup>Incubation temperature in °C.<sup>c</sup>Enzyme concentrations in footnote a w/v%: Weight per volume, ppm: parts per million, %: Percentage on pulp basis.<sup>d</sup>Clarity in Abs: Absorbance, T: Transmittance.

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**Table 3** Effect of Enzyme concentration with Incubation time and Temperature on Viscosity of Different Juices

Fruit/vegetable	Enzymes	Incubation time <sup>a</sup>	Incubation temperature <sup>b</sup>	Enzyme concentration <sup>c</sup>	Viscosity <sup>d</sup>	References
Bael ( <i>Aegle marmelos</i> (L.) Correa)	Pectinase	210	35	24 mg/100 gm	1.35	Singh et al. (2012)
Soursop ( <i>Annona muricata</i> L.)	Pectinase	180	35–40	0.05%	4.68	Yusof and Ibrahim (1994)
Apricot	Pectinase	240	40	2.5%	1.11	Joshi et al. (2011)
Pear	Pectinase	240	40	2.5%	1.17	Joshi et al. (2011)
Banana ( <i>Musa sapientum</i> cv. Berangan)	Pectinase	240	44	0.4%	14.2	Shahadam and Abdullah (1995)
White grape ( <i>Vitis vinifera</i> )	Pectinase	30	27–30	0.048%	1.05	Sreenath and Santhanam (1992)
Banana ( <i>M. sapientum</i> cv. Berangan)	Pectinase	80	43.2	0.084%	1.89	Lee et al. (2006)
Sapodilla ( <i>Achras sapota</i> )	Pectinase	120	40	0.1%	1.37	Sin et al. (2006)
Carambola ( <i>Averrhoa carambola</i> L.)	Pectinase	20	30	0.1%	1.33	Abdullah et al. (2007)
Kiwi ( <i>Actinidia deliciosa</i> )	Pectinase, amylase, and cellulase	120	50	0.06, 0.025, and 0.025 g kg <sup>-1</sup> , respectively	5.43	Vaidya et al. (2009)
Date (Variety <i>Birhi</i> and <i>safrî</i> )	Pectinase and cellulase	60–300	40	1% (1:1)	17.6 ( <i>Birhi</i> ) and 14.8 ( <i>safrî</i> )	Al-Hooti et al. (2002)
Carrots ( <i>Daucus carota</i> )	Pectinase and cellulase	30	50	2% (3:2)	2.75	Anastasakis et al. (1987)

<sup>a</sup>Incubation time in minutes.<sup>b</sup>Incubation temperature in °C.<sup>c</sup>Enzyme concentrations in %: Percentage on pulp basis, mg/100 gm: milligram per 100 g of fruit/pulp.<sup>d</sup>Viscosity in cps: centipoises.

Sharma, H.P., Patel, H., Sugandha, 2017. Enzymatic added extraction and clarification of fruit juices—A review. Crit. Rev. Food Sci. Nutr. 57 (6), 1215–1227.

alternative to reduce turbidity, in many studies (Kashyap et al., 2001; Landbo et al., 2007). Pectinases degrade pectin, hence resulting in a reduction in viscosity and cluster formation, which facilitates separation through centrifugation or filtration. As a result, the juice presents higher clarity, as well as more concentrated flavor and color (Abdullah et al., 2007; Kaur et al., 2004). Pectinase enzymes used in grape juice macerate increased the juice clarity and filterability by 100% according to Brown and Ough (1981). For clarified fruit juices, a juice that has an unstable cloud or whose turbidity is considered “muddy” is unacceptable to be marketed as clear juices (Viquez et al., 1981).

## Proteins as Clarifying Agents

All proteins have an isoelectric point, which is a pH at which they carry no net charge when they are in solution. Most of the proteins are least soluble at their isoelectric point. For instance, casein precipitates when it attains neutrality under acidic conditions. However, certain proteins such as ovalbumin form stable solutions when they attain their neutrality (Hornsey, 2007). Attraction of positively charged proteins such as gelatin, isinglass, and casein toward negatively charged materials forms the basis for clarification. Proteins have a high affinity for polyphenols and interact with phenolic compounds by hydrogen bonding between the phenolic hydroxyl and the carbonyl oxygen of the peptide bond (Zoecklein et al., 1995).

### Gelatin

Gelatin is prepared from hydrolysis of collagen from skin and bones of animals. Collagen can either be acid treated (Type A gelatin) or alkali treated (Type B gelatin). Enzymatic hydrolysis of collagen is also one of the methods to obtain gelatin. In food industries, for many years, gelatin has been used as a clarifying agent, stabilizing agent, and as a protective coating material (Salgues and Bidan, 1984; Adler-Nissen, 1985).

During juice and beverage processing, gelatin aids clarification by forming flocs in the liquids. The isoelectric point of gelatin is pH 4.7 and hence carries positive charge in juice or wine and thereby attracts the negatively charged phenolic substances and suspended particles, which then causes neutralization and settling of heavy flocs. These heavy flocs are later removed by sedimentation, centrifugation, or any other filtration technique. To retain a certain level of astringency in the beverages, little quantities of tannins should be added by the manufacturers before addition of gelatin. The concentration of gelatin to be added plays a crucial role in successful clarification of beverages, as the excess or insufficient amounts of gelatin can lead to the problems such as overgluing, undesirable color loss, or retention of haze. Different grades of gelatin are available in both solid as well as liquid forms. Commercially, gelatin is rated depending on its purity and ability to absorb water "bloom". A bloom number of 75 or 100 is suitable for wines, and the usage levels for fining of wines usually range from 0.2 to 0.8 lb (1000 gallons)<sup>-1</sup> (or 0.024–0.096 g L<sup>-1</sup>). However, much larger doses, up to 4 lb (1000 gallons)<sup>-1</sup> (or 0.48 g L<sup>-1</sup>), are required for juice fining (Morris and Main, 1995). The positive aspect of using gelatin is that it can be used in additive-free products, as it is categorized as an ingredient and not an additive.

### Isinglass

Isinglass, protein fining agent, is produced from sturgeon collagen (Boulton et al., 1996). It is available in sheets or flocculated forms. It is beneficial to work with flocculated form of isinglass as it does not require rinsing to remove fishy odors. For efficient clarification, the triple helical structure of isinglass plays a vital role (Jackson, 2000). Keeping in mind, the denaturation temperature of this structure (29°C), maintaining low temperatures during its preparation, is very important (Boulton et al., 1996). However, if hot water is used for preparation, it undergoes partial hydrolysis and thereby resulting in the formation of smaller molecules. The molecular weight is reduced from 140 000 to 15–58 000, resulting in the differences in fining characteristics and a product that is more gelatinlike in its activity (Rankine, 1984).

Isinglass is principally used in white still and sparkling wines for improvement in clarity, cleaning up the aroma, and modifying the finish to give brilliant and softer wines without significantly modifying tannin levels with typical usage levels in the range of 0.2–0.5 lb (1000 gallons)<sup>-1</sup> (or 0.024–0.06 g L<sup>-1</sup>) (Morris and Main, 1995). With an isoelectric point 5.5, it carries a net positive charge at the pH of the wine, which electrostatically attracts negatively charged particles (Rankine, 1984; Margalit, 2004; Troost, 1988).

Isinglass removes phenolics and tannins from beverages without significantly affecting color, astringency, and body than most other protein fining agents because it is less active toward condensed tannins than either gelatin or casein (Rankine, 1984). Use of isinglass is most suitable for obtaining brilliant, softer wine, and it also reinforces the yellow color in case of white wines (Ribéreau-Gayon et al., 2006). Other advantages over gelatin include the enhanced clarification at lower concentrations with lesser dependability on temperature (Ribéreau-Gayon et al., 1972).

Despite its advantages, there are certain issues with the use of isinglass. Many people wish to avoid drinking alcoholic beverages that have been clarified with isinglass because of the specific allergic reactions toward fish and fish products, and therefore, alternatives to isinglass are sought for (Walker et al., 2007). Also, it produces excessive lees (sediments that form during and after fermentation, which may include grape cell remains, seeds, dead yeasts and bacteria, precipitated tannins, and tartarate salts), and when the contact period of isinglass with wine increases, it tends to impart objectionable fishy odor (Zoecklein et al., 1999; Zoecklein, 2002).

### Casein

Casein is a heterogeneous group of four phosphoproteins and phosphoglycoproteins ( $\alpha_1$ -casein,  $\alpha_2$ -casein,  $\kappa$ -casein, and  $\beta$ -casein) with molecular weight ranging from 11.6 to 24.1 kDa and an average isoelectric point of 4.6 (Evans, 1982; Fox and Mulvihill, 1982). This protein is characterized by sequences of hydrophobic and hydrophilic amino acids, resulting in an amphiphilic character with micelle-forming properties (Weber et al., 2007). Being an insoluble protein at 4–5 pH, it is prepared and added to wines in the form of alkaline solution (O'Neal et al., 1950; Boulton et al., 1996). Caseinates are readily soluble in water and hence wine-makers generally prefer potassium or sodium caseinate. However, when it is used in the form of sodium caseinate, it results in the wine with increased contents of sodium. With an isoelectric pH of 4.6, casein carries a positive charge, which causes flocculation in acidic media such as wine. It adsorbs and mechanically removes suspended materials as it settles (Marchal et al., 2002b).

Caseins are most commonly used to reduce haziness and tannin concentrations. Also, it is used to remove undesirable odors and is sometimes used as a substitute for carbon for color modification of juice and white wines. The usage levels for wines typically ranges from 0.2 to 2 b (1000 gallons)<sup>-1</sup> (or 0.024–0.24 g L<sup>-1</sup>) (Morris and Main, 1995).

Caseins are not as effective as gelatin or bentonite for stabilization of wines. Greater stability was reported by Cruess (1963) for Muscatel and sherry wines fined with bentonite as compared with those fined with casein. But, if caseins are used along with other fining agents, it can improve the quality of beverage. For example, Dambrouck et al. (2005) reported that casein assisted in producing better quality of champagne than using bentonite alone. Manufacturers should also consider allergic problems faced by some consumers toward casein fractions.

### Egg Albumen

Egg albumen is one of the oldest fining agents used for reducing the harshness of red wines. Peynaud (1984) reported that about 12.5% (w/w) protein can be found in fresh egg whites. The principal proteins in egg white are albumen (water soluble) and globular proteins (soluble in neutral dilute salt solutions). To aid solubility of egg white, addition of small amounts of sodium chloride is a common practice.

Albumen is primarily used to remove excess tannins and thereby reducing astringency in wines. Studies have been conducted on the ability of albumen clarify wines and its impact on finished quality (Meunier, 2003; Bonerz et al., 2004; Castillo-Sánchez et al., 2006). Having an isoelectric point between 4.55 and 4.90 (Boulton et al., 1996), albumen carries positive charge that attracts negatively charged tannins. Egg albumen is suitable for red wines but not for white wines, as high levels of tannins are required for flocculation. Usage level for wines varies from 1 to 8 egg whites per 60 gallon barrel, with an average of 2–4 (Morris and Main, 1995). However, care has to be taken to avoid excessive frothing and warming while addition to avail better dissolution in wines.

### Other Proteins

Increasing consumer concerns about possible allergies from animal-based proteins, stricter rules imposed by regulatory agencies altogether with the risk of bovine spongiform encephalopathy has prompted manufacturers and researchers to look for the alternatives to animal-based proteins for the purpose of clarification. In view of this, plant- and vegetable-based fining agents as alternative to animal-based proteins have started gaining attention from many researchers. Potatoes and peas have already been accepted and allowed in some winemaking regions (Iturmendi et al., 2013). Marchal et al. (2002a) showed very good clarification of Burgundy wine with the help of gluten proteins. Zeins as potential clarifying agents for red wine have also been reported (Simonato et al., 2013). Wheat gluten and isolates from pea, lentil, and soybean, used as fining agents, have shown to effectively reduce turbidity in white wine (Granato et al., 2014).

## Polysaccharides as Clarifying Agents

Many plant- and seaweed-based polysaccharides function as stabilizers, gelling agents, and viscosity modifiers in a wide range of food products. Polysaccharides such as alginates, carrageenan, and other seaweed-based extracts attain negative charge at lower pH and hence can bind and precipitate positively charged proteins from aqueous solutions (Cabello-Pasini et al., 2005). This forms the basis for clarification of juices and other beverages.

### Alginate

The most abundant polysaccharide which is extracted from brown seaweed is alginate. On dry basis, alginate comprises about 18%–40% of the biomass in brown algae (Moe et al., 1995; Whistler and BeMiller, 1997). It is present in seaweed as a mixed salt of sodium, potassium, calcium, and magnesium (Draget, 2009). *Ascophyllum nodosum*, *Durvillaea antarctica*, *Durvillaea potatorum*, *Laminaria digitata*, *Laminaria hyperborea*, *Laminaria saccharina*, *Laminaria japonica*, *Ecklonia maxima*, *Macrocystis pyrifera*, *Lessonia nigrescens*, and *Lessonia trabeculata* are some of the common species of algae, which are cultivated to produce alginate (McHugh, 2003; Helgerud et al., 2009). In its structure, alginic acid contains three kinds of polymer segments, viz., D-mannuronic acid units, L-guluronic acid units, and alternating D-mannuronic acid and L-guluronic acid residues (Black, 1950; Hoppe and Schmid, 1962). The proportion of these polymer segments also varies depending on the type of brown algae used.

Alginic acid is insoluble, but sodium, potassium, or ammonium salts are readily soluble and hence are preferred for most food and industrial uses. Among the most widely used compound is the sodium alginate, referred as algin. Alginates have found numerous applications in the food industry, and it can act as thickening, stabilizing, emulsifying, suspending, film-forming, or gel-forming agents without affecting the flavors. Gibsen and Rothe (1955) have reported many such applications of alginate. Apart from these applications, alginates can also be used during juice processing (Moncrieff, 1953), beer (Steiner, 1953), and winemaking (Kulzhinskaya, 1951) in the form of clarifying agent.

### Other Polysaccharides

Other polysaccharide-based clarifying agents may include gum arabic (Vivas et al., 2001), guar gum (Wang et al., 2010), carob gum (Cairns et al., 1986), xanthan gum (Erkan-Koç et al., 2015), carboxymethylcellulose (Genovese and Lozano, 2001), agar-agar (Rehman et al., 2014), gellan, λ-carrageenan, and xanthan (Ghafoor et al., 2008), and many others.

## Synthetic Polymers as Clarifying Agents

### Polyvinylpolypyrrolidone

PVPP is a synthetic, high-molecular-weight clarifying agent made up of cross-linked monomer of polyvinylpyrrolidone. PVPP has long been used in the beverage industry as a polyphenol adsorbent (Siebert and Lynn, 1997, 1998). Although, it has been called as a “protein-like” fining agent, insoluble PVPP interacts with only few reactive groups. Hence, PVPP is used for binding and removing smaller phenolic compounds such as catechins and anthocyanins, which are responsible for causing browning and bitterness in wines. However, PVPP, along with charcoal and casein, can remove resveratrol, a component that imparts certain health benefits (Castellari et al., 1998). Typical usage level of PVPP for wines ranges from 1 to 6 lb (1000 gallons)<sup>-1</sup> (or 0.12–0.72 g L<sup>-1</sup>) (Morris and Main, 1995).

### Nylon

Nylon is a synthetic fiber that comprises of repeating polymer units linked by peptide bonds. It is mainly used for clarification of wines, and its use is legal in many countries. On its surface, nylon has carbonyl functional groups, which can adsorb phenols and similar compounds. Successful reduction in color of white table wines and slowed browning with very little effect on the quality of wine has been reported by Fuller and Berg (1965). However, it is less preferred to PVPP because of its lesser efficiency of adsorption (Caputi and Peterson, 1965; Fuller and Berg, 1965; Rossi and Singleton, 1966; Boulton et al., 1996).

## Tannins as Clarifying Agents

For clarification, tannins made from oak wood or grape pomace are used (Zoecklein et al., 1995; Jackson, 2000; Lee and Noble, 2003). In solution, tannin is negatively charged and forms complex with positively charged gelatin to form flocs and thereby cause clarification. When compared with bentonite, tannins are less popular because of its lower adsorptive capacity (Ribéreau-Gayon et al., 2006). Tannin is also used for adjusting astringency in juices and wines.

## Bentonite as a Clarifying Agent

Bentonite is arguably the most frequently used fining agent. It is volcanic clay discovered in Wyoming. When hydrated, it can grow 20 times its size. Bentonite must be hydrated before being added to the wine or fruit juices, otherwise it would just sink to the bottom and absorb all parts of the wine and not just the particles intended. After hydration, bentonite is able to attract positively charged particles because it has a negative charge itself.

When a 5% (w/v) suspension of bentonite is made up with warm water, a pale green–gray milky liquid results if the concentration of bentonite reaches 10%–15% (w/v), forming a gelatinous paste. These thicker slurries will form a gel when left to stand but will liquefy on agitation, i.e., they are thixotropic. This is due to the presence of positive charges on the edges of the plates, which are electrostatically drawn to the negatively charged faces of adjacent plates and thus form a contact at an angle. This means that although bentonite primarily acts by an adsorptive interaction between the flat, negatively charged platelet surfaces and positively charged proteins, a certain amount of binding with negatively charged wine constituents may occur. Bentonite is now widely used for the adsorption of proteinaceous material from wines but was originally used for clarifying vinegar.

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## Dietary Fiber (Psyllium, $\beta$ -Glucan)

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### Overview

$\beta$ -glucans and psyllium are recognized as functional and bioactive ingredients and they are included in the complex dietary fiber group.  $\beta$ -glucans are found in bacteria, edible mushrooms, yeast and grains, such as oat, barley, rye, and wheat (Mittmesser and Combs, 2017). It can be purchased like a food additive (VITACEL<sup>®</sup> Oat Fibre, Germany) or it can be extracted from above-mentioned sources for scientific studies. Psyllium is a term used to designate both plantago plants and a mucilaginous material prepared from seed husks of *Plantago* genus including *Plantago ovata*, *Plantago psyllium*, *Plantago indica*, etc., which grow in certain temperate and subtropical regions (Yu et al., 2008; Cui and Roberts, 2009). It has a long history of use as a dietary fiber supplement to promote the regulation of large bowel function, and the reduction of blood cholesterol levels (Fischer et al., 2004). It can be acquired alone (as particles or powder form, Frontier, USA) or accompanied by other additives like sucrose (Metamucil<sup>™</sup>, USA). Nowadays,  $\beta$ -glucans and psyllium are employed by the food industry and are studied by the world research community in several fields of science (chemistry, food science, nutrition, medicine, materials science, microbiology, etc.).

The basic structures of  $\beta$ -glucans and psyllium are known from past decades. However, small variations, continue to be reported year after year. From a functional point of view,  $\beta$ -glucans and psyllium are soluble in water and they are form gels. Accordingly, they could classify as viscous soluble fibers (Dello Staffolo et al., 2012). Jenkins et al. (1986) mentioned the term viscous soluble fiber in their works about fibers and health benefits. Then, several researchers advance in this topic (McRorie and McKeown, 2017).  $\beta$ -glucans and psyllium differ in that the first is a single chemical substance, while psyllium is a mixture of chemical substances since it is the yield of seed milling.

$\beta$ -glucan and psyllium have been utilized in food industry owing to their health benefits and technological properties. This is consistent with the global trend in creating healthy foods, also called functional foods, to incorporate into the diet of consumers who are concerned about reducing diseases and/or improving their life quality.

### Chemical Structure and Molecular Characteristics

Cereal  $\beta$ -glucan is a polysaccharide that occurs in the subaleurone and endosperm cell walls of cereals seeds, including oats, barley, rye, and wheat. The level of  $\beta$ -glucans in cereals varied between species, as shown in Table 1. Cereal  $\beta$ -glucans are linear homopolymers of glucose bonded by  $\beta$ -(1  $\rightarrow$  4) and  $\beta$ -(1  $\rightarrow$  3) glucosidic linkages. About 90% of the glucose units are arranged in blocks of two or three consecutive (1  $\rightarrow$  4)-linked units separated by a single (1  $\rightarrow$  3)-linkage, which forms the two building blocks of cereal  $\beta$ -glucans: a cellotriosyl (DP3) unit and a cellotetraosyl (DP4) unit, as shown in Fig. 1. The (1  $\rightarrow$  3)-linkages act as kinks to disrupt the regularity of the (1  $\rightarrow$  4)-linkages (Nie et al., 2018a). The remaining less than 10% of the polymer chain is mainly composed of longer cellulosic sequences ranging from 5 up to 14  $\beta$ -D-Glcp residues (Fig. 2).

The DP3/DP4 ratio (the fingerprint of each grain) in oat  $\beta$ -glucan is typically 2:1; in barley and wheat are 3:1 and 4:1, respectively. However, small variations in the trisaccharide–tetrasaccharide ratio has been reported within the same species, which may arise from several factors including differences between species, growing-conditions, extraction-conditions, and analytical methods. Some studies have suggested the presence of amino-acid residues and inner C-6 carbon-bound phosphomonoesters in the oat  $\beta$ -glucan molecule although, these additional structures have not always been observed (Wang and Ellis, 2014; Comino et al., 2016).

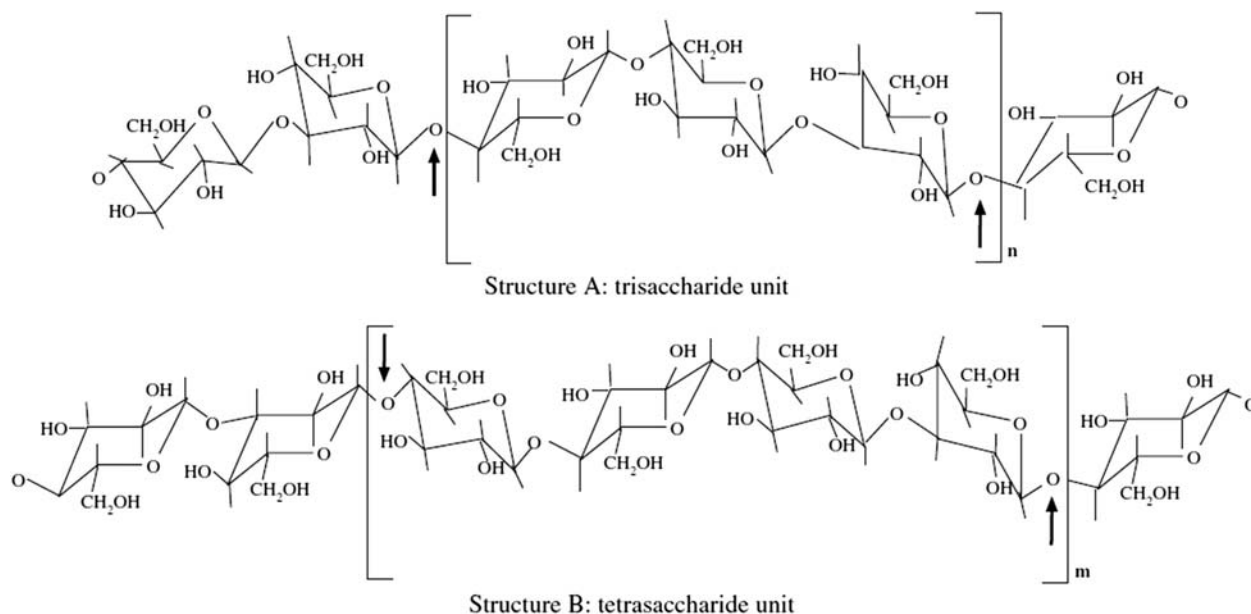
Cereal  $\beta$ -glucans in aqueous solution adopt a disordered random coil conformation with an expanded, semi-flexible chain conformation whose shape fluctuates continually under Brownian motion. In addition, there is molecular aggregation among  $\beta$ -glucan molecules through hydrogen bonding, which imparts great stiffness (thus high solution viscosity) to the polymer. These aggregates could be effectively eliminated in 0.5 M sodium hydroxide, but not with other treatments (6 M urea or ultrasonic treatment). The success of obtaining an aggregate-free solution allowed the measurement of true molecular weight and conformational

**Table 1** Amount (w/w) of  $\beta$ -glucans in four common cereals

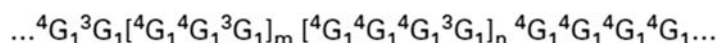
Cereal	$\beta$ -Glucan content
Barley	3%–11%
Oat	3.2%–6.8%
Rye	1%–2%
Wheat	0.5%–1%

From Cui and Roberts (2009).





**Figure 1** Building blocks (trisaccharides and tetrasaccharides) of cereal  $\beta$ -glucans. From Cui and Roberts (2009).



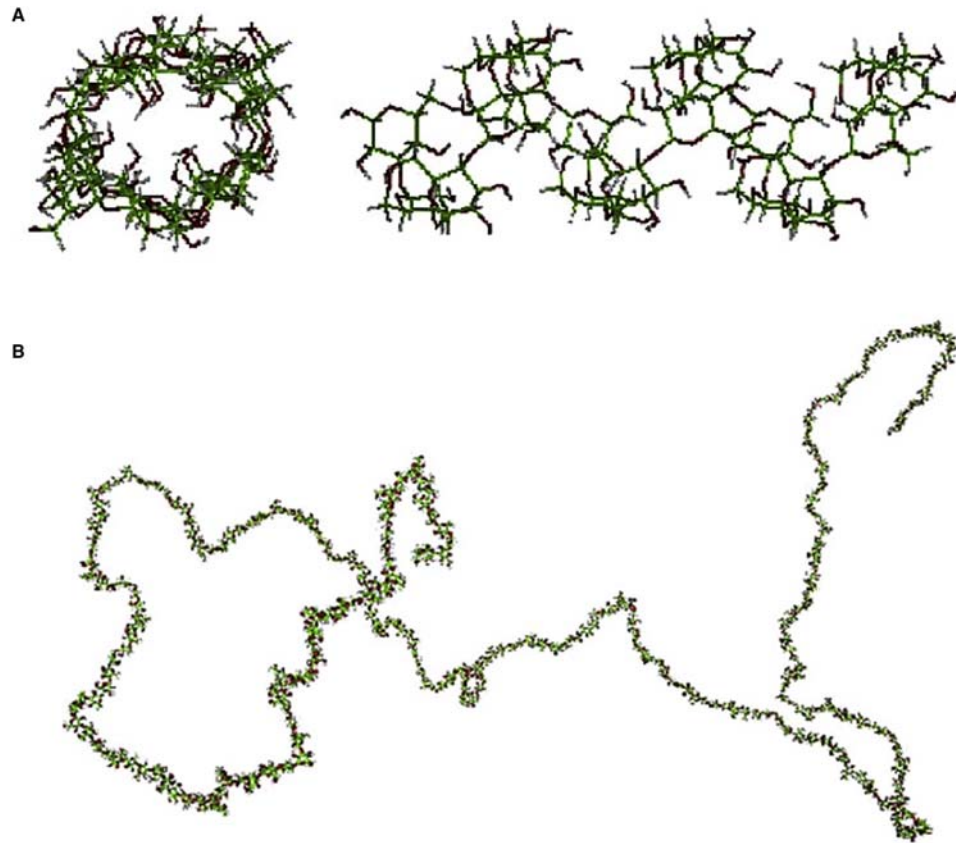
**Figure 2** Chemical structure of oat  $\beta$ -glucan. G represents a glucose unit and the numbers indicate the linkage sites; the ratio of  $m/n$  is approximately 2. From Wang and Ellis (2014).

properties of single molecules (Li, 2007; Nie et al., 2018a). The aggregation behavior was also confirmed by NMR relaxation techniques (Wu et al., 2017). The oat  $\beta$ -glucan had an average molecular weight of  $2.0 \times 10^3$  kDa whereas barley  $\beta$ -glucan had  $1.79 \times 10^3$  kDa average molecular weight (Shah et al., 2017). Even so, this value could vary to some extent owing to the complexity and polydispersity of natural polysaccharides (Nie et al., 2018a). Li et al. (2012) proposed that as molecular weight increased, the degree of aggregation decreased, due to the lower diffusion rate of large molecules. These authors also used computer modeling techniques to calculate those molecular parameters and visualize the shape of the random coil polysaccharide in aqueous solution. Fig. 3A illustrates the active site for the formation of junction zones between two  $\beta$ -D-glucan molecules. Fig. 3B shows the 3D molecular model with a moderately extended sinuous chain conformation. The conformational parameters of cereal  $\beta$ -glucans were calculated by RMMC simulation, which are in good agreement with experimental results and also revealed that chain stiffness increased with the tri/tetra ratio. Furthermore, the conformation of  $\beta$ -glucans could be modified by physical (ultrasonic, irradiation, microwave, extrusion, dough formation, baking, cooking/boiling) and chemical (carboxymethylated, sulfated and phosphorylated) methods (Comino et al., 2016; Honcu et al., 2016; Wang et al., 2017).

Psyllium material obtained by milling psyllium seed husks mainly contains hemicellulose, cellulose, and lignin (Dello Staffolo et al., 2011). In most of the *Plantago* species analyzed to date, it has been found that psyllium hemicellulose is a highly branched heteroxylan which consists of a xylan backbone with both (1 $\rightarrow$ 4) and (1 $\rightarrow$ 3) linkages and side chains attached to the O-2 or O-3 position (Fig. 4). Psyllium polysaccharide is composed of xylose (57%), arabinose (22%), 10%–15% uronic acid, and trace amounts of galactose, rhamnose, glucose, and mannose. However, these percentages fluctuate depending on *Plantago* genus species from polysaccharides were extracted as well as, the extraction method employed (Nie et al., 2018b). Although most psyllium polysaccharides found are heteroxylans, there are still some exceptions. Biringanine et al. (2012) described a *Plantago palmata* polysaccharide that presents an unbranched galacturonan domain.

The different extraction techniques (water or alkaline) produce several heterogeneous fractions of psyllium polysaccharides: some fractions contain uronic acids while other fractions are neutral polysaccharides as noted by different research groups. The presence of soluble and gel fractions observed by Guo et al. (2009) is in agreement with the findings of Al Assaf et al. (2003) who fractionated psyllium polysaccharides into three fractions: a water-soluble fraction (Mw 1–3 million Dalton), a gel fraction (Mw 9–20 million Dalton), and a matrix fraction (insoluble). Edwards et al. (2003) reported molecular weights of  $2.2 \times 10^6$  for water extracted and  $1.6 \times 10^6$  for 0.1 M NaOH extracted psyllium polysaccharides using size exclusion chromatography coupled with multiangle laser light scattering. Comparing the data of Al-Assaf's group and Edward's group, it was found that the water-extractable polysaccharides were in the same order and the difference existed between the polysaccharides extracted from alkaline solutions (Cui et al., 2013).

Currently, most commercial products containing ground psyllium seeds husk utilize the *P. ovata* species. Saghir et al. (2008) studied the psyllium polysaccharide extracted from *Plantago ovata* and found it a main chain consisted of  $\beta$ -(1 $\rightarrow$ 4)-linked Xylp;



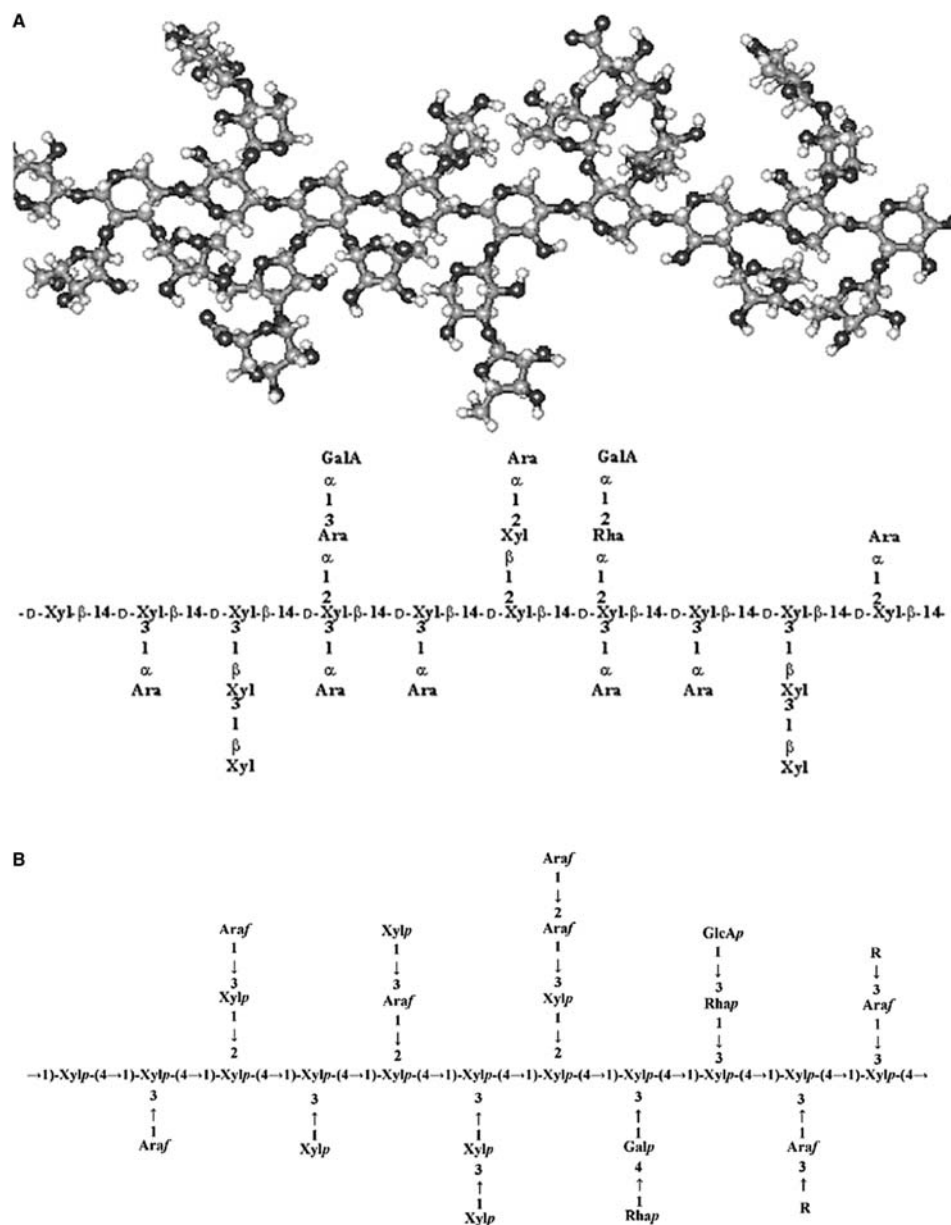
**Figure 3** (A) Side and top view of single helix structure of six consecutive cellotriosyl units. (B) Snapshot view of a  $\beta$ -D-glucan molecule in random coil shape (Li et al., 2012).

some Xylp residues carried a single Xylp moiety at its O-2, and other Xylp residues have trisaccharide branches at position 3 with a sequence of L-Araf- $\alpha$ -(1 $\rightarrow$ 3)-D-Xylp- $\beta$ -(1 $\rightarrow$ 3)-L-Araf.

Psyllium polysaccharides prepared from different species have in common the gelling and strong water-absorbing capacities. These features can be explained because psyllium gum is an anionic polysaccharide which could bear a negative charge due to ionized carboxyl groups. Therefore, the intermolecular electrostatic repulsion owing to homo-charges makes the polysaccharide chains fully extended and interpenetrated with each other to form intermolecular cross-linking which induces gelation (Farahnaky et al., 2010). Moreover, the swelling behavior could be a result of the entanglement of polysaccharide chains and development of inter- and intramolecular hydrogen bonds between the polysaccharide and water, which would cause more and more water to be trapped within the polysaccharide chains (Rao et al., 2011). It is noteworthy that psyllium polysaccharide is rich in calcium, which has a significant influence on its gelling properties (Yin et al., 2015).

## Functional Properties

Likewise, the chemical structure, molecular weight, and molecular weight distribution are fundamental  $\beta$ -glucans properties that regulate their overall functionality: solubility, hydration, conformation, and gelation in aqueous systems, which is relevant for the food industry and physiological behavior (Nie et al., 2018a). A higher (DP3/DP4) ratio causes reductions in solubility, promoting the gel-forming properties (Brennan and Cleary, 2005). High-molecular-weight oat  $\beta$ -glucan solutions exhibit typical viscoelastic flow behavior and do not form a gel within a reasonable time period. Freshly prepared barley and wheat  $\beta$ -glucan solutions are also viscoelastic fluids as demonstrated in Fig. 5 (Cui and Roberts, 2009).  $\beta$ -glucan gelation capacity is in the order of wheat > barley > oat, which is the same as the order of the tri/tetrasaccharides ratio of these cereals. The gelation rate of  $\beta$ -glucan solutions usually decreases with increased molecular  $\beta$ -glucan weight as shows Fig. 6 (Cui et al., 2013). Subsequent findings allowed researchers to propose the following hypothesis. The gelation rate and strength of cereal  $\beta$ -D-glucans are determined by a combination of the mobility of the polymer chains and the entanglement density of the “junction zone” of the polymer chain in solutions. An increase in the tri/tetra ratio in the  $\beta$ -D-glucans leads to a more rigid conformation in dilute solutions, and easier formation of gels at higher concentrations; smaller molecular weight  $\beta$ -D-glucans have higher mobility, which have more opportunity to interact with neighboring chains to form entanglements; this results in a higher degree of aggregation. The association of

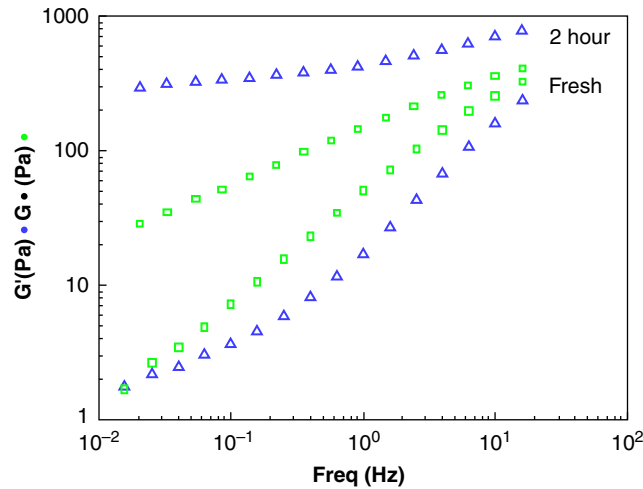


**Figure 4** (A) Proposed consensus psyllium structure extracted from ispaghula husk (*Plantago ovata*) (B) The possible structure of polysaccharide (PLP-2) from the seeds of *Plantago asiatica* L. Xylp: xylopyranose, Araf: arabinosefuranose, Galp: galactopyranose, Rhap: rhamnopyranose. R, may be T-linked GlcAp, 1,6-linked Glcp, 1,4-linked Glcp, 1,3,6-linked Glcp or 1,3,4-linked Galp. (A) From Edwards et al. (2003). (B) From Nie et al. (2018b).

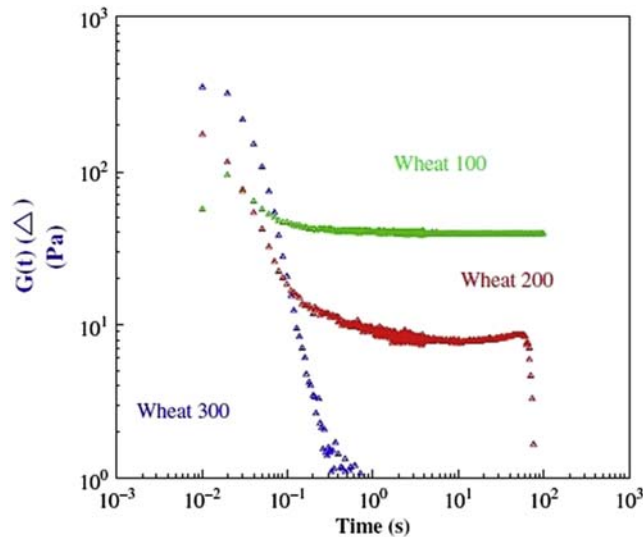
three-dimensional networks (entanglements) or the aggregates at higher concentrations leads to the formation of gel (Nie et al., 2018a).

Psyllium polysaccharide could be dissolved in strong ammonia solution and dimethyl sulfoxide. However, it is insoluble in acetone, alcohol, ether, chloroform dichloromethane, dimethylamine, trimethylamine, diethylether, and ethylacetate (Nie et al., 2018b). Besides, psyllium gum does not completely dissolve in water due to its high molecular weight. It forms a gel-like paste or dispersion when hydrated (Cui et al., 2013). Freshly prepared psyllium gum dispersion (1%) exhibits a Newtonian plateau at low shear rates and shear thinning flow behavior at higher shear rates. However, upon aging, psyllium gum dispersions form cohesive gels (Izydorczyk et al., 2005a,b). Psyllium gel is thermally stable, with  $G'$  and  $G''$  decreasing with increased temperature. No melting is observed unless the temperature exceeds 80 °C (Fig. 7), where a deeper decrease of  $G'$  was observed (Haque et al., 1993). It was also found that the psyllium gel exhibits an extensive swelling property in water and 0.1 M HCl solution, whereas no swelling was observed in 0.5 M NaOH solution (Rao et al., 2011).

Farahnaky et al. (2010) investigated the effect of concentration, temperature, and pH on the dynamic rheology of psyllium gels. The results showed that psyllium gel at 2%-3% concentration was a weak gel, since the dynamic storage modulus ( $G'$ ) was larger



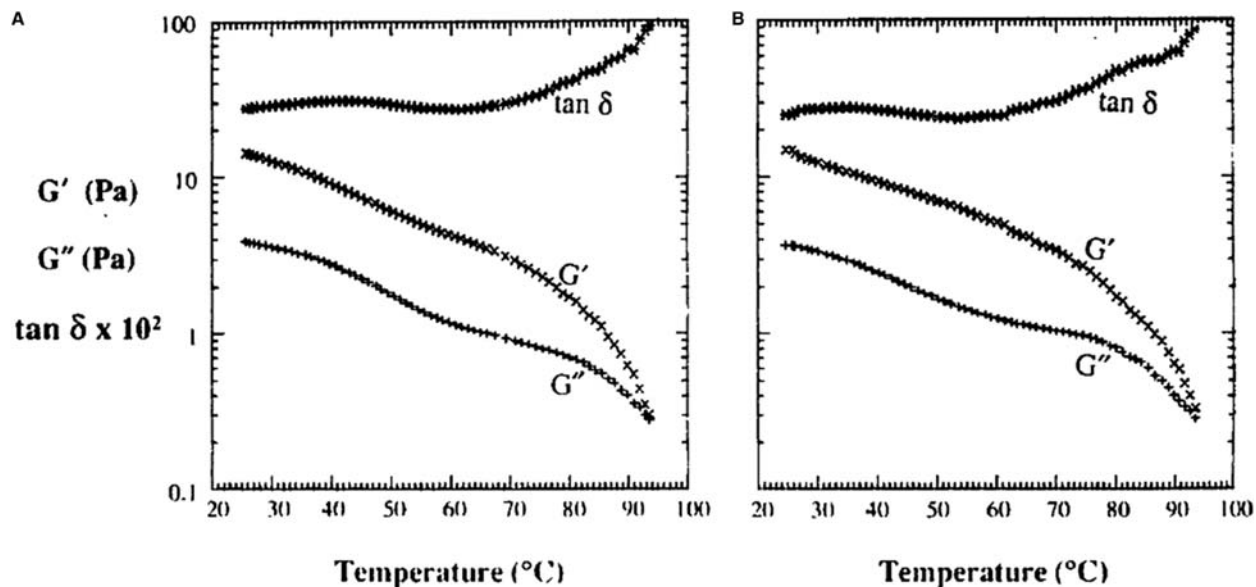
**Figure 5** Viscoelastic properties of wheat b-glucan: freshly prepared versus standing for 2 hours at room temperature (25 °C) ( $M_w = 3.4 \times 10^4$ , measured at 25 °C). From Cui and Roberts (2009).



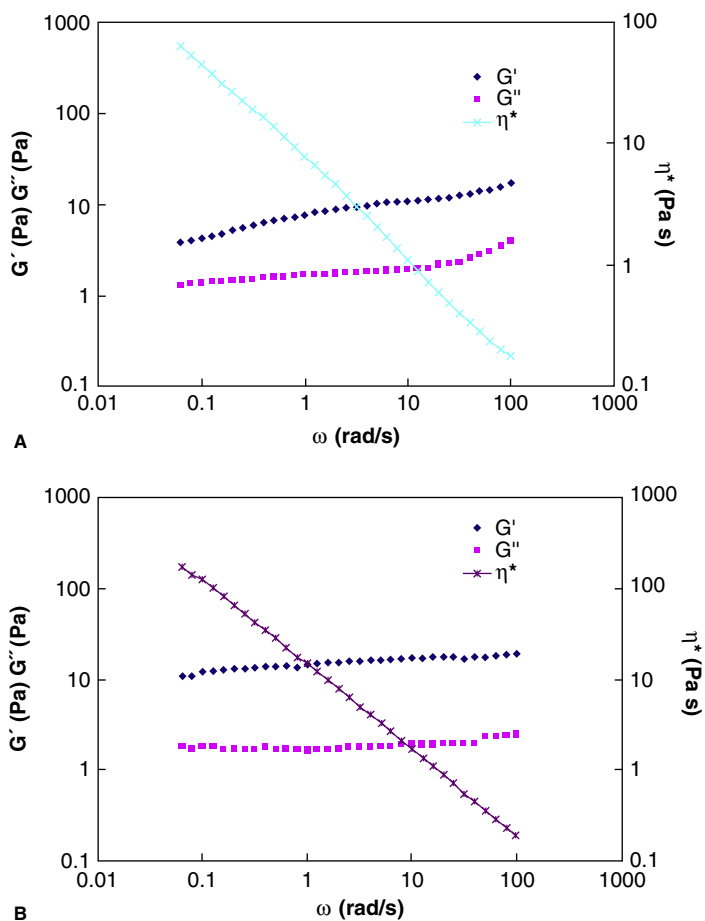
**Figure 6** Increase in gelation rate corresponding to decrease in molecular weight. From Cui and Roberts (2009).

than the viscous modulus ( $G''$ ), and there was no obvious melting point found for psyllium gel, indicating that the melting process was a continuous and long process. As for the pH value, the maximum functional properties of psyllium gels were observed at a pH of 4 and 7. It can be explained as net electrostatic repulsion decreases at low pH values, and the interaction of molecules also decreases, which leads to a reduction in gel elasticity. In addition, that the gel elasticity decreases in a higher pH value environment might be attributed to an alkaline depolymerization reaction, which results in fewer junction zones.

When  $\text{Ca}^{2+}$  is added to the gel, the gel becomes more resistant to temperature; meanwhile, its bioavailability is reduced (Cho et al., 2001). Guo et al. (2009) reported that when the psyllium polysaccharide solution was added to with 50 mM  $\text{Ca}^{2+}$ , the spectrum of  $G'$  and  $G''$  showed less frequency dependency, and lower  $\tan \delta$ , which meant the psyllium gel appeared more typical of a true gel in the presence of  $\text{Ca}^{2+}$ . On the contrary, by adding  $\text{Ca}^{2+}$ , the gel strength became much stronger and it was more tolerant of higher temperatures and the microstructure of the gel changed from fibril strand gel to aggregated gel (Fig. 8). Yin et al. (2015) treated psyllium gels with ethylenediaminetetraacetic acid (EDTA) to remove calcium ions. They found that its intrinsic viscosity decreased significantly; however, adding  $\text{Ca}^{2+}$  increased the apparent viscosity of psyllium gels increased considerably. In addition to modifying the rheological behavior of psyllium dispersions and gels, calcium binding also causes a decrease in its bioavailability and absorption in the digestive tract (Rodríguez et al., 2008). A reduction in the availability of glucose and iron has also been observed in chemical digestive models (Rodríguez et al., 2008; Dello Staffolo et al., 2011).



**Figure 7** Temperature dependence of  $G'$ ,  $G''$  and  $\tan$  for 1% (w/v) isabgol (from *Plantago ovata*) on (A) heating and (B) cooling at  $1 \text{ deg. min}^{-1}$ . Measurements were made at  $10 \text{ rad s}^{-1}$  and 2% strain. From Haque et al. (1993).



**Figure 8** Mechanical spectra (1% strain;  $25^{\circ}\text{C}$ ) showing the variation of  $G'$ ,  $G''$  and  $\eta^*$  with frequency ( $\omega$ ) of AEG (0.5%, w/v), (A) without  $\text{Ca}^{2+}$ , (B) with  $50 \text{ mM } \text{Ca}^{2+}$ . From Guo et al. (2009).



## Functionality and Health Promotion Relationship

$\beta$ -glucans and psyllium are viscous soluble fibers inducing thickening when mixed with liquids. An increase in the viscosity of food may have several effects, including slowing down the small intestinal transit time, reducing the interaction between food nutrients and digestive enzymes, and increasing the time for nutrient diffusion, by increasing the thickness of the unstirred water layer. A mechanism for viscous dietary fibers in appetite regulation has been reported. Viscous soluble fibers can absorb large quantities of water, and increase stomach distension, which may trigger afferent vagal signals of fullness. Besides, more time and effort are required to masticate foods rich in dietary fibers, and this allows more signals mediating satiety sensations to the brain. Viscous dietary fibers increase the viscosity of the digesta in the small intestine and therefore prolong small intestine transit time and absorption rate of nutrients. These, in turn, affect gastric emptying and signaling to the central nervous system (Brownlee, 2011; Nie et al., 2018b; McRorie and McKeown, 2017).

This mechanism that influences the absorption of nutrients in the first portion of the digestive system, generates effects related to health promotion and life quality improvement such as: regulation of glycaemia (FDA, 2017; Wood, 2011; Gibb et al., 2015), hypocholesterolemia (Wolever et al., 2011; Wei et al., 2009), hypolipemia (Choi et al., 2010; Rahbar et al., 2014), body weight decrease (Salas-Salvadó et al., 2008; Galisteo et al., 2010). Likewise, these effects have secondary consequences as decreased risk of cardiovascular disease (Code of Federal Regulations, 2010; FDA, 1998; McRae, 2017) and cancer prevention (Dahm et al., 2010; Kaczmarczyk et al., 2012; Sohn et al., 2012).  $\beta$ -glucan and Psyllium possess other abilities in relation with health benefits such as immune enhancement (Zhao et al., 2014) and antioxidant activity (Kofuji et al., 2012; Gong et al., 2015).

## Functional Foods

In recent years  $\beta$ -glucans as emulsion stabilizers, bulking agents, fat replacers, and healthy ingredients, have been incorporated and studied in the following food groups. For example, in bakery products have been developed two-layer flat bread (Izydorczyk et al., 2008), cookies and peanut spreads, muffins (Moriarty et al., 2011), and batter and sponge cake (Majzoobi et al., 2015). However, in yeast-leavened bread,  $\beta$ -glucan could produce high water retention, poor crumb texture, and loaf volume. These effects are dependent on the concentration of  $\beta$ -glucan in the bread as well as the molecular weight (Cui and Roberts, 2009). It has been also studied durum wheat pasta and noodles (Izydorczyk et al., 2005a,b). In this type of foods, slight differences in color have been observed as a disadvantage, in comparison with pasta without fiber (Quinde-Axtell et al., 2005). Among milk products, it has been developed: yogurts (Brennan and Tudorica, 2008), cheeses, and ice creams. Chicken meat patties and sausages (Huang et al., 2011) are examples of processed meats products. In beverages, Lyly et al. (2009) studied the perceived satiety with healthy volunteers. Currently, the best-selling beta glucan-containing foods are bread, cereals bars, breakfast cereals, snack foods, and oat and barley-based products, such as whole oats, oat bran, oat meal, barley flour, etc. However, from time to time other types of foods with beta glucans, as functional ingredients appear but, they do not achieve to stabilize in the market. An exception could be pasta including cereal bran, which can be found in the market with some discontinuity, many years ago.

Psyllium has been used for decades, in beverages (orange juice, apple, etc.) to which pharmaceutical formulas like Metamucil are added (Chan and Wypyszyk, 1988). Probably, the first use of Psyllium as a food additive was into confectionery and bakery products (Czuchajowska et al., 1992) through its ability to mimicking gluten network. Then, it is continued to work in this area (Zandonadi et al., 2009; Cappa et al., 2013; Raymundo et al., 2014; Fratelli et al., 2018). Among milk products, it has been studied: yogurts (Rodríguez et al., 2008; Ladjevardi et al., 2015; Yadav et al., 2016), dairy desserts (Dello Staffolo et al., 2017). Additionally, psyllium has been employed in confectionery jams (Figueroa and Genovese, 2018) as a stabilizer and a gel filling.

It is estimated from a western diet that the average daily consumption of arabinoxylan in the absence of fiber supplements ranges from 1–5 g/day. To obtain a beneficial effect on post-prandial glucose excursions, the conditions of use for the claim are that 8 g of arabinoxylan-rich fiber derived from wheat endosperm (which is at least 60% arabinoxylan by weight) per 100 g of available carbohydrates should be consumed per quantified portion as part of a meal. To achieve this recommended intake, consumption of fortified foods or arabinoxylan fiber supplements will be required (Kellow and Walker, 2018). In consequence, incorporating psyllium into food, especially at the level required to allow a health claim, is challenging due to its strong water-absorbing and gelling properties. In addition, an unpleasant slimy mouthfeel and undesirable flavor characteristics were reported for some foods containing psyllium. To promote the application of psyllium in foods, some researchers developed an enzyme treatment method to produce psyllium with improved sensory properties and reduced water-adsorbing capacity without altering its hypolipidemic capacity (Allen et al., 2004).

## Conclusions

$\beta$ -glucans and psyllium are viscous soluble fibers with the ability to form gels, which gives them several interesting properties for the food industry, life quality improvement, and treatment of certain diseases. Cereal  $\beta$ -glucans are linear homopolymers of glucose bonded by  $\beta$ -(1  $\rightarrow$  4) and  $\beta$ -(1  $\rightarrow$  3) glucosidic linkages with repeated building blocks of tri and tetrasaccharides. Psyllium hemicellulose is a highly branched heteroxylan which consists of a xylan backbone with both (1  $\rightarrow$  4) and (1  $\rightarrow$  3) linkages and side attached chains.  $\beta$ -glucans and psyllium promote health because they regulate glycemia, cholesterolemia, lipidemia, body weight and

decrease the risk of cardiovascular and cancer diseases. These effects have been well documented and they continue to study for both polysaccharides. A wide range of food products with  $\beta$ -glucans and psyllium have been developed and studied to satisfy functional foods demand by consumers and reach their recommended intakes. In this process, some sensory disadvantages have appeared in some foods that have begun to be solved but even today are the future challenge for food scientists and technologists with the aim of achieving the highest levels of acceptability by consumers.

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## Diglycerides

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### Diglyceride Structure

Diglycerides or diacylglycerols (DAG), are composed of two fatty acids esterified to the trihydric alcohol glycerol. Since glycerol has three hydroxyl groups available as binding sites, there are two possible structural isomers. A 1,2-DAG has fatty acids esterified to the *sn*-1 and *sn*-2 positions on the glycerol. A 1,3-DAG has fatty acids linked to the *sn*-1 and *sn*-3 positions. Acyl migration occurs within DAG molecules and results in an equilibrium with 1,3-DAG making up 60%–70% of the total concentration (Lo et al., 2008; Matsuo, 2004; Siew and Ng, 1999). This class of molecule is amphiphilic in nature because of the lipophilic fatty acids and the hydrophilic hydroxyl group. As a result, DAGs have surface active properties.

### Synthesis in Plants and Animals

DAGs can be produced in plant and animal cells as a by-product of triglyceride (TAG) biosynthesis (Siew and Ng, 1995). TAGs are synthesized by the addition of fatty acids to glycerol-3-phosphate with the help of fatty acid-CoA esters and acyltransferase enzyme complexes (Belitz et al., 2009). During this process DAGs are produced just prior to the synthesis of TAGs. Therefore, in the event that this process is not completed, a DAG may be formed. Additionally, the hydrolytic activity of lipase enzymes during maturation or harvesting of oil fruits and seeds as well as during the storage of an edible oil can lead to increased levels of DAGs. These enzymes hydrolyse the ester linkages within TAGs, producing DAGs and monoglycerides (MAGs) along with free fatty acids (Cheng et al., 2017). DAGs are typically found within edible oils at very low levels, but depending on the type of oil, method of extraction, and storage conditions they may be found at concentrations up to 10% (Lo et al., 2008; Matsuo, 2004). Edible oils originating from oil fruits, as opposed to oil seeds, are known to contain higher levels of DAGs because they are exposed to water for a longer time, meaning the TAGs are more prone to undergoing hydrolysis reactions (Cheng et al., 2017).

### Industrial Production

While there are many different ways through which DAGs can be formed, DAGs for commercial use are typically produced one of two ways. Either through a direct esterification reaction between glycerol and fatty acids, or by performing glycerolysis between glycerol and a fat or blend of fats. With both of these processes, the amount of glycerol in the reaction will determine the ratio of MAG/DAG/TAG produced. The production of DAGs can be optimized for a specific process by changing the amount of glycerol added as a reactant. A high concentration of glycerol will lead to an increase in the concentration of MAGs produced. Alternatively, performing these reactions with a low amount of glycerol results in a product high in TAGs. Furthermore, glycerolysis is a more cost-effective option because the cost of a fat or oil, whether it be natural or hydrogenated, is less than that of the fatty acids used in a direct esterification process. Additionally, a higher quantity of glycerol is required to perform a direct esterification. This increases the cost further and because lipids have a low solubility in glycerol, the reaction must be performed at a higher temperature (Hasenhuettl, 2008; Lo et al., 2008).

Both processes can be performed either chemically or enzymatically. Chemical esterification and glycerolysis reactions are often faster and more reproducible compared to the enzymatic processes. In addition, in terms of material cost, it is more cost effective to perform these reactions chemically, because the cost of enzymes greatly exceeds that of the chemical catalysts (Gibon and Kellens, 2014). However, with chemical processing, the reaction is run at high temperatures (>200 °C). Besides the energy costs associated with these high reaction temperatures, side reactions occur which lead to colour changes and the production of off-flavours. This decreases the overall quality of the oil, which must be improved through further processing steps (Hasenhuettl, 2008). Furthermore, the lipase enzymes used to catalyze these reactions typically have optimal temperatures in the range of 60–80 °C (Kristensen et al., 2005). Since the reaction temperatures are relatively low when using enzymes, the quality of the oil remains high. As a result, the high cost of enzymes and the lower reaction rates may be offset by the improved quality of the oil (Phuah et al., 2015). Therefore, the use of enzymes represents a very viable means of producing DAGs. In addition, with the push for clean label products in recent years, the use of a biocatalyst is an attractive alternative to chemical catalysts.

When performing enzymatic modifications on lipids, it is important to consider using immobilized enzymes, particularly, enzymes immobilized on a hydrophobic support. Immobilized enzymes are both easier to disperse in oil and easier to separate and recover compared to free lipases. The use of a hydrophilic support encourages the formation of a layer of glycerol surrounding the lipase particles. This creates a barrier, decreasing contact between the lipase and the lipids, reducing the performance of the enzyme. It is for these reasons that lipases immobilized on hydrophobic supports have been known to perform more effectively (Fregolente et al., 2008; Kristensen et al., 2005).

## Diglycerides as Emulsifiers

DAGs are frequently used in food products for emulsification purposes. They are typically found as a mixture of MAGs and DAGs (Kim and Akoh, 2015). In fact, MAGs and DAGs are some of the most commonly used emulsifiers in the food industry. The DAG molecule has only one hydroxyl group, whereas MAGs have two, and so DAGs are therefore more lipophilic compared to MAGs and thus have a lower HLB value. Because of these differences, MAGs are more surface active and are a more sought-after ingredient for emulsification purposes. However, the distillation process used to purify the MAGs, after a mixture of partial glycerides has been produced, adds to the ingredient cost. For this reason, a mixture of MAGs and DAGs is used more frequently. In many cases, a blend of emulsifiers with different HLB values will perform better than one single emulsifier as the emulsifiers work synergistically (ICI Americas Inc., 1976). Therefore, it is often beneficial for food systems to contain a blend of MAGs and DAGs besides simply as a means of reducing cost. While MAGs and DAGs may be found in a wide range of food products as an emulsifier, they are predominantly used in bakery products (i.e. breads and cakes), frozen desserts, and sauces/dressings (Nash and Brickman, 1972). This emulsifier is typically used to increase the volume and improve the crumb structure of baked goods and to extend their shelf life, by preventing staling. In sauces and dressings, this emulsifier acts to keep the water and oil phases from separating, thereby improving the appearance and shelf life.

In addition to DAGs being used directly as an emulsifier, these molecules can be esterified to organic acids, producing a wide range of emulsifiers with different HLB values that can be used for a variety of food applications. In most cases, it is again a mixture of MAGs and DAGs used to produce these esters and they are named accordingly. These include, diacetyl tartaric acid esters of mono- and diglycerides (DATEM), citric acid esters of mono- and diglycerides (CITREM), lactic acid esters of mono- and diglycerides (LACTEM), acetic acid esters of mono- and diglycerides (ACETEM), and tartaric acid esters of mono- and diglycerides (TATEM).

## Proposed Health Benefits

There are several well researched health benefits pertaining to the consumption of DAGs. These include reduced serum TAG levels and anti-obesity effects. Although there has been conflicting research on these health benefits, due mainly to differences in the levels of DAGs consumed and the condition of the test subjects, it is generally regarded that the consumption of DAGs in place of TAGs is beneficial (Phuah et al., 2015). Consumption of DAGs has been shown to reduce fat stores within the body, helping to reduce body mass index and waist circumference. Additionally, several animal studies have demonstrated increased rates of  $\beta$ -oxidation as well as increased activity of the enzymes involved in this process (Lo et al., 2008; Matsuo, 2004).

The energy value and digestibility of DAG oil has been shown to be similar to that of traditional TAG oil (Taguchi et al., 2001). It is therefore believed that the different metabolic fates of DAGs compared to TAGs bring about these positive health effects. The effects arise mainly from the 1,3-DAGs rather than the 1,2-DAGs. When TAGs are metabolized, two of the ester bonds are cleaved to form free fatty acids and 2-MAGs. These molecules are then able to pass through the intestinal wall where they are reassembled into TAGs based on a 2-MAG pathway. They are then packaged into chylomicrons for transport throughout the body. In the case of 1,2-DAGs, the ester bond at position *sn*-1 is cleaved to form 2-MAG and a free fatty acid. These components are therefore able to be used towards assembling TAGs for packaging into chylomicrons. 1,3-DAGs however, will be metabolized to form 1-MAGs and free fatty acids. The body is not efficient at assembling TAGs from 1-MAGs, and so following absorption in the gastrointestinal tract the components are transported to the liver for metabolism rather than being transported and stored throughout the body in adipose tissue. This difference in metabolic pathway between TAGs and 1,3-DAGs is believed to cause the reduced post-prandial serum TAG levels and the decreases in body fat stores observed when DAG oil is consumed in place of traditional TAG oils (Flickinger and Matsuo, 2003; Kim and Akoh, 2015; Lo et al., 2008; Maki et al., 2009; Matsuo, 2004; Teramoto et al., 2004).

## Diglyceride Oil

As a result of the health benefits associated with the consumption of DAGs, oils containing high levels of DAGs have been commercialized. KAO Corporations developed a cooking oil known as Econa oil in Japan and released it to the market in 1999 (Matsuo, 2004). This oil contained around 80% DAGs, compared to conventional cooking oils that contain less than 10%. Enova oil later became available in the United States after Kao Corporations partnered with Archer Daniels Midland (Lo et al., 2008). These products were found to contain high levels of glycidyl esters and were voluntarily removed from the market in 2009 (Gibon and Kellens, 2014).

Glycidyl fatty acid esters (GEs) are classified as a “possible human carcinogen” (group 2) by the International Agency for Research on Cancer because they are hydrolysed to form the carcinogen glycidol in the intestinal tract. GEs are formed because of the high temperatures of the deodorization step during the refining of oils. Furthermore, DAGs are known to be a precursor to GE formation. As a result, most edible oils contain some level of glycidyl esters. Furthermore, oils containing a DAG content of greater than 6% are known to have considerable amounts of glycidyl esters (Cheng et al., 2017).

## Crystallization of Lipids

Pure DAGs crystallize differently from pure TAGs. Additionally, the isomeric form of the DAG changes the crystallization behaviour. When considering DAGs and TAGs that contain the same fatty acids, the melting point of the 1,3-DAG is generally around 10 °C higher than that of the TAG. While the melting point of the 1,2-DAG is approximately 10 °C lower than that of the 1,3-DAG, meaning that it has a similar melting point to that of the TAG (Lo et al., 2008). In the case of TAGs and DAGs containing palmitic acid, 1,3-dipalmitin (1,3-PP) has a melting point of 72.5 °C, tripalmitin has a melting point of 65.5 °C, and 1,2-dipalmitin (1,2-PP) has a melting point of 64.0 °C (Belitz et al., 2009). Furthermore, the two isomeric forms of DAG crystallize in different polymorphic forms. 1,2-DAG can crystallize in either the  $\alpha$ -form or the  $\beta'$ -form, while 1,3-DAG can crystallize in two types of the  $\beta$ -form (Lo et al., 2008).

Besides simply crystallizing differently in the purified form compared to TAGs, small amounts of DAGs have an effect on the crystallization of TAGs in lipid mixtures. Palm oil is notorious for crystallizing slowly. This is believed to be caused by the high DAG content (typically 5%–8%). Siew and Ng (1999) investigated the effects of various DAGs containing palmitic and oleic fatty acids on the crystallization of purified palm oil TAGs. In this study, DAGs were first removed from the palm oil and specific DAGs were later added back to the purified palm oil TAGs at various concentrations (1, 2.5, 5, and 10%). It was found that many of the different types of DAGs increased the induction of crystallization period of the palm oil TAGs. Both isomeric forms of diolein (1,2-OO and 1,3-OO) along with 1,3-PO increased the induction period at all tested concentrations, while addition of 1,2-PO reduced the induction period. This reduction in the induction period was attributed to the ability of the asymmetrical 1,2-PO to form mixed crystals with the palm oil TAGs. Interestingly, at low concentrations, the increase in the induction period was amplified by the disaturated DAGs (1,3-dipalmitin and 1,2-dipalmitin), however, higher concentrations (5%) of 1,3-PP resulted in rapid crystal growth. It was suggested that this was caused by the difference in melting point between 1,3-PP and the TAGs. Upon crystallization, 1,3-PP provides a nucleation site for TAG crystal growth. Furthermore, palm DAGs slowed the rate of crystallization of the palm TAGs. When looking at the effects of the individual DAGs on the crystallization rate, it was observed that low concentrations (1%) of 1,3-PP greatly reduced the rate of crystallization, while higher concentrations (5%) increased the crystallization rate. 1,2-PP decreased the rate of crystallization only slightly when added at low concentrations, but at higher concentrations the rate of TAG crystallization was greatly reduced. Again 1,2-PO appeared to assist in the crystallization process, increasing the rate of crystallization, just as it had reduced the induction period.

Wright and Marangoni (2002) investigated the effects of milk fat DAGs on the crystallization of milk fat TAGs. DAGs were found to delay crystallization by both inhibiting nucleation and reducing the crystal growth rate. These effects also demonstrated a concentration dependence. It was suggested that DAGs primarily composed of the fatty acids that make up the high melting fraction of the milk fat have a greater effect on TAG crystallization because they would become incorporated into the solids portion and disrupt the TAG crystal network. Furthermore, DAGs that contain fatty acids differing from those of the high melting fraction TAGs also have an inhibitory effect on crystallization, however, they do not affect the nucleation because they do not become incorporated into the TAG crystal network in this stage. Additionally, with regards to the different isomers of DAGs containing palmitic and oleic fatty acids, it was determined that the 1,2-DAGs (1,2-PP, 1,2-OO, and 1P,2O) delayed milk fat TAG crystallization at 25 °C while mixtures of 1,2-DAGs and 1,3-DAGs enhanced the crystallization slightly.

Silva et al. (2014) studied the effects of DAGs on the crystallization of TAGs by adding dipalmitin, distearin, and diolein to pure tripalmitin, tristearin, and triolein. Each of the three DAGs delayed the crystallization of both the saturated TAGs, tripalmitin and tristearin. As a result, the crystallization temperature was reduced. The opposite effect was observed with the unsaturated TAG triolein. In this case, each of the DAGs accelerated the crystallization process, with distearin causing the largest rise in crystallization temperature. Furthermore, it was determined that both dipalmitin and distearin acted to stabilize the  $\alpha$  polymorphic form in both tripalmitin and tristearin, effectively delaying the transition to the  $\beta$ -form. Diolein, however, was seen to accelerate the  $\alpha$  to  $\beta$  polymorphic transition of both TAGs.

Because of their influence on the crystallization of TAGs, DAG addition represents a viable method of improving the quality and extending the shelf life of margarine. Hernqvist and Anjou (1983) demonstrated the effectiveness of DAGs at stabilizing the  $\beta'$  polymorph. In this study, the addition of 5% DAGs to margarines made with hydrogenated rapeseed and soybean oils delayed the  $\beta'$  to  $\beta$  polymorphic transition from four weeks to 44 weeks.

In summary, DAGs are a partial glycerol naturally present in edible oils, that can also be produced industrially in high concentrations. This class of lipid is widely used in food products as an emulsifier, and because of the well researched health benefits associated with DAGs, oils high in DAGs have been produced and sold as a healthy cooking oil in both Japan and the USA. DAGs have been shown to have either inhibitory or enhancing effects on TAG crystallization depending on the isomeric form of the DAGs and the fatty acid profile of both the DAGs and the TAGs. Therefore, there are some instances in which it is beneficial to remove DAGs in order to improve the physical properties of the oil and other cases in which adding DAGs to an oil may improve its crystallization behaviour.

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## Egg Proteins

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### Glossary

**Amphipathic** a chemical compound containing both hydrophobic/lipophilic and hydrophilic properties

**Apolipoproteins** proteins that specifically bind lipids to produce lipoproteins. Their major role is to carry lipids through the circulatory and lymphatic system

**Calcification** impregnation with calcium or calcium salts

**Glycation** formation of a covalent bond between a sugar molecule (such as fructose or glucose) to a protein or lipid molecule

**Isoelectric point** refers to the pH at which a specific molecule bears no net electrical charge

**Lectin** any protein or glycoprotein that is capable of binding to the sugar moieties of glycoproteins and glycolipids present on the surface of cells in most organisms. These proteins help in stimulating lymphocyte proliferation

**Polymorphism** refers to the branching of the genetic tree which is because of two or more alleles present at one DNA position or in one DNA region, each with significant frequency in the population

**Reactive oxygen species (ROS)** chemical species which contains oxygen and are chemically reactive, for instance, superoxide, peroxides, singlet oxygen

### Nomenclature

**TSAA** Total Sulfur Amino acids, i.e. (Methionine + Cystine)

**Quercetin** Plant polyphenol belonging to the flavonoid group and has a bitter taste. It is present in many vegetables, fruits, grains and leaves

## Introduction

The chicken eggs have always been recognized as an excellent source of human nutrition, especially for the dietary protein. The chemical composition of an egg has always fascinated human mind, may be due to the compartmentalized structure of the whole egg. However, only in recent years, with the help of innovative research tools, scientists have revealed the structural and functional diversity of the different components in eggs. These revelations diversify the use of eggs in various processed food products and increase the commercial value of an egg. Egg proteins are well-known for its functional properties and are massively used as an ingredient to enhance the texture or flavor of variety of food products. Additionally, egg proteins are also a potential source of bioactive proteins and peptides. The bioactive egg proteins and peptides can exhibit health beneficial effect above and beyond their known nutritional value. Research of last two decade has highlighted some bioactivity of egg proteins and broadened the use of egg as a critical ingredient of functional foods or nutraceuticals. Subsequently, egg proteins, especially proteins from egg white, are known as a major allergen affecting nearly 2.5% young children in the USA (Caubet and Wang, 2011). Therefore, this chapter briefly discusses the different proteins of egg and their functional and biological properties.

The egg consists of three major portions; a) eggshell, b) egg white, and c) egg yolk. Protein is one of the major component present in all three parts of the egg, and egg white is the prime source of proteins. The following section discusses the major proteins found in each part of the egg.

## Egg Shell Proteins

The eggshell is the outermost layer of an egg and is mainly composed of a foamy layer of cuticle, a calcium carbonate layer, and then two flexible membranes (inner and outer membrane). This whole structure together retains the egg white or albumen and egg yolk inside the egg and also prevent the invasion of any pathogenic bacteria (Burley and Vadehra, 1989).

The organic matter of chicken eggshell and eggshell membrane comprises of a complex mix of proteins and polysaccharides, out of which proteins constitute almost 70% of the total organic matter (Tullet, 1987). The eggshell membrane has been found to contain many bacteriolytic enzymes, such as N-acetylglucosaminidase and lysozyme, and other components which might have a role in preventing the invasion of Gram-negative and Gram-positive bacteria. Moreover, the eggshell membrane hydrolyses



have been found to contain hydroxyproline suggestive of the presence of collagen in the membrane layers (Nakano et al., 2003), the collagen constitutes almost 10% of the total proteinaceous matter in the eggshell.

Eggshell mainly comprises of various ubiquitous proteins which are extensively expressed in different organs. Osteopontin, a phosphorylated glycoprotein, is found in kidney, bones and many body secretions and it was found to be expressed in the uterine epithelial cells during the calcification of the eggshell (Pines et al., 1994). This protein is mainly confined to the mammillae, core of the non-mineralized shell membrane fibers, and the outermost segment of the shell palisade layer (Fernandez et al., 2003). Osteopontin inhibits the precipitation of calcium carbonate in the eggshell, and the protein loses its inhibitory activity upon dephosphorylation with alkaline phosphatase. This suggests that osteopontin might have a role as a modulator in the precipitation of calcium carbonate in the uterine fluid or even as an inhibitor during the termination of the calcification process (Hincke and St Maurice, 2000).

Clusterin is another ubiquitous eggshell secretory protein which is a heterodimeric glycoprotein bonded with disulfide linkages (Mann et al., 2003). This particular protein is found in many tissues and is also present in all the calcified regions of the eggshell. In the uterus, it is secreted by the tubular gland cells into the uterine fluid, irrespective of the shell calcification stage. Clusterin might have a role in preventing premature aggregation and precipitation of eggshell proteins by acting as an extracellular chaperone in the uterine fluid (Mann et al., 2003). There are specific proteins which are unique to the eggshell and are secreted only by the tissues present in the shell. These proteins have been identified in domestic hens only.

Ovocleidin-17 (OC-17) is a 142 amino acid long phosphorylated protein having a C-type lectin domain, and it was the first protein to be purified to homogeneity. This protein is also present as a 23 kDa glycosylated protein in a minor form (Mann, 1999). The tubular gland cells of the uterus secrete OC-17 throughout the calcified part of the shell during the entire calcification period (Hincke et al., 1995; Mann and Siedler, 1999; Reyes-Grajeda et al., 2004). OC-17 modifies the shape of calcium carbonate crystals in vitro (Reyes-Grajeda et al., 2004).

Ovocleidin-116 (OC-116) is an 80 kDa protein with 742 amino acids, and it was the first eggshell protein which was cloned (Hincke et al., 1999). The protein comprises of two disulfide bonds and two N-glycosylations (Mann et al., 2002). OC-116 was named ovoglycan (Fernandez et al., 2001, 2003) and it forms the protein core of the primary proteoglycan of the shell (Carrino et al., 1997). The carbohydrates of OC-116 comprises of 17 different oligo-structures (Nimtz et al., 2004). Eight of them were hybrid-type, four were the high-mannose type, and the rest five had a complex-type structure. When present in the uterine fluid, OC-116 exists as a 116 kDa protein due to glycosylation modification and as a 190 kDa protein after glycation (Arias et al., 1992; Fernandez et al., 1997). This protein is thought to have a role in the modulation of calcite growth.

Ovocalyxin-32 (OCX-32) is released in the uterine fluid by the surface epithelial cells of the uterus during the end of the calcification phase and thus it is mainly confined to the outer regions of the shell, i.e., the vertical crystal layer, cuticle, and the palisade layer (Gautron et al., 2001b, 2003; Hincke et al., 2003). Therefore, it has been suggested that this protein has a role in the termination of the calcification process of the eggshell.

Ovocalyxin-36 (OCX-36) is found in abundance in the uterine fluid during the calcification process, and the expression of this protein is highly upregulated. This protein has been cloned as well (Gautron et al., 2007). It has been reported that OCX-36 has homologous similarities with proteins involved in innate immune responses such as bactericidal permeability increasing proteins, lipopolysaccharide binding proteins, and Plunc family proteins, which suggests the fact that OCX-36 may also be involved in the defence mechanisms to keep the egg pathogen free (Gautron et al., 2007).

Ovocalyxin-25 and -21 are also two other eggshell proteins which are exclusively detected in tissues undergoing mineralization. Database analysis reported that Ovocalyxin-21 has remarkable homologies with brichos domain-containing proteins. The Brichos domain consists of nearly 100 amino acids and comparing the similarities, several functions of the ovocalyxin-21 proteins were postulated including chaperon-like functions (Sanchez-Pulido et al., 2002). Ovocalyxin-25 contains two protease inhibitory domains, one of which is the WAP-type. The matrix protein from the nacreous layer of the pearl and shell of molluscs, known as lustrin A, also has the same inhibitory WAP-type domains (Shen et al., 1997).

## Egg White Proteins

The egg white is made up of four individual layers: chalaziferous layer, thin layer, thick layer, and the chalazae cord. The thin layer accounts for about 23.3% of egg white, which is further separated into two layers, i.e., inner and outer thin layers. The thin inner layer (16.8% of egg white) is attached to the chalaziferous layer, which accounts for about 2.7% of egg white, whereas, the thin outer layer is connected to the inner eggshell membrane. The outer and inner thin layers are separated by the thick or viscous layer which accounts for the most substantial portion of egg white, i.e., 57.3% (Brake et al., 1997; Conrad and Philips, 1938; Li, 2006).

Water is the primary constituent of egg white which accounts for about 84% to 89% of the total egg white or albumen weight. Among albumen solids, proteins are the major constituents (10%–11%), while the minor components include carbohydrates (0.9%), lipids (0.03%), vitamins and minerals (Li-Chan and Nakai, 1989).

Egg proteins are well known for their high nutritional quality, excellent digestibility and comprise of all the essential amino acids necessary for the human nutrition and development (Friedman, 1996). Egg albumen consists of several different protein components which have been identified and characterized through modern high-resolution analytical techniques (Raikos et al., 2006). In a study, 78 egg white proteins were detected using 1-dimensional electrophoresis and liquid chromatography tandem mass-spectrometry (LC-MS/MS) (Mann, 2007). However, among egg white proteins, ovalbumin, ovotransferrin, ovomucoid, ovomucin,

**Table 1** Physio-chemical properties of major and minor egg white proteins

<i>Protein</i>	<i>dry weight of albumen (g/kg)</i>	<i>Isoelectric point (pI)</i>	<i>Molecular weight (kDa)</i>	<i>T<sub>d</sub> (°C)</i>
<b>Major proteins</b>				
Ovalbumin	540	4.5	45	84
Ovotransferrin	120	6.1	76	61
Ovomucoid	110	4.1	28	77
Ovomucin	35	4.5–5.0	5500–8300	–
Lysozyme	34	10.7	14.3	75
<b>Minor proteins</b>				
Ovoglobulin		(6.1–5.3)		
G <sub>2</sub> globulin	40	5.5	30–45	92.5
G <sub>3</sub> globulin	40	4.8	–	–
Ovoinhibitor	15	5.1	49	–
Ovoglycoprotein	10	3.9	24.4	–
Ovoflavoprotein	8	4.0	32	–
Ovomacroglobulin	5	4.5	769	–
Cystatin	0.5	5.1	12.7	–
Avidin	0.5	10	68.3	–

‘–’ represents not determined.

Modified from Powrie, W.D., Nakai, S., 1985. Characteristics of edible and fluids of animal origin: egg. In: Fennema, O. (Ed.), Food Chemistry. New York, Marcel Dekker, pp. 829–855 and Data compiled from Li-Chan, E.C.Y., Powrie, W.D., Nakai, S., 1995.

and lysozyme have been studied extensively due to their abundant presence in egg albumen. Physicochemical characteristics of major egg white protein is provided in **Table 1**. The structure and chemical composition of these proteins are described in details in the following section.

### Ovalbumin

Ovalbumin constitutes about 54% of the total egg albumen and thus it is the primary protein present in egg white. It is a phosphorylated glycoprotein made up of complete three subunits having different phosphate groups along with a carbohydrate group attached to its N-terminal (Li-Chan et al., 1995). Ovalbumin is a member of the serpin (serine protease inhibitors) superfamily despite lacking the inhibitory activity unlike the other serpin-like proteins (Huntington and Stein, 2001). The molecular weight of ovalbumin is 45 kDa and is composed of 386 amino acids along with two genetic polymorphisms seen at 290 (Glu → Gln) and 312 (Asn → Asp) (McReynolds et al., 1978). Among all other egg albumen proteins, ovalbumin is a unique protein as it contains six cysteine residues, two of which are involved in a disulfide bond between Cys74 and Cys121, while the rest four include free sulfhydryl (–SH) groups. One out of the four –SH group is reactive only when the protein is denatured, however, the other three are masked in the native state (Fothergill and Fothergill, 1970).

The amino acid composition analysis of egg albumen revealed that 50% of the total amino acids are hydrophobic while 30% are acidic and charged amino acids, the latter contributing to the acidic isoelectric point (pI) of 4.5. Ovalbumin has a unique amino acid distribution as compared to other glycoproteins. It lacks an N-terminal ladder sequence but contains an acetylated glycine and proline in the N- and C-terminal, respectively, along with carbohydrate moiety attached to the amino acids in the N-terminal (McReynolds et al., 1978; Huntington and Stein, 2001).

Ovalbumin-Y is a chimeric glycoprotein with an amino acid sequence very similar to the native ovalbumin and a carbohydrate group identical to ovomucoid (Hirose et al., 2006). Ovalbumin-Y protein was first identified and characterized by Nau et al. (2005) by 2-Dimensional PAGE and peptide mass fingerprinting. Ovalbumin Y is not phosphorylated unlike ovalbumin; however, it is glycosylated. Three isoforms of ovalbumin Y protein and five isoforms of ovalbumin-related Y protein have been identified via electrophoresis, each protein differing in their pIs. However, this polymorphism could not be explained by genetic variations or by phosphorylation or glycosylation levels (Guérin-Dubiard et al., 2006).

Ovalbumin in its native form is resistant to digestion by trypsin but once it is heat denatured or given an acid or pH change treatment, it becomes susceptible to trypsin digestion. With high pH and temperature dependent denaturation, ovalbumin converts into a thermally stable form known as S-ovalbumin (Pelegrine and Gasparetto, 2006). Sugimoto et al. (1999) demonstrated that storage temperature influences this conversion more than the storage time, where longer storage time at 20–25 °C did not affect the conversion. However, Huang et al. (2012), demonstrated that during storage, the conversion of ovalbumin to S-ovalbumin had been attributed to an increase of pH and change of pH has a direct effect on the conversion rather than temperature. Therefore, despite temperatures of 4 °C, long time storage of eggs may increase the conversion of native ovalbumin to S-albumin by 81% (Huang et al., 2012).

Ovalbumin is also the primary allergen in egg white which is responsible for IgE-mediated allergic reactions (Caubet and Wang, 2011). The ovalbumin epitopes which bind IgE consists of mainly polar, charged and hydrophobic amino acids and these

sequences are mostly made up of  $\beta$ -sheet and  $\beta$ -turn structures. The only allergenic epitope which comprises of an alpha helix is Asp95-Ala102 (Kim, 2002).

### Ovotransferrin

Ovotransferrin is a monomeric glycoprotein which is involved in the transfer of ferric ions to the developing embryo from the hen's oviduct. Ovotransferrin accounts approximately 12% of the entire egg white protein (Desert et al., 2001; Abdallah and El Hage Chahine, 1998). Ovotransferrin belongs to the transferrin protein family and has been reported to have around 50% homology with mammalian lactoferrin and transferrin (Mazurier et al., 1983). Ovotransferrin can bind 2 mol of different metal ions per mole of protein. For, ovotransferrin, lactoferrin and serum transferrin, each lobe binds one carbonate anion and one  $\text{Fe}^{+3}$  atom (Lambert et al., 2005). Such iron complex formation in ovotransferrin inhibits microbial growth that requires iron.

The N and C lobes of ovotransferrin consist of a single iron-binding site located in a deep cleft along with 15 disulfide bridges which maintain the globular structure of the protein (Kurokawa et al., 1999). The N- and C- terminal lobes of ovotransferrin form two sulfide bonds where Ala1-Tyr72 in the N-terminal segment acquires a local-native like confirmation (Mizutani et al., 1997). This interaction between the two lobes is very critical for iron acquisition (Alcantara and Schryvers, 1996). The various metal- and anion-binding properties of the iron binding sites of ovotransferrin can be attributed to the presence or absence of basic amino acid residues (Nadeau et al., 1996).

The process of in-vitro  $\text{Fe}^{+3}$  uptake and release by ovotransferrin are reported to be very similar, but not identical, to lactoferrin and serum transferrin. The  $\text{Fe}^{+3}$  is bound very tightly by the four protein ligands (Tyr92, Asp60, His250 and Tyr191 present in the N-lobe) in a closed interdomain cleft (Abdallah and Chahine, 1999). Ovotransferrin efficiently binds  $\text{Fe}^{+3}$  at pH greater than 7 and releases any bound  $\text{Fe}^{+3}$  at a pH lesser than 4.5 (Guérin-Dubiard et al., 2006). On uptake of  $\text{Fe}^{+3}$ , the transferrins undergo a major conformational transition from the apo structure (open-form/iron-free) to the closed/iron-bound holo formation, suggesting that initial binding occurs in the open form (Mizutani et al., 1999). Both these forms have significantly different physiochemical properties, for instance, the holo-form emits a salmon pink color due to the presence of iron whereas the apo-form does not have any color. Moreover, the apo-form is more prone to physical and chemical changes as compared to the holo-form (Kurokawa et al., 1999).

Apart from the iron-binding capacity, several recent studies have investigated other structural and functional features of ovotransferrin that might be associated with various biological properties. For instance, the embryos of mammal and bird's egg are susceptible to oxidative stress, and thus maintenance of a constant, reducing environment during the development of the embryo can provide protection and although much knowledge is not present currently, however egg white is suggested as the primary target for this. Ibrahim et al. (2006) reported that ovotransferrin is capable of autocleavage at specific sites once it gets reduced by thiol reducing agents. This autocleavage occurs due to a unique chemical reaction between the four tripeptide motifs present on both sides of the two disulfide domains (115–211 and 454–544 residues) of ovotransferrin protein. It has been found that many auto-processing proteins contain these reduction-scissile sequences (His/Cys-X-) which suggest that this sequence is evolutionarily conserved.

### Ovomucoid

Ovomucoid is a glycoprotein which belongs to the Kazal family of protein inhibitors. It constitutes 11% of the total egg white proteins and is thermally stable (Li-Chan and Nakai, 1989). The protein consists of 186 amino acids with a molecular mass of 28 kDa (Kovacs-Nolan et al., 2000). It consists of 9 disulfide bonds and has three different domains which are crosslinked only by the intra-domain disulfide bonds. Ovomucin has trypsin inhibitory effect, and the active site for the trypsin inhibitory activity lies within the Domain II of the protein. However, considerable variations in the inhibitory activities and specificities of the domains have been reported from different avian species (Li-Chan and Nakai, 1989). Chicken ovomucoid is one of the significant egg white allergens, and it plays a crucial role in the pathogenesis of IgE-mediated allergic reactions (Mine and Zhang, 2001, 2002a; Mine and Rupa, 2003a, 2004). This allergenic potential could be attributed to its higher stability towards gastrointestinal digestion and heat treatment (Hirose et al., 2005). Yoshino et al. (2004) reported that the digestibility of ovomucoid by pepsin is much better over the pH range of 1.5–2.5. However, the digestibility loses at a pH of 3.0 or higher. Moreover, pepsin-digested fractions of ovomucoid retain its trypsin-inhibitory activities. Besler et al. (1997) reported that the epitopes on ovomucoid which were responsible for the IgE binding were present only on the protein backbone and not the carbohydrate groups. Nine IgE epitopes (5–16 amino acids) and eight IgG epitopes (5–11 amino acids) were identified within the primary ovomucoid sequence. Through mutational studies of the epitopes, it was found that charged amino acids (lysine, glutamic acid, and aspartic acid), polar amino acids (cysteine, tyrosine, threonine, and serine), and hydrophobic (glycine, leucine, and phenylalanine) are crucial for antibody binding (Mine and Zhang, 2002b). Numerous studies have been conducted to alter the composition and structure of the ovomucoid epitopes responsible for the allergenicity (Mine and Rupa, 2003b). Some of the attempts made include heating in the presence of wheat flour (Kovacs-Nolan et al., 2000; Kato et al., 2001), gamma irradiation along with heating (Lee et al., 2002), deglycosylation by endo-beta-N-acetylglucosaminidases (Yamamoto et al., 1998), and genetic modifications (Rupa and Mine, 2006a). However, none of these modifications made any significant changes to the allergenic epitopes of ovomucoid, suggesting that the epitopes were extremely resistant to any modifications.

### Ovomucin

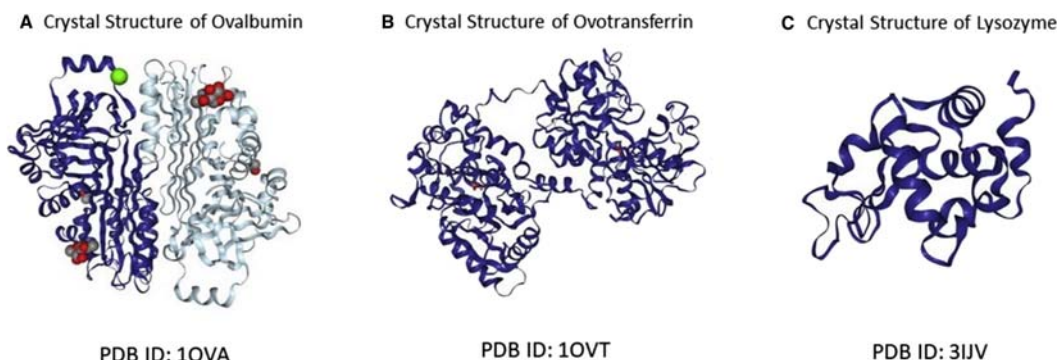
Ovomucin contributes to about 3.5% of the total egg white proteins. It is a sulfated glycoprotein which is responsible for the jelly-like structure of egg white. The protein consists of two parts: soluble part (8,300 Da), which is the main component of the inner and outer egg white, and the insoluble part (220–270 kDa), which is responsible for the insoluble gel-like fraction of thick albumen (Omana and Wu, 2009). Both fractions are made up of two subunits,  $\alpha$ -ovomucin and  $\beta$ -ovomucin, but have different carbohydrate contents. The soluble fraction consists of 40  $\alpha$ - and three  $\beta$ -subunits, while the insoluble fraction contains 84  $\alpha$ - and 20  $\beta$ -subunits (Robinson and Monsey, 1971; Omana and Wu, 2009). There are two distinct subunits of the  $\alpha$ -subunit, i.e.,  $\alpha 1$  and  $\alpha 2$ , and both the  $\alpha$ -subunits have lesser carbohydrate groups than  $\beta$ -subunits. Acidic amino acids such as glutamic acid and aspartic acids mainly make up the  $\alpha$ -subunit (Omana and Wu, 2009), whereas, serine and threonine primarily make up the  $\beta$ -subunit (Robinson and Monsey, 1971). A study by Toussant and Latshaw (1999) reported that the quality of eggs could be positively correlated with the amount of ovomucin present in the thick albumen. However, a higher concentration of highly glycosylated  $\beta$ -ovomucin containing hexoses, sialic acid, and hexosamines, signifies the inferior quality of eggs. Ovomucin is responsible for many of the functional and biological properties of egg white. It has a significant role in thinning of egg white during prolonged storage. Studies have shown that thinning of egg white can be either due to disruption of the ovomucin-lysozyme complex or the reduction of disulfide bonds leading to the degradation of ovomucin (Abeyrathne et al., 2014). Moreover, it is known for its exceptional emulsifying and foaming properties (Mann, 2007).

### Lysozyme

Egg white lysozyme consists of 129 amino acids and is a 14.4 kDa protein with a pI of 10.7. Lysozyme present in the egg is unique as it is highly soluble and stable as compared to lysozyme present in other foods. Although the lysozyme present in egg exists as a monomer, it is also frequently found as a dimer which leads to its thermal stability. Four unique disulfide bonds stabilize the tertiary structure of egg lysozyme (Kato et al., 2006). Lysozyme tends to bind to negatively charged proteins in the egg albumen such as ovalbumin, ovomucin, and ovotransferrin (Abeyrathne et al., 2014). The chalaza and the chalaziferous layer mainly consists of the lysozyme–ovomucin complex. The complete structure with a resolution of 2 Å and the amino acid composition of lysozyme had been established in the 1960s (Li-Chan and Nakai, 1989). However, still many research is carried out to investigate its structure and function further. Three-dimensional structural analysis, at the resolution of 1.46 Å, revealed the hexagonal crystal form of lysozyme (Blake et al., 1965; Kato et al., 1992). Various studies have also been conducted to investigate the structural changes of lysozyme induced by different conditions, such as aqueous-organic solvent mixtures (Griebenow & Klibanov, 1996), pH variations (Babu and Bhakuni, 1997), co-crystallization in presence of different alcohols (Deshpande et al., 2005), sorbitol (Petersen et al., 2004), in presence of thiol reagents (Raman et al., 1996), and supercritical CO<sub>2</sub> treatment followed by heat treatment (Liu et al., 2004). The first purification of lysozyme was done using the high concentration of ammonium sulfate, however, it led to the modification of the characteristics and morphology of the protein due to the high salt concentration used during extraction (Liu et al., 2004; Abeyrathne et al., 2013). The purification technique which is commonly used nowadays for lysozyme is cation exchange chromatography which makes use of the high pI value of the protein (Abeyrathne et al., 2013). However, due to the small size of the resin granules used in the chromatography, the flow rate is low which makes it a time-consuming process. Crystallographic structures of some of the three major egg white proteins are listed in Fig. 1.

### Minor Proteins in Egg White

Egg white contains more than 50 proteins (Mann, 2007), among which the five major ones are described above. However, there are some other proteins, which although are present in very small amount, play a crucial role in determining the physicochemical and structural properties of the egg. Four of such proteins are described in the following section.



**Figure 1** Crystallographic structure of major egg white proteins. Protein Data Bank.



### Ovoglobulin

Ovoglobulin constitutes 4% of the total egg white proteins and is a minor protein. The protein is made up of two subunits G2 and G3, with molecular weights of 36 kDa and 45 kDa respectively (Ogawa and Tanabe, 1990; Damodaran and Razumovsky, 1998). Ovoglobulin was reported to be completely soluble in high and low ionic strength salt solution and coagulated with heat treatment; these properties were very similar to ovalbumin (Damodaran and Razumovsky, 1998). Ovoglobulin G2, present in chicken eggs, shows polymorphism (Asal et al., 1993). Ovoglobulin has been reported to be crucial for the foaming properties of egg white, even though other biological functions are not well known yet (Sugino et al., 1997b). In a study by Damodaran and Razumovsky (1998), the competitive adsorption of the five major egg white proteins described above, along with ovoglobulin was studied, at low and high ionic strengths, and it was found that at 0.1 ionic strength, only ovoglobulin and ovalbumin were able to adsorb at the interface, while the other proteins were excluded from the interface.

### Cystatin

Cystatin is a cysteine proteinase inhibitor which inhibits the action of thiol proteases, for instance, papain and ficin. Cystatin of the chicken egg contains two disulfide bonds near the carboxy-terminal along with a reactive site which is highly conserved. The pI of phosphorylated cystatin is 5.6, whereas it is 6.5 for the non-phosphorylated form (Guérin-Dubiard et al., 2006), with a molecular weight of 13kDa (Li-Chan and Nakai, 1989). In a study, the effects of storage conditions of eggs, the age of hens, and heat treatment of albumen on the cystatin activity was investigated. The cystatin present in eggs laid by hens, either older than 60 weeks or younger than 30 weeks, had the lowest activity, while eggs laid by hens aged 40–50 weeks had the highest cystatin activity. Furthermore, the eggs stored for 28 days at 15 °C showed a 4%–12% decrease in the cystatin activity. Thermal treatments also decreased the activity (Trziszka et al., 2004). Apart from having proteinase inhibitor activity, cystatin has been reported to have bioactive properties, such as, antimicrobial activity against bacterial pathogens (Wesierska et al., 2005), and inhibitor of bone matrix degradation in the resorption lacunae adjacent to osteoclasts (Brand et al., 2004).

### Avidin

Avidin is well known for its biotin-binding activities and is essentially a tetrameric glycoprotein from egg albumen. All the four monomers of the protein are capable of binding biotin and establishing a strong interaction with a dissociation constant of ~10–15 M (Stadelman et al., 1995; Mine and Yang, 2010). The protein constitutes approximately 0.05% of the total proteins in egg white. Each avidin chain comprises of 128 amino acids which are arranged as eight-stranded antiparallel beta-barrel, where the D-biotin binding site is defined by the inner regions. The secondary structure of avidin mainly comprises of beta-sheets and extended beta-turns (66%), while the rest is made up of  $\beta$ -turns and disordered structures (Stadelman et al., 1995). Avidin has been shown to have insecticidal and antimicrobial activity. The insecticidal activity has been used with genetically engineered and host plant resistance against Colorado potato beetle (Rupa and Mine, 2006a). Bacteriostatic effect of avidin has been reported against *Salmonella typhimurium*. However, the effect is lost on biotin addition (Stadelman et al., 1995). Terminal mannose and N-acetylglucosamine residues of avidin are capable of binding to lectins. Tumor cells express lectins on their cell surface at various levels, which acts as an important biomarker. Thus binding of avidin and cytotoxic agents can be easily detected via cell surface lectins. This mechanism was considered for tumor treatments, and avidin served as a potential vehicle for transport of toxins, drugs, therapeutic genes, and radioisotopes (Yao et al., 1998).

### Ovoflavin

Ovoflavin, also known as riboflavin-binding protein or ovo-flavoprotein, is a phosphoglycoprotein which is present in both egg yolk and white in equal amounts. This protein binds with riboflavin or vitamin B2 and the protein contains the highest selenium (Se) content (1800 ng/g) as compared to other egg proteins (Kiliç et al., 2002). Ovoflavin in egg white constitutes 219 amino acids with the presence of pyroglutamic acid at the amino terminus (96). Although the biological function of ovoflavin is not fully understood its ability to bind minerals and vitamins suggests that it may be involved in nourishing the embryo with vitamins and minerals during development (Hamazume et al., 1984).

## Egg Yolk Proteins

Egg yolk is composed of plasma and granules, located between the thin and thick albumen, supported by the chalazae. Yolk plasma constitutes 80% of the yolk fraction, and its protein content is of 23% on a dry basis (Freschi et al., 2011), composed of Low-Density Lipoprotein (LDL) (15%) and globular glycoproteins (15%) (Laca et al., 2015). Proteins such as livetins present as  $\gamma$ -livetins are mainly IgY antibodies (Chalamaiah et al., 2017), whose functions have been applied as immunological supplements in foods (Yang et al., 2014) and several other industries. Yolk granules nonetheless have a higher concentration of lipids (33%) and proteins (58%) in comparison with plasma (Laca et al., 2014). Their structure formation is mainly composed of non-soluble HDL-phosvitin complexes. Therefore a medium ionic strength has shown to modify its solubility. Protein composition is also dependent on factors

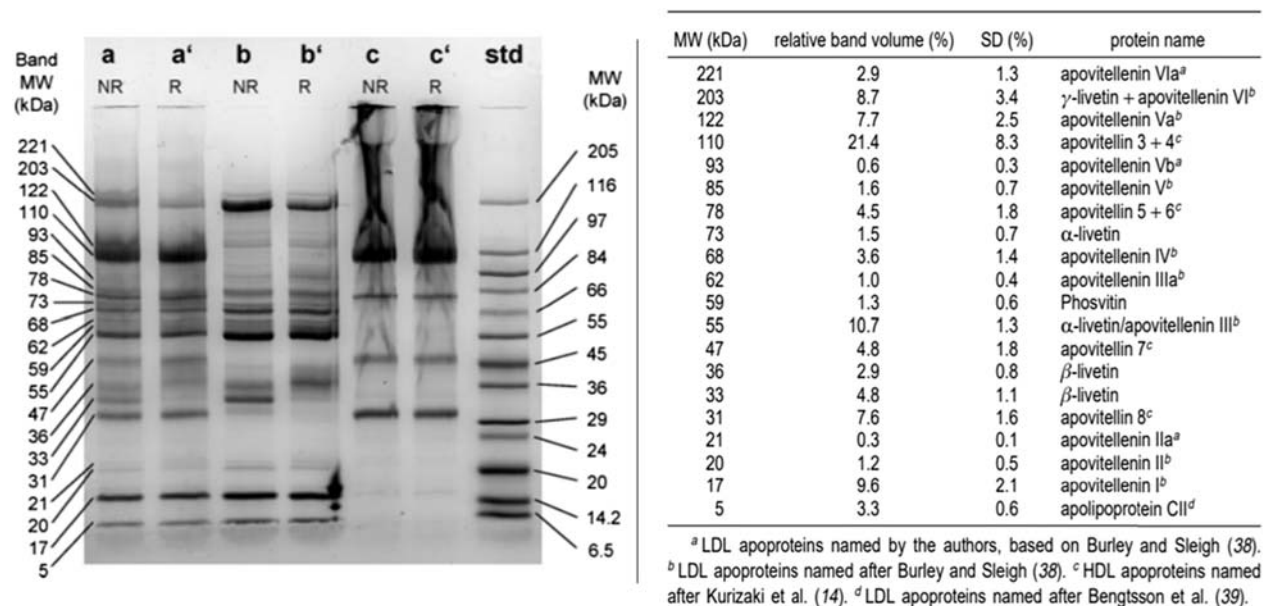
such as feed intake and environment during the hen's productive life. Molecules such as pigments or vitamins determine the color of the egg yolk. Studies supplementing herbs, quercetin, and TSAA with lysine, improved the color, oxidative stability, and reduced yolk protein respectively (Simitzis et al., 2018; Hammershøj and Johansen, 2016; Novak et al., 2004). Also, egg lipid and protein content vary throughout the productive cycle of the hen. In order to maintain egg weight homogeneity, a reduction in the amino acid content is realized. Furthermore, alterations in the environment such as heat and feed intake undermine lipid content in the organism. Heat stress initiates lipid peroxidation in cell membranes due to the release of hormones corticosterone and catecholamines (Asli et al., 2007). The following section describes the three major egg yolk proteins and Fig. 2 depicts all the egg yolk proteins present under reducing and non-reducing conditions.

### Low-Density Lipoprotein (LDL)

LDL proteins are predominant in yolk plasma with an 85% protein composition. Its structure is characterized by a micelle nano-structure containing triglycerides and cholesterol (Anton et al., 2003; Martin et al., 1964). Emulsification properties of LDL is an important application for the food industry. Factors such as heat treatment, ionic strength, particle dispersion and defatting induced protein changes in the structure affecting its functionality (Kiosseoglou, 2003). For instance, amphipathic side chain structure makes the protein interact with hydrophobic and hydrophilic interfaces such as W/O or O/W. Apolipoproteins in LDL micelle adsorb faster than low molecular weight proteins, helped by its structure flexibility (Anton et al., 2003; Mine, 1997, 1998; Martinet et al., 2003). In contrast, granules protein has shown an emulsification property dependent of pH. Specifically, at pH 4, its emulsification capacity is reduced as a result of protein dimer formation (Aluko and Mine, 1998). Due to pH changes and an ionic strength increase, lipoproteins tend to minimize the interaction of granules proteins in an emulsion. The delivery mechanism is among other potential applications of LDL when combined with polysaccharides (Zhou et al., 2016).

### High-Density Lipoprotein (HDL)

HDL is mainly composed of protein, lipids, cholesterol, and minor lipids. Its structure functionality includes antioxidant activity and protective effects against pathogens (Yamamoto et al., 1990; Kassafy et al., 2005). Lipid moiety in HDL influence its electron donating properties which can reduce the generation of reactive oxygen species (ROS). Mechanisms proposed to include the reduction of lipid hydroperoxides to non-reactive species through the electron transfer of methionine amino acid, interfering with lipid oxidation propagation (Elias et al., 2008).



**Figure 2** SDS PAGE profile of egg yolk proteins under non-reducing (NR) and reducing (R) conditions (a and a') whole EY (b and b') EY plasma (c and c') EY granule (std) MW standard. From Guilmineau, F., Krause, I., Kulozik, U., 2005. Efficient analysis of egg yolk proteins and their thermal sensitivity using sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing and nonreducing conditions. J. Agric. Food Chem. 53, 9329–9336.



## Phosvitin

Phosvitin is a highly phosphorylated molecule with 124 of 217 amino acids binds to phosphate through covalent bonds, being serine the predominant amino acid (Byrne et al., 1984; Lei and Wu, 2012). This characteristic yield a high mineral binding capacity, interacting with 95% of the iron present in egg yolk (Grogan and Taborsky, 1986). The addition of ascorbic acid release the phosvitin bonded iron, thereby increasing the lipid peroxidation in egg yolk (Nielsen et al., 2000). On the other hand, its chemical properties derive from the emulsifying functional property. When in complete protein moiety, emulsifying properties are conferred by the interaction of the protein charged N- and C- termini. Further, glycosylated phosvitin has shown to improve the viscoelastic layer (Khan et al., 1999).

## Livetin

Livetin is a globular water-soluble glycoprotein which makes up 30% of the total egg yolk plasma proteins. The protein is present in three forms, namely,  $\alpha$ -livetin,  $\beta$ -livetin, and  $\gamma$ -livetin (Sugino et al., 1997b).  $\gamma$ -livetin is primarily immunoglobulin Y (IgY) which is an ideal substitute for mammalian IgG (Laca et al., 2015). The reported molecular weights of  $\alpha$ -livetin,  $\beta$ -livetin, and  $\gamma$ -livetin are 80,000, 45,000, and 170,000 respectively.

$\alpha$ -livetin has been reported to be the primary allergen responsible for the bird egg syndrome, and the protein is partially heat-labile inhalant. The reactivity of IgE towards  $\alpha$ -livetin was reported to reduce by almost 90% when it was heated for 30 minutes at 90 °C. Only a partial cross-reactivity was observed between  $\alpha$ -livetin and conalbumin (Quirce et al., 2001). Martin et al. (1957) reported that although the molecular weights and tyrosine: tryptophan ratios of  $\alpha$ -livetin and serum albumin were similar, they were not identical proteins because of the solubility. However, newer studies reported that although there was some precipitation of  $\alpha$ -livetin at lower concentrations of  $(\text{NH}_4)_2\text{SO}_4$ , most of it was not precipitated by 50% saturation. Moreover, the peptide patterns and immunological results suggested that both  $\alpha$ -livetin and serum albumin were the same protein.

$\beta$ -livetin contains 7% hexose. This protein is distinct from ovalbumin, although the molecular weights of both the proteins are very similar. Because the immunological patterns of  $\beta$ -livetin were identical to serum protein, it was considered an  $\alpha$ 2-glycoprotein, from its carbohydrate content and electrophoretic mobility. The sedimentation coefficient of 3S of  $\beta$ -livetin was similar to human serum, although the sialic content was lower in  $\beta$ -livetin as compared to human serum proteins (Schmid and Burgi, 1961; Burgi and Schmid, 1961).

The  $\gamma$ -livetin or IgY is derived from IgG of hen's serum, although it differs in from mammalian IgG in many of its chemical and structural properties. Yolk IgY consists of Asn-linked oligosaccharides like IgG. However, the oligosaccharide composition is different in both the immunoglobulins. The molecular weight of the heavy chains of IgY is greater than mammalian IgG (Kovacs-Nolan and Mine, 2004). Along with compositional differences, IgG and IgY also have functional differences. For instance, the isoelectric point of IgY is lower, and it is incapable of association with the mammalian complement, Protein G, Protein A, or rheumatoid factors. IgY also shows lesser binding capabilities with bacterial and human Fc receptors (Kovacs-Nolan and Mine, 2004).

## Conclusion

Hen's egg has been considered as one of the most nutritious source of food as it can sustain both life and growth. The proteins present in the egg are nutritionally complete with a great balance of essential amino acids. Although the egg proteins are present in all parts of the eggs, but the major concentration lies in the egg white (50%) and egg yolk (40%), while the remaining proteins are distributed in the egg shell and egg shell membrane. Each protein component of the egg white and egg yolk is responsible for imparting a specific physical and chemical characteristic towards the entire functionality of an egg. Moreover, environmental conditions such as ionic strength, pH, and temperature treatments can modulate the functional properties of these proteins. Therefore, a greater understanding of the egg proteins could help in the development of functional foods, as egg proteins, particularly egg white proteins, have gained a lot of research interest in the recent years. Apart from academic research, food industries have also shown a lot of interest in egg proteins to explore the various functional properties of eggs, which could provide them many commercial benefits.

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# Enzyme Applications in Food Processing: Traditional Uses to New Developments

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## Glossary

**Active packaging** Not only segregating the foods from environment, active packaging aims to selectively induce certain events in the food contents. The events include oxygen elimination, pH indication, and microbial decontamination.

**BAP** Bioactive peptides. During digestion of milk, meat, and plant proteins, smaller oligopeptides (10 to 15 amino acid units) are yielded. Some of them have positive effects on our health, such as lowering blood pressure, reducing the risk of blood clotting, and preventing microbe growth. The studies to intentionally enrich BAP in foods are recently focused in functional food research.

**Catalysis** Acceleration of chemical reactions by a catalyst that is involved in the reaction without changing its chemical structure before and after the reaction. Thus the catalyst can catalyze the reaction multiple times.

**Cellulose** A major poly-sugar in plant. Basic chemical component is glucose that is also the basic unit of starch. While starch is a storage compound in plant, cellulose is form physical structure of plant body. It is a plant fibre in foods.

**Esters** Chemical compounds where an acid (typically  $R-COOH$ ) is condensed with an alcohol ( $R'-OH$ ) forming  $R-COO-R'$ .

**Fatty acids** Acids made of a carboxyl group with an alkyl group, i.e., generally expressed as  $R-COOH$ . They are found in triglycerides in the form of esters. Small fatty acids can evaporate and we can smell them. Some larger fatty acids are essential nutrients.

**Fermentation** It is scientifically defined as metabolic activities where their metabolites are used as final accepters of electron in the metabolic reactions. If electrons are accepted by external compounds, such as molecular oxygen, it is called respiration. Meanwhile, in the food industry, fermentation is commonly defined as processing of food materials with the activities of microbes. Typical example is alcohol beverage, cheese, and pickles.

**Free radicals** Electrons occupy an electron orbit as a pair. But occasionally only one electron occupies an electron orbit. This unpaired electron is extremely reactive. It does not diminish until it meets another radical, so that a free radical can create a chain reactions in many molecules.

**HFCS (high fructose corn syrup)** A common sweetening agent utilized in foods. Unlike sugar (sucrose), it is a chemically processed product through hydrolysis of starch. Through glucose isomerase activity, glucose in hydrolyzed starch is converted into fructose. Fructose is much sweeter than sucrose or glucose, thus high fructose substances can show the same sweetness with smaller amounts.

**$K_m$  value (Michaelis Constant)** It is a parameter to define the affinity of a compound to an enzyme in catalytic reactions. Small  $K_m$  value represents better affinity, leading faster reactions.

**Lactose** The major carbohydrate in mammal milk. It is almost exclusively found in milk in nature. Chemically it is a conjugated molecule of glucose and galactose.

**Maillard Reaction** Reaction between amine group and hydroxyl group. Proteins contain multiple amine groups, and sugar molecules contain multiple hydroxyl group. Thus Maillard Reaction is a common reaction in foods, leading brown colour in foods. A typical example is colour formation of bread crust.

**Oligosaccharides** Sugar molecule polymers made with less than 10 to 15 sugar molecule units. In recent years, their biological activities are studied as functional food ingredients. GOS (galactose oligosaccharide) is one of the examples.

**Pectins (pectic substances)** Poly-sugar compounds found in plant body. It is a major compound in the plant cell wall to give physical strength in plant body. In nutritional view, it is considered as a plant fibre.

**Peroxides** Compounds that have  $-O-O-$  groups. This two oxygen structure is very reactive. It can oxidize another compound or form a free radical.

**Poly-phenols** Typical bitter compounds found in plant products. In recent years, its biological activities draw attentions of food industry for their health benefits.

**Prebiotics** Compounds that assist the growth and maintenance of probiotic microbes in our intestine.

**Probiotics** Gastro-intestinal microbes that show positive effects to our health.

**Proteins** polymers of amino acids. They are a main nutrients in foods, and also major functional compounds found in living organisms.

**Saccharification** Degradation of polymer of sugars, such as starch, into smaller units of sugar. For example, starch is degraded into maltose (two-glucose unit) and glucose.



**Starch** Polymer of glucose. It is a storage material in plant and human can use it for energy generation through hydrolysis into glucose and metabolizing glucose.

**Triglycerides** A major compound found in fats (lipids, oils). They are ester compounds between a glycerol and three fatty acids. Glycerol has three hydroxyl ( $-\text{OH}$ ) groups and each of them is condensed with a fatty acid. If only one or two hydroxyl group is esterified, the compound is called monoglyceride or diglyceride.

## Introduction

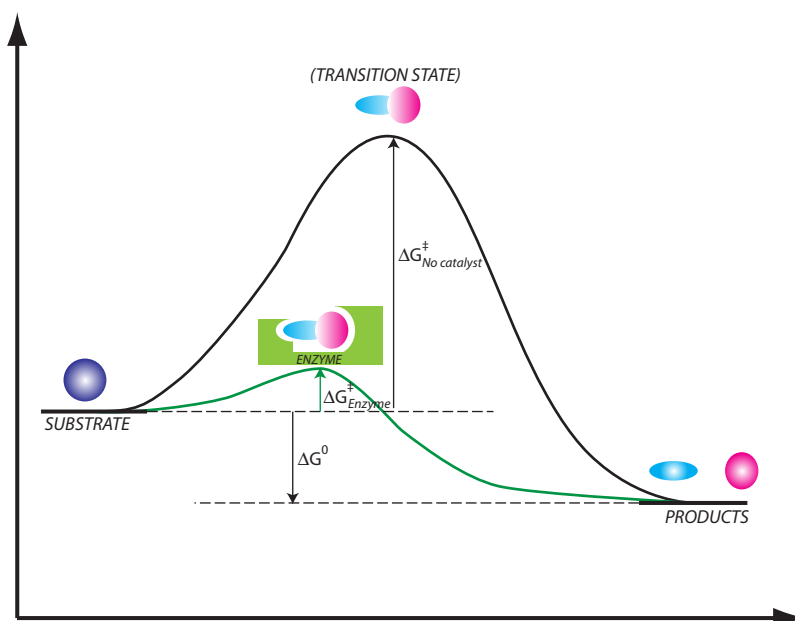
A majority of foods are processed from their raw materials before they are in grocery stores. The processing can occur at various stages in the food supply chain, and it utilizes many different means to achieve required/desired modifications in food materials. The processing is considered as controlled chemical and/or physical modifications of raw materials in order to change the properties of materials. In the chemical modifications, enzymes can play important roles to determine the direction of modifications and, therefore, the products. Moreover, the chemical modifications can lead to physical changes. The utilization of enzymes varies from traditional uses, modern applications to experimental approaches in developing stages.

## What Is the Enzyme?

Majority, if not all, of reactions associated with the living activities are mediated by enzymes. One of the advantages of enzymes is that they 'catalyze' the reactions to accelerate them. There is another big advantage of enzyme: the enzymes can distinguish specific molecules, allowing specific reaction and specific products. These two advantages (catalysis and substrate-product specificity) make the enzymes essential in the metabolism of living organisms.

## What Is Catalysis?

Many chemical substances are reasonably stable under the physiological conditions, and generally require a large energy input to convert them into others. This energy is called activation energy ( $\Delta G^\ddagger$ ) to bring the energy level of substance up to go over the energy barrier between substances (Fig. 1). Enzymes can bring this activation energy level low so that the energy available under physiological conditions is sufficient to go over the barrier to produce products. This lower activation energy also accelerates the rate of reaction drastically. Some enzymes can accelerate the reaction by  $10^{20}$ -times, and typically acceleration rates are  $10^6$ – $10^8$ -times



**Figure 1 Free Energy Profiles of Enzyme Reactions.** The enzymes stabilize the transition state of their reactions, lowering the free energy of transition state, i.e., low activation energy ( $\Delta G^\ddagger$ ). It allows the reaction from substrate to product easy to occur with a low energy input. It should be noted that the enzymes do not change the free energy of substrate or product, thus free energy difference ( $\Delta G^0$ ) remain the same, keeping the equilibrium between substrate and product.



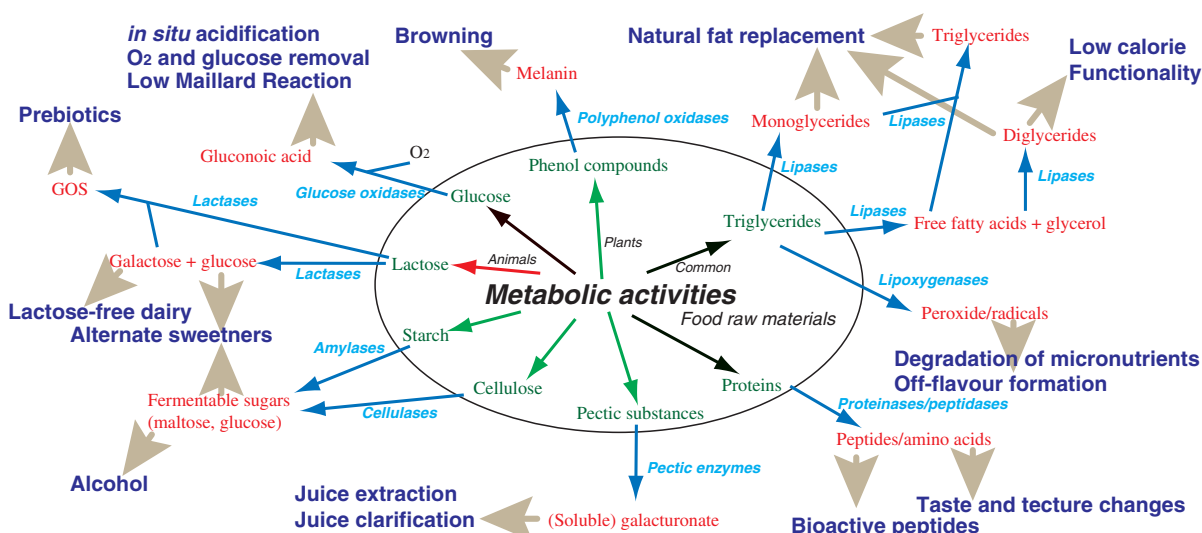
of spontaneous reaction under given conditions. This acceleration can be reproduced with cell-free systems as far as the available conditions are similar to physiological conditions, making enzyme ideal to accelerate reactions in many processing systems.

## Traditional Utilization of Enzymes in Foods

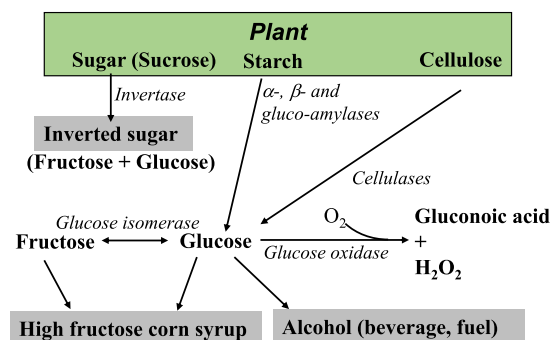
Enzymes were found and defined in 19th century as mediators in biological activities. They might not had been defined until that time, but the utilization of enzyme itself has a long history in food processing. In this section, such applications and their extensions are briefly summarized (Fig. 2).

### Amylases

One of traditional utilizations of enzyme is to use amylase (EC. 3.2.1.1 and EC.3.2.1.2) in cereal processing, especially cereal-based alcohol beverages (Fig. 3). Cereals are plant seeds, and are rich in starch. The seeds use their starch to provide the initial energy for germination and shoot/root formation until the plants can start photosynthesis. Metabolism in plant use glycolysis system to generate ATP as the energy source. A common starting material for this energy production is glucose, and starch provides glucose through their hydrolysis in the germination stage. Amylases are enzymes that catalyze this hydrolysis and are activated and synthesized in the event of germination when water, oxygen, light and temperature conditions meet the requirements (Georg-Kraemer et al., 2001; MacGregor, 1977).



**Figure 2** Enzyme Influences and Applications in Food Processing. Blue arrows indicate the enzyme reactions by indigenous enzymes and that we utilize. They give the influences on foods as shown by gray arrows. Green, black and red arrows indicate plants-specific, common and animal specific activities in general metabolisms.



**Figure 3** Enzyme Reactions of Plant Carbohydrate Processing. Plant carbohydrates (starch, sugar, and cellulose) are processed using enzymes to produce value-added food products.

From an ancient time, human have utilized amylases to saccharify the seed starch, notably to produce alcohol beverages from cereals (Damerow, 2012; Legras et al., 2007). Archaeologists speculate that beer was brewed in Mesopotamia where cultivation of wheat was first adapted as early as 10,000 BCE, and written evidences are found in Sumer/Mesopotamia and Egypt that are dated back at least 4000 years ago. As in many other traditional enzyme utilizations, they obviously did not know why cereals could be converted into alcohol beverages, but they knew that the malting process followed by a boiling step can ferment cereal extracts to beer.

In the traditional brewing, all amylase activities come from the cereal itself. Soaking seeds in water can stimulate the amylase activity in cereals and start to convert starch into maltose (glucose disaccharide). Fortunate coincidence for human being is the fact that amylase enzymes in cereal are fairly heat stable (Bush et al., 1989). This property allows us to extract converted sugars and residual starch from cereals in hot water (65 °C), yet keep the activity of amylase to process more starch into fermentable sugars during and after extraction of soluble materials in cereals. Quality of beer also heavily depends on the water quality. It is partly because amylase is activated and stabilized in the presence of calcium cation (Bush et al., 1989).

While traditional brewing only uses amylases in cereal, contemporary brewing may use exogenous amylases to ensure the saccharification of starch to maintain the consistency of beer quality among batches (Guerra et al., 2009). A major source of exogenous enzyme is aspergillus mould. This mould is traditionally used in brewing rice wine. The rice wine is made from rice, but unlike beer, raw materials are polished to remove kernel and germ, i.e., enzymes are removed. So that the soaking does not stimulate the saccharification. Instead *Aspergillus oryzae* is inoculated on the steamed rice to saccharify starch (Teramoto et al., 1993). Aspergillus amylases are commercially available for industrial applications and are used as exogenous enzymes in the process of beer making as adjunct materials for brewing (Guerra et al., 2009). In addition to aspergillus mould, *Bacillus amyloliquefaciens* and *B. licheniformis* are used to provide thermo-stable amylases (Guerra et al., 2009).

In addition to amylase enzymes, hemicellulases, cellulases, and proteinases are often used to increase the efficiency of saccharification and filtration and to allow the use of adjunct starchy materials (such as other cereals and low-quality barley malt) (Briggs et al., 2004). The addition of these enzymes can degrade cellulose/hemicellulose matrix to expose more starch to amylase activities.

### Proteinases

Proteinases (EC. 3.4.x.x) are often found in the traditional food processing. A representative proteinase application is the cheese-making process. Cheese is a product made of aged milk protein coagulum. Main milk proteins are grouped into two: caseins and whey proteins (Walstra et al., 2005). Casein proteins are found as micelles (colloidal particles) in milk, where hydrophobic  $\alpha$ - and  $\beta$ -caseins form the core and hydrophilic  $\kappa$ -casein is concentrated on the surface of micelles (Walstra et al., 2005). This structure allows water-insoluble  $\alpha$ - and  $\beta$ -caseins to be suspended in milk. Chymosin proteinase hydrolyzes  $\kappa$ -casein into hydrophobic para- $\kappa$ -casein, making micelles instable in the water phase (Walstra et al., 2005). This results in the coagulation of casein micelles to form cheese curds. Chymosin is the enzyme found in calf's stomach juice, and has a high activity to coagulate milk casein proteins to assist the digestion of the protein. Chymosin is collected from stomach juice of young cow after slaughter. Thus, the supply of chymosin depends on supply of calf meat. In 70's to 80's, the demands for beef and cheese went up. Since the cows were raised to fully-grown cows to yield more meat to meet these demands, the number of calf stomach supply was not sufficient to cover the required amount of chymosin for cheese making (Harris et al., 1982; Flamm, 1991). The hunt for alternative enzymes were extensively conducted, but none of them could produce the cheese as high in quality as using calf chymosin. Today chymosin are produced from cloned bacteria, and it makes the supply and price of chymosin more stable (Flamm, 1991).

Another traditional utilization of proteinases is found in meat tenderization. Meats are aged for 5 days to 2 weeks after the animals are slaughtered (Huff Lonergan et al., 2010; Ouali, 1990). During this stage, muscle is ripened into meat with the indigenous enzyme activities. In muscle, many different proteinases are present, and their specificities towards different meat proteins and their working pH ranges vary (Huff Lonergan et al., 2010; Ouali, 1990). As the ageing proceeds, remaining metabolic activities in meat produce lactic acids and the pH is gradually lowered. During this process, indigenous proteinases work on a variety of meat proteins, developing texture and flavour changes in meat. This process can be enhanced by injecting exogenous enzymes into meat during ageing (Huff Lonergan et al., 2010; Ouali, 1990).

Furthermore, many fermented foods develop their distinctive flavour due to protein hydrolysis by proteinases secreted by microbes. For example, Asian fermented soybean products (natto, tempeh, soy sauce, miso, and so on) and many types of cheese use *Bacillus subtilis*, *Aspergillus* spp., *Rhizopus* spp., *Penicillium* spp., and lactic acid bacteria as fermenting microbes (Rhee et al., 2011; Batra and Millner, 1974; Aidoo et al., 2006; Mine et al., 2005). These microbes yield proteinases in the fermentation, and hydrolyze proteins in the materials. While proteins are generally tasteless and odour-less, their hydrolysate shows a variety of taste based on the peptide profile generated by the microbial proteinases.

### Lipases

Lipases (EC. 3.1.1.3) are another group of enzymes that influences many types of fermented products. During fermentation, lipases secreted from microbes to hydrolyze triglycerides (Seitz, 1974). While triglycerides are odour-less, their hydrolysis products (free fatty acids) have distinctive smell because of liberated short-chain, volatile fatty acids. A small amount of hydrolysis can generate the smell in food products. In fermented foods, the developed smell is considered as their aroma, and can stimulate our appetite. Aroma of cheese is one of such examples and cheese is loved for their aroma as well as taste. Meanwhile, the excess smell development in fresh fluid milk and butter are called as rancidity and are considered as food spoilage by most.

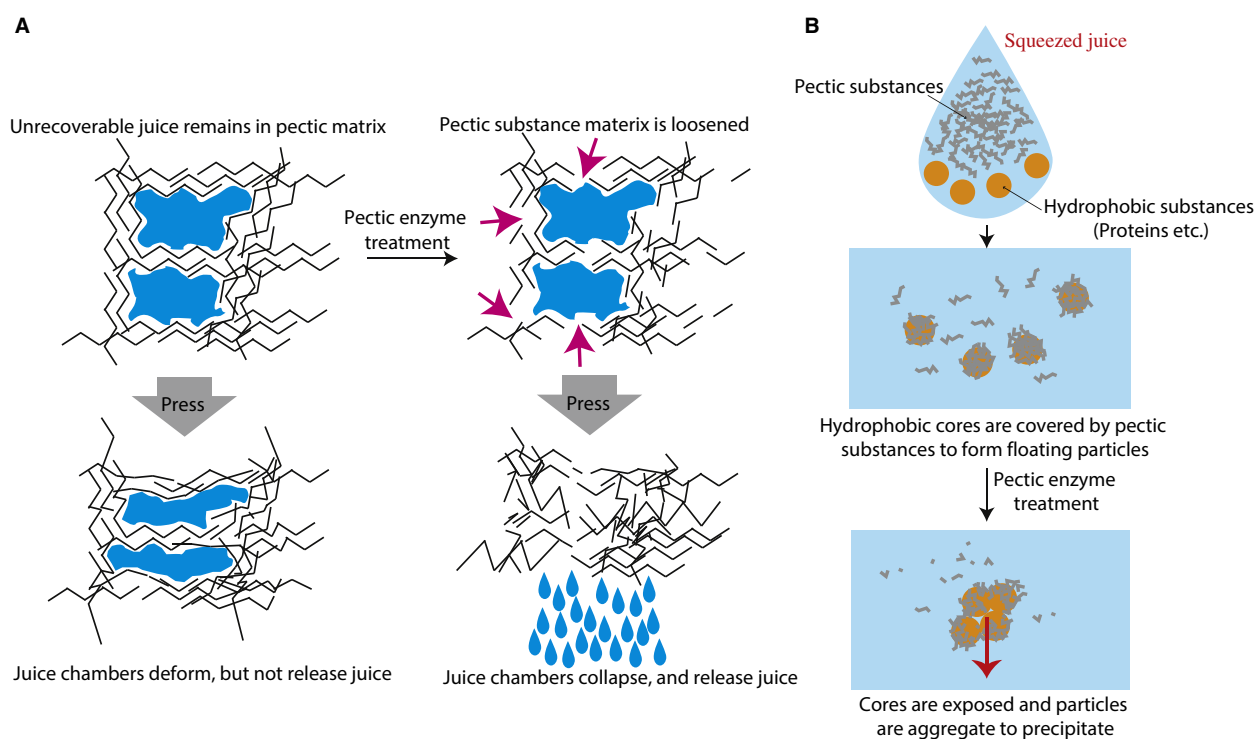
## Modern Enzyme Utilization in Foods

In addition to the traditional utilizations of enzymes, we now use enzymes intentionally to achieve certain effects in food products. The advancement of science allows us to rationally introduce enzymes in the food system and give a desired result within a short time and with a reasonable efficiency and costs. In this section, some of such applications are summarized.

### Pectinases

Fruit juice is a common commodity in the food supply and have been consumed for many centuries. As far as the juice produced at home, many people do not mind about the moderate yields and cloudiness after the squeezing of fruits. However, the juice industry needs high yields from fruits and crystal-clear juice for some juice (such as apple cider). The influential factor in these issues is the presence of pectin (Pinelo et al., 2010; McLellan et al., 1985). Pectin (pectic substances) is polymers of saccharides including galacturonans, rhamnogalacturonans, arabinans, galactans, and arabinogalactans (Thakur et al., 1997). These polymers are made of galacturonic acid, L-rhamnose, arabinose, and galactose. Among them, galacturonic acid consists of >97% of pectic substances, i.e., mostly galacturonan or rhamnogalacturonan (Thakur et al., 1997). Galacturonic acid has a carboxyl group on its sugar ring (i.e., 2,3,4,5-tetrahydroxy-6-oxo-hexanoic acid), and 75% of carboxyl group are esterified with methanol. The degree of methoxylation is a determinant of hydrophobicity of pectic substances (Thakur et al., 1997). Pectin forms skeletal matrix of fruit body. The matrix entraps the juice and often resists to the pressure (Fig. 4A). The other issue, juice cloudiness, is caused by liberated pectic substances (Pinelo et al., 2010; McLellan et al., 1985; Ceci and Lozano, 1998). The juice press squeezes the fruits and a part of pectic substances are liberated from fruits flesh. Due to their hydrophobicity, liberated methoxy-galacturonan can aggregate with other galacturonan and hydrophobic substances, and form colloidal particles. These particles give the cloudy looking in the juice (Fig. 4B) (Pinelo et al., 2010; McLellan et al., 1985; Ceci and Lozano, 1998).

To address these issues, pectic enzymes have been utilized in the juice industry (Fig. 4) (Pinelo et al., 2010; McLellan et al., 1985; Ceci and Lozano, 1998). Pectic enzymes are a family of three enzyme groups that are classified according to their substrate specificity and reaction mechanisms. The three groups are polygalacturonases, pectin esterases, and pectate lyases (Jayani et al., 2005; Kashyap et al., 2001). Pectin esterase (EC. 3.1.1.11) is a hydrolase that cleaves off a methoxy group from a methoxylgalacturonate unit in galacturonan, and polygalacturonase (EC. 3.2.1.15) hydrolyzes galacturonan (poly-(methoxy)galacturonic acid) into smaller



**Figure 4 Pectic Enzyme Utilization.** (A) When fruits are squeezed, pectin/cellulose flesh matrix are distorted without allowing the entrapped juice sipping out. When fruit flesh is treated with pectic enzymes, the wall of juice chambers is disintegrated to allow juice squeezed out from the chamber upon pressure application. (B) Pectic substances tend to aggregate around hydrophobic substances, such as denatured proteins, once pectin are liberated from plant flesh matrix. The aggregation particles of pectin are small enough to be suspended in the juice as colloids, showing cloudiness in juice. Pectic enzymes can increase the solubility of pectic substances through removal of methoxy group and fragmentation of long chains. These degradations allow the colloid particles aggregating between them through exposure of hydrophobic core of particles.

pieces. Non-methoxy galacturonan is hydrophilic; whereas methoxy groups give galacturonate hydrophobicity, leading to aggregation of pectin in fruits juice processing. A combination of two enzymes allows floating particles of pectin aggregate (cloudiness of juice) becoming soluble (Pinelo et al., 2010; McLellan et al., 1985; Ceci and Lozano, 1998), which clarify juice (Fig. 4B). The third pectic enzyme (pectate lyase; EC 4.2.2.2) also truncates the galactronan with a non-hydrolysis mechanism. These pectic enzymes can be utilized to loosen the structure of pectic skeleton of fruits fresh. The pectic skeleton of these fruits often restricts the recovery of juice from the fruits (Fig. 4A). It was shown that even pulp parts of fruits, which usually yield little juice by simple pressing, can yield 82% of weight as juice after treatment with pectic enzymes (Chauhan et al., 2001).

### Cellulases

For juice extraction, there is another substance that affects the fruits flesh matrix: cellulose. The hydrolysis of cellulose is shown to increase the yields of fruit juice (Kashyap et al., 2001). When using cellulases (EC. 3.2.1.4) in the combination with pectic enzymes, the enzyme mixture can totally liquefy the fruits. The process can be utilized to make juice without producing pomace (Kashyap et al., 2001).

Cellulase is also an important enzyme for sustainability of Earth's resources. As everyone now realizes, fossil energy emits carbon dioxide. One of the countermeasures for the excess CO<sub>2</sub> emission is to use biofuels. Bioethanol is one of the most common biofuels in today's technology basis. Cellulase enzyme is utilized to provide fermentable sugar from inedible materials, and this second-generation bioethanol is becoming an industrial choice for bioethanol production systems (Sun and Cheng, 2002; Cannella and Jørgensen, 2014; Tabka et al., 2006).

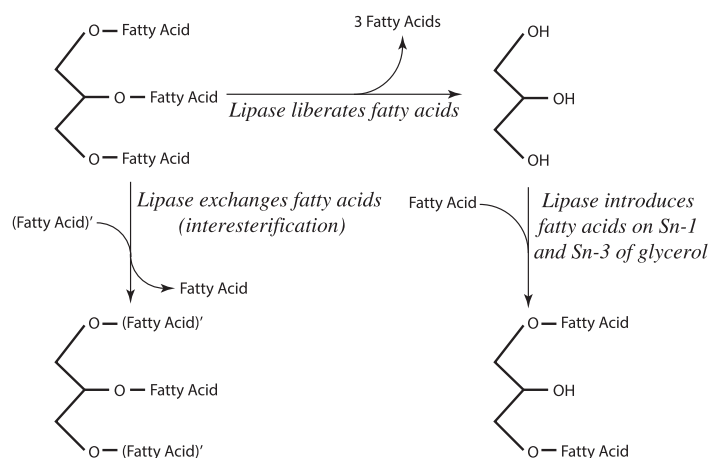
### β-Galactosidases

Dairy products are one of the largest sectors in the food industry in many developed countries. Milk is a 'perfect' food that contains all required nutrients to support infant growth; while many adults have issues with consuming dairy products. Two major issues of dairy products are food allergy and lactose intolerance (Prentice, 2014; Bordoni et al., 2017). Lactose is the major carbohydrate found in milk, and virtually it is exclusively produced as a milk ingredient in nature. Once children are weaned off from mother's milk and start to eat other foods, they lower the production of enzyme to hydrolyze lactose. How low this enzyme production becomes in adults depends on ethnic (genetic) groups and food consumption customs. If an individual does not produce enough of this enzyme, ingested lactose (in dairy products) reaches his large intestine. The assimilation of disaccharides is slower than monosaccharides, and therefore the remaining lactose is rapidly consumed by intestinal bacteria, producing carbon dioxide gas and lactic acid that stimulate the bowl movement to cause diarrhea (Deng et al., 2015).

β-galactosidase (aka lactase; E.C.3.2.1.35) is the enzyme that hydrolyzes lactose into galactose and glucose. To address the lactose intolerance issue, this enzyme is used in two ways: as a processing agent and as digestion assisting pills. By addition of β-galactosidase in the milk processing, lactose can be hydrolyzed in the milk (Deng et al., 2015; Corgneau et al., 2017). Resulted monosaccharides are easy to assimilate in our intestinal system and cause no diarrhea. Further this enzyme can be used to produce sweetening agents from a byproduct of dairy processing (i.e., whey) (Li et al., 2015).

### Lipases

As mentioned in an earlier section, lipase (E.C.3.1.1.3) catalyzes the hydrolysis of triglycerides to yield three fatty acids and glycerol. In the recent decades, the utilization of lipase has been developed to produce foods with less obesity issues (Fig. 5). One of such



**Figure 5 Interesterification and Esterification by Lipases.** We can manipulate the fatty acid moieties on triglycerides through lipase activities. Depending on the free fatty acid availability, lipase can replace or introduce fatty acids on glycerides (glycerol). Utilization of these reactions can yield specific-triglycerides and diglycerides with desired fatty acids.

applications is to form diglycerides (Singh and Mukhopadhyay, 2016; Berger et al., 1992). Many lipases have preferences in the position on triglycerides; while the length of fatty acid does not affect the hydrolysis much. The most preferred position is Sn-1 and Sn-3 positions (Xu, 2000). Since enzymes catalyze chemical reaction, but does not change the equilibrium between substances, most of enzymes can catalyze reverse reactions. Mixture of glycerol and fatty acids can yield diglycerides which have fatty acids on Sn-1 and Sn-3 positions when catalyzed by lipase. Diglycerides have similar properties to triglycerides as food ingredients, but ingestion of diglycerides produce less energy.

Lipases are also utilized for interesterification to replace fatty acids of triglycerides with different fatty acids (Fig. 5) (Macrae, 1983). This reaction can be achieved by chemical catalyst ( $\text{NaOCH}_3$ ) or by lipase enzymes. Chemical interesterification is a random reaction and is not suitable for position specific interesterification; whereas the position-specific interesterification by lipases is useful to mimic natural triglycerides from less expensive triglycerides. For example, lipase is utilized to produce cocoa butter alternatives. Cocoa butter triglycerides are mostly made of three fatty acids: oleic acid, stearic acid, and palmitic acid, and their simple compositions make the melting temperature range of cocoa butter narrow: at body temperatures, cocoa butter melts. A substitute of cocoa butter was developed using lipase on sunflower oil. Sunflower oil is an inexpensive oil and is widely available (the fourth common plant oil product). A sunflower cultivar has 50% ~ 60% oleic acid contents in its oil. Using lipases, interesterification of sunflower oil is processed with excess stearic acid. This interesterification introduces stearic acid on Sn-1 and Sn-3 position (i.e., 1,3-stearic acid-2-oleic acid). This chemical structure is the same found in cocoa butter, and thus this interesterified sunflower oil product shows the similar properties to cocoa butter (Chang et al., 1990). Another example of interesterification is found in human milk fat simulated oil. Baby formula is formulated to include all necessary nutrients for baby, and is made of commonly available materials, such as milk ingredient. Since milk fat is an expensive fat and milk is known to cause allergy, development of substitute from vegetable oil was desired. Vegetable oil often contains high amount of saturated fatty acids, meanwhile human needs higher amount of unsaturated fatty acids. Using oleic acid, linoleic acid, and linolenic acid, palm oil is interesterified. The resulted oil has unsaturated fatty acids on Sn-1 and Sn-3 position and palmitic acid on Sn-2 position. This composition is similar to human milk fat (Lai et al., 2000; Quinlan et al., 1995; Ghazali et al., 1995).

### Lipoxygenases

While the unsaturated fatty acids are beneficial and essential for our health, they can act negatively on the preservation of certain foods, leading to formation of off-flavour and discolouration. This is especially critical for vegetables and legume since they have high activities of lipoxygenase (EC 1.13.11.12) (Hsieh, 1994; Eskin et al., 1977). Lipoxygenase is an enzyme to catalyze the addition of molecular oxygen on to a double bond in the fatty acid. Resulted peroxide molecular species are a powerful oxidative agent in vegetables and legume (Brecht, 1995; Rolle and Chism, 1987). The peroxides also cleave themselves and yield an alcohol and a shorter fatty acid (for example, linoleic acid becomes hexanol and 12-oxo-*trans*-10-dodecanoic acid, yielding different smell from the original material). But more critical factors in off-flavour formation and discolouration are that lipoxygenase can yield radicals (Donnelly and Robinson, 1995). The radical reaction does not diminish radical; it passes the free radical to another molecule until it reacts with another radical. Thus, one radical affects many molecules including vitamins and proteins. It results in low vitamins and high volatile compounds such as sulfur compounds. Lipoxygenase is not only found at high activities in vegetables and legumes, it is also a hardy enzyme that can survive in many different treatments and they can even work at a very low temperature, i.e., frozen vegetables can develop off flavour during the frozen preservation (Sheu and Chen, 1991; Güneş and Bayindirli, 1993; Rodriguez-Saona et al., 1995; Velasco et al., 1989). The removal of lipoxygenase can be achieved by exposure to ethanol, mechanical milling, deblanching and alkali-heat treatment.

### Glucose Oxidases

While oxidation is considered as food deterioration, some oxidases are utilized in the food processing for the positive effects. Glucose oxidase (E.C.1.1.3.4.) is utilized for elimination of molecular oxygen, *in situ* production of hydrogen peroxide, and *in situ* production of a weak acid (Fig. 3).

Molecular oxygen is one of the most common oxidative agents abundant in the processing and storage. While we can pack foods in air-tight packages (such as cans) to prevent the oxygen exposure, the existing oxygen in the package and materials can be enough to cause oxidation of foods during the storage. The removal of molecular oxygen in the packages is a critical factor to attain a long shelf life. Glucose oxidase can use glucose to capture oxygen. A small dose of glucose oxidase can use glucose in foods to deplete molecular oxygen completely from air-tight packages (Dondero et al., 1993; Field et al., 1986). Moreover, the products of this enzyme is gluconolactone (spontaneously becomes gluconic acid) and hydrogen peroxide, thus we can use the reaction of glucose oxidase positively for *in situ* production of acid (Fox and Stepaniak, 1993) and hydrogen peroxide (Dobbenie et al., 1995). An issue of food processing is browning through Maillard reactions that is accelerated with heat (even at Pasteurization temperature), leaving brown colour in the food products. Glucose oxidase can be utilized to remove glucose to suppress the Maillard Reaction (Sankaran et al., 1989; Low et al., 1989).

Glucose oxidase is also used for glucose quantification (Wilson and Turner, 1992). This enzyme can be immobilized without sacrificing much of its activities (Wilson and Turner, 1992). The immobilized glucose oxidase is set with platinum, then hydrogen peroxide generated from glucose oxidase can be reduced into water and oxygen with the platinum catalyst. This reduction releases an electron that can be detected as an electric signal. The system is widely used as glucose electrodes in the processing and research.



### Glucose Isomerase

Another industrial enzyme that takes glucose as the substrate is glucose isomerase (E.C. 5.3.1.5) (Fig. 3). This enzyme is physiologically found as xylose isomerase (Bhosale et al., 1996). The difference between xylose and glucose is the presence of 6'-carbon and hydroxyl group in glucose. Xylose isomerase does not severely recognize the presence of 6'-carbon, and can take glucose as the substrate at a lower reaction rate. While  $K_m$  value (Michaelis Constant) for xylose is reported as 5 ~ 93 mM, the  $K_m$  value for glucose is 86 ~ 920 mM (Chen, 1980). In physiological settings, a high  $K_m$  towards glucose suppresses the glucose reaction and rather xylose is selectively isomerized. However, if xylose does not exist and glucose is abundant, this enzyme can isomerize glucose to fructose, and *vice versa*. The importance of this enzyme in industrial settings is in processing of high fructose corn syrup (HFCS) (Bhosale et al., 1996). Hydrolysis of starch, as discussed earlier in the amylase section, can generate glucose. Starch hydrolysate has been known as a sweetener for a long time. However, glucose is not as sweet as sugar (sucrose). Meanwhile, fructose is about 1.5-times sweeter than sucrose. When applying glucose isomerase on a glucose solution, the conversion of glucose to fructose proceeds and eventually reaches an equilibrium at 45:55 between fructose and glucose. This product is commonly referred as HFCS45 (using corn starch as the starting material). Theoretically this mixture shows sweetness about 10% to 20% above the same weight of sucrose. In the last few decades, more individuals pay attentions to calorie intake, and HFCS45 are utilized as an alternative sweetener. It is also inexpensive and supply is stable, compared to sucrose. Thus, certain foods (such as soda pops) use HFCS45 (or its higher-fructose derivative, HFCS55) as a substitute of sugar to achieve the same sweetness at a lower cost and calories.

### Polyphenol Oxidases

Browning of fresh plant products is a critical issue for food supply (Lee and Whitaker, 1995). Consumers tend to choose the products with the appearance, and brown vegetables are not their preferred choices. The browning of fresh products is due to the enzyme within the plant and molecular oxygen in the atmosphere. The enzyme is polyphenol oxidases (PPO; E.C.1.10.3.1 and E.C.1.14.18.1) (Yoruk and Marshall, 2003; Mayer, 2006; Queiroz et al., 2008). There are two enzymes that are referred as PPO: catechol oxidase and tyrosinase. The former enzyme catalyzes oxidation of catechol and *o*-diphenol to a corresponding quinone (Yoruk and Marshall, 2003), whereas tyrosinase catalyzes phenol to *o*-diphenol as well as further oxidation to quinone (Mayer, 2006). The resulted quinone substances spontaneously polymerize into polyphenol, i.e., melanin. This tannic substance shows brown colour in the food products. Prevention of PPO-browning can be achieved in two ways: elimination of oxygen and reduction of enzyme activities (Yoruk and Marshall, 2003; Sapers and Miller, 1998). The elimination of oxygen is achieved by physical separation of the fresh products from molecular oxygen. For enzyme inhibition, addition of salt is known as an effective measure. The browning also has an issue in the browning during fruit juice processing. We can add antioxidants to prevent the browning, but there is a limited effect with a limited dose of inhibitory compounds. Thus for the juice making, another countermeasure is often considered. PPO is mostly found as in the form associating with pulp portion of fruits. Therefore, the removal of pulp as much as possible can make the PPO activities low, and combined use of inhibitors can reduce the browning defects drastically (Yoruk and Marshall, 2003; Sapers and Miller, 1998).

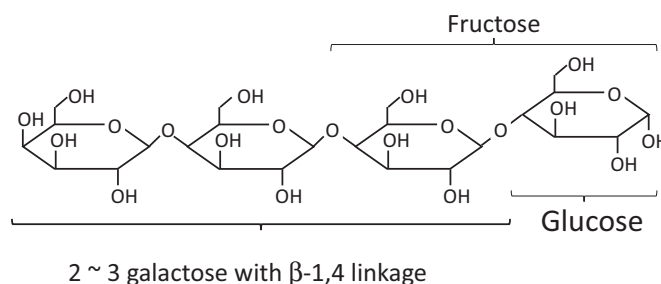
### Recent Developments of Food Enzyme Applications

The above section has discussed the development/utilization of enzymes in the past decades, and they are currently employed in the food processing practice. In this section, we look into more recent R&D to use enzymes in food processing.

### Prebiotics and Lactases

Prebiotics promote probiotic microbes' activities. There are many prebiotics that have been reported, including resistant starch, pectin, and  $\beta$ -glucans (Lomax and Calder, 2008; Andersson et al., 2001). Majority of prebiotics are so-called dietary fibre, but there are other smaller prebiotics compounds. Such example is oligo-saccharides. Many of oligosaccharides are found in nature and by synthesis, and they can be taken as purified preparations, synthetic additives, and natural ingredients in food materials. For synthesis of oligosaccharides, enzyme activities can be utilized. Sialidases (E.C.3.2.1.18),  $\alpha$ -1-fucosidases (E.C.3.2.1.51) and  $\beta$ -galactosidases (E.C.3.2.1.23) are studied for their ability to synthesize oligosaccharide prebiotics (Zeuner et al., 2014). Human milk oligosaccharides made through transsaccharification by sialidases and fucosidases have been extensively reviewed (Bode, 2015). While transsialylation and transfucosylation naturally occurs in human milk,  $\beta$ -galactosidase mediated oligosaccharides are studied to produce oligosaccharides *in vitro* (Vera et al., 2012; Yu and O'Sullivan, 2014; Rodriguez-Colinas et al., 2012). As discussed earlier,  $\beta$ -galactosidases (lactases) are the enzymes which hydrolyze lactose into galactose and glucose. When lactose concentration is high,  $\beta$ -galactosidase can catalyze the transgalactosidation to produce a prebiotic compound, galactooligosaccharides (GOS), where galactose of lactose is transferred onto another galactose moiety of lactose and GOS. This reaction produces a trisaccharide Gal- $\beta$ -(1 $\rightarrow$ 4)-Gal- $\beta$ -(1 $\rightarrow$ 4)-Glc and a tetrasaccharide Gal- $\beta$ -(1 $\rightarrow$ 4)-Gal- $\beta$ -(1 $\rightarrow$ 4)-Gal- $\beta$ -(1 $\rightarrow$ 4)-Glc (Fig. 6).  $\beta$ -galactosidase studied in the production of GOS are from *Bifidobacterium longum* (Hsu et al., 2007), *Kluyveromyces lactis* (Rodriguez-Colinas et al., 2011), *Aspergillus oryzae* (Vera et al., 2012; Albayrak and Yang, 2002), *Lactobacillus pentosus* (Maischberger et al., 2010), *Lactococcus lactis* (Yu and O'Sullivan, 2014), and *Bacillus circulans* (Rodriguez-Colinas et al., 2012). The conversion of lactose has reached GOS at ~50% of total





**Figure 6 Galactooligosaccharides.** The galactose moiety in lactose is transsaccharified onto another lactose, and tri-saccharides (and another transsaccharification onto tri-saccharides for tetra-saccharides) are produced through  $\beta$ -galactosidase activities.

carbohydrates (Vera et al., 2012; Yu and O'Sullivan, 2014; Rodriguez-Colinas et al., 2012) with initial lactose concentrations at 40% ~60%. When lactose concentration is within the order observed in skimmed milk (4.6%), the conversion rates of lactose can reach 50% (Rodriguez-Colinas et al., 2012) to 72% (Yu and O'Sullivan, 2014).

### Bioactive Peptides and Proteinases/Peptidases

In the last twenty years, proteolysis of food proteins is found to have additional potential health benefits. The proteolysis yields smaller peptides and some of these peptides can have physiological activities: such as antihypertensive, antioxidative, calmodulin-modulating, anticancer, immunoregulatory, anti-inflammatory, and antithrombotic activities. These peptides are now classified as "bioactive peptides (BAP)". It was first observed in the milk proteins, and then expanded to egg, beef, fish meat, sea shell proteins, legume protein and so on (Udenigwe and Aluko, 2012). Many proteinases are artificially employed to hydrolyze these proteins, and the peptides in hydrolysate are examined for physiological activities. In order to increase the yields of BAP, many efforts have been conducted including denaturation of proteins (Le Maux et al., 2016; Korhonen et al., 1998) and prediction of potential BAP activities (Gu et al., 2011). A critical issue in the production of BAP is how to separate it from the hydrolysate. Most products claiming BAP activities use hydrolysates as the ingredients, and the effects per protein amount is still limited (Agvei and Danquah, 2011).

### Active Packaging

Another recent development is active packaging of foods. The packaging materials have been inert by nature. It is effective to protect foods from mechanical damages, dirt contamination, distribution ease, and so on. Meanwhile, they may not prevent growth of already-contaminated microbes, oxidation by atmospheric oxygen, and influences from ethylene for plant produces. Active packaging materials address these issues. For example, ethylene, which is a phyto-hormone to ripen fruits, can be scavenged by the presence of potassium permanganate in the packaging materials (Vermeiren et al., 2003), extending the shelf life of fruits. There are many materials and methods proposed for active packaging: two types of active packaging use enzyme activities for their actual active factors. One of them is oxygen scavenging and the other is antimicrobial packaging (Vermeiren et al., 1999). Oxygen scavenging packaging uses oxidases, such as glucose oxidases (Fortier and Bélanger, 1991; Wong et al., 2008), as the active ingredient and consume available oxygen in the package. It is, however, less cost effective than to use inorganic materials such as ferrous cations. For the antimicrobial packaging, enzymes such as lactoperoxidase (Min and Krochta, 2005), lysozyme (Padgett et al., 1998), glucose oxidase (Fortier and Bélanger, 1991; Wong et al., 2008) and lactoferrin (Min et al., 2005) are shown for their positive results. An issue of enzyme usage in the package is their stability towards temperature, moisture, and pH. It is still underdevelopment, but in future, more enzyme utilization in the active packaging is expected.

### Food Wastes and Enzymes

Lastly, the enzyme utilization in the food waste treatment should be mentioned. Foods are organic materials, and their production has certain impacts on the environment. Meanwhile, about 30% of foods are reportedly wasted. A majority of food waste is carbohydrates, and its utilization can contribute to the sustainable food supply and environmental protection. Many enzymes are available to hydrolyze carbohydrates, such as amylases, pectinases and cellulases. Along with hydrolases of other food waste components, such as proteinase and lipases, enzyme reactions can be utilized to degrade food waste to provide the feedstock for other purposes, including biofuel production (Uçkun Kiran and Liu, 2015; Uçkun Kiran et al., 2014).

## Conclusion

Enzymes are considered as efficient tools to decrease food processing costs, to reduce the waste and to provide more safe and healthy foods. Many of applications have been utilized and are being developed. In next decades, we expect to see more enzyme utilizations in the processing of foods, in the analyses of foods and in the reduction of impacts on the natural environment.

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# Encyclopedia of Food Chemistry: Fat replacers

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## Glossary

**Fat extender** used to reduce the concentration of fats by replacing them with a standard lipid combined with other ingredients.

**Fat mimetic** used to replace fat however it requires a high-water content for to attain its desired functionality.

**Fat Substitute** used to replace fat on a weight-by-weight basis but is resistant to hydrolysis by digestive enzymes.

**Low-calorie fat** used to substitute fat with a synthetic fat with one or more of the fatty acids on the glycerol backbone being replaced with something other than a fatty acid.

## Introduction

Reduced fat products are common place in the food landscape of today; consumers often choose these products to control or restrict caloric intake or to reduce the perceived threat of coronary heart disease (CHD). Messages centering on lipids, especially around saturated and *trans* unsaturated, has led a large cohort of consumers to shift their diet towards consuming more low-fat options. However, the paradigm that all saturated and *trans* fats have deleterious biological consequences has shifted once again and it is now being suggested that saturated fat and *trans* fats from ruminants may not be implicated in CHD as once believed (Chavan et al., 2016; de-Souza et al., 2015). In practice, because of recommendations to limit saturated fats to no more than 10% and *trans* unsaturated fats to no more than 1% of a person's dietary energy, consumers often choose low-fat options. Although mandates exist to reduce hardstock fats from foods, a simple fat reduction is associated with undesirable changes to texture and sensory perception of fat products (Liu et al., 2016b). Up to now, there has been no single fat replacer identified that is able to mimicking the diverse functionalities of fat across a wide range of foods therefore fat replacers are targeted to specific applications (Table 1).

Replacement of the fat component of foods is often difficult because they have multi-functional roles in foods as they influence chemical, physical and sensory attributes of foods as well as their processing characteristics. Simply reducing the content of fat causes quality losses that decrease the acceptance of foods (Peng and Yao, 2017). Therefore, depending on the role that fat has in determining the quality of these product it will affect the complexity of the system that must be implemented to replace that lipid fraction (Jones, 1996). Fat replacer is a broad term to describe any technology used to reduce or eliminate fat and can be subdivided into the following categories (Jones, 1996):

Fat Substitute: used to replace fat on a weight-by-weight basis but is resistant to hydrolysis by digestive enzymes;

Fat mimetic: used to replace fat however it requires a high-water content for to attain its desired functionality;

Low-calorie fat: used to substitute fat with a synthetic fat with one or more of the fatty acids on the glycerol backbone being replaced with something other than a fatty acid.

Fat extender: used to reduce the concentration of fats by replacing them with a standard lipid combined with other ingredients.

Fat replacers can also be sub-divided based on the primary reason for the removal of fat. Caloric reducers replace a fraction of all lipids, while hardstock fat replacers only replace a fraction of the *trans* unsaturated and saturated fats (Fig. 1) (Akoh and Decker, 1995; Rogers, 2011). Caloric reducers include olestra (Bergholz, 1992), non-digestible carbohydrates (Peng and Yau, 2017), proteins (Singer, 1996), while *trans* unsaturated and saturated fat replacers are often small molecules such as sorbitan tristearate, phospholipids, monoglycerides, and modified fatty acids (Rogers et al., 2014, 2009; Wang and Rogers, 2015).

## Carbohydrate-Derived Fat Replacers

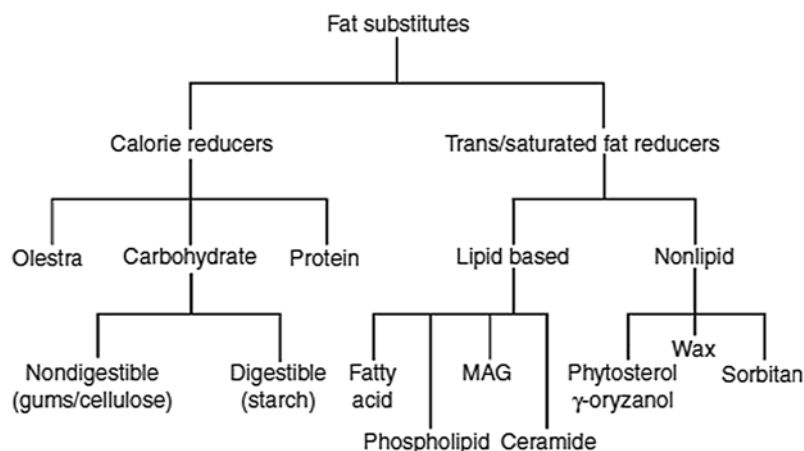
The molecular diversity of carbohydrates gives rise to various structural and physicochemical properties allowing formulation chemists to tailor the physical attributes that need replaced upon the removal of fat (de Souza et al., 2015; Peng and Yau, 2017). Furthermore, methods are available to modify carbohydrates; for example, substituted starch using octenyl succinate or cross-linked starches are commonly used as a fat replacer because of the indigestibility and further caloric reduction. When a fraction of fat is removed, it is most commonly replaced with carbohydrates such as starch, maltodextrin, gums, or dietary fiber with or without additional water.

Starch is typically used in its non-gelatinized form because the starch granules are on the same order of magnitude as fat crystals or fat emulsion droplets which emulates the mouthfeel imparted by lipid droplets (McClements et al., 2017). Because of the size and physical attributes of starch granules, they are commonly cross linked to prevent swelling and leaching of the amylose and in numerous cases amylopectin is debranched (Klaohanpong et al., 2017). Starch based gels have been proven capable of replacing

**Table 1** Classification of fat replacers by nutrient source and function properties

Type of fat substitute	Nutrient source (energy density)	Functional properties	Use in food
Derived from carbohydrate Polydextrose	Water-soluble polymer of dextrose (1 cal g <sup>-1</sup> )	Bulking and retaining moisture	A wide range of foods, including baked goods, confections, and frozen desserts
Modified food starch	A variety of starch sources (1–4 cal g <sup>-1</sup> )	Modifying texture, gelling, thickening, and stabilizing	Processed meats, salad dressing, baked goods, frozen desserts, etc.
Dextrin and maltodextrins	A variety of starch sources (4 cal g <sup>-1</sup> )	Modifying texture and bulking	Baked goods, dairy products, salad dressing, sauces, and spreads
Gums and pectin	Xanthan, guar, locust bean, carrageenan, alginates and fruit	Retaining moisture, mouthfeel and modifying texture	Wide range of products, including baked goods, sauces, and salad dressings
13-Glucan	Various plant sources (virtually noncaloric)	Modifying mouthfeel, texture, and pourability	Dairy products
Derived from protein	Soluble fiber extracted from oats (1–4 cal g <sup>-1</sup> )	Adding body and texture	Baked goods and a variety of other food products
Microparticulated protein and modified whey	Denatured or microparticulated protein from egg/milk (1–4 cal g <sup>-1</sup> )	Modifying mouthfeel	Dairy products, spreads, and bakery products
Derived from fat Olestra	Sucrose polyester with triglycerides (noncaloric)	Modifying texture and mouthfeel	Savory snacks (stable for fried foods)
Caprenin and salatrim	Caprylic, capric, behenic acid, and glycerine, or triglyceride of short- and long-chain fatty acids (5 cal g <sup>-1</sup> )	Simulating properties of cocoa butter	Confections, baked goods, and dairy foods
Mono- or diglycerides	Derived from vegetable oil and emulsified with water (9 cal g <sup>-1</sup> )	Adding moisture and modifying texture and mouthfeel	Baked goods, vegetable dairy replacers

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**Figure 1** Primary strategies to substitute fats in edible food applications. Reproduced from Rogers, M.A., 2011. Novel lipid substitutes. In Moo-Young, M. (Ed.), Comprehensive Biotechnology, second ed., vol. 4. Elsevier, pp. 603–616. Reproduced with permission from Elsevier.

numerous attributes which fats contribute including: increased water-holding capacity, gel firmness and viscosity and these changes are often perceived as an increase in creaminess in liquid formulation such as low fat salad dressing (Peng and Yau, 2017).

Starch can also be partially hydrolyzed using enzymes or acid to form maltodextrin. Maltodextrins with dextrose equivalents (DE) between 2 and 4 are most commonly used as fat replacers between 1 and 5 wt% because of their ability to gel and thicken (McPherson and Seib, 1997). At 1 to 5 wt% maltodextrin, 25 to 35 wt% of the fat can be replaced in cookies while maintaining a full-bodied texture and desirable mouthfeel (Chavan et al., 2016). By selecting the appropriate DE the melting point of the maltodextrin gel can be tailored to simulate the oral melt down properties of fat (Peng and Yau, 2017). Not only does the DE effect the ability to mimic the physical properties of fats, but so does the source of the maltodextrin (Peng and Yau, 2017). Maltodextrin fat replacers are commonly employed in dairy products, confectionary, frozen desserts, cereal baked goods, and meat products because they form soft, spreadable, thermo-reversible gels with melt-in-the-mouth properties. However, maltodextrins increase



the glycemic load of the food and post meal glycemia, which are less desirable for health (Hofman et al., 2016). There are also physical limitations in its application due to the amylopectin which has a tendency to retrograde, causing setback in low-fat salad dressings and their poor freeze-thaw stability and unreliable heat and acid stability (Chavan et al., 2016).

Non-digestible polysaccharides may also be utilized as fat replacers. Microcrystalline cellulose (MCC) is partially depolymerized cellulose synthesized from  $\alpha$ -cellulose precursor. MCC is a modified form of cellulose that is composed mainly of anhydroglucose units with  $\beta(1-4)$  glycosidic bonds (Chavan et al., 2016). The majority of colloidal grades have microcrystals less than 0.2  $\mu\text{m}$  in length, that when added to an aqueous medium disperse rather than hydrate (Lucca and Tepper, 1994). MCC is typically processed with adjuvants such as guar gum which act as a 'glue' to hold the insoluble cellulose crystals in a 3D network that is thixotropic mimicking the body and mouthfeel of lipids. Other non-digestible polysaccharides such as pectin,  $\beta$ -glucan, inulin, locust bean gum have all shown promise to be used as fat replacers.

Pectin is composed of a galacturonan backbone which may be methyl-esterified giving rise to low and high-methoxy pectin. Pectin is able to gel and thicken foods; however, because it forms strong gels, there are challenges in incorporating high concentrations of pectin into foods (Min et al., 2010). The functionality of pectin is influenced by the source, which gives rise to variations in molecular weight and degree of methyl esterification. High methoxy pectin gels in the presence of high sugar concentrations in acidic conditions, while low methoxy pectin requires the addition of divalent ions such as calcium in basic conditions (Durand et al., 1990). Slendid is a citrus pectin that when combined with calcium is able to replace up to 100% of the fats in a variety of processed foods ("Low-calorie fat replacer is based on pectin," 1991). Recent work with pectin fat replacers has focused on reducing the amount of fat in processed meats, salad dressings and ice cream (Maneerat et al., 2017; Tekin et al., 2017).

Inulin is a fructosyl-fructose-linked oligomeric carbohydrate with a polymerization degree  $\approx 10$ . Inulin is a fructosyl-fructose-linked oligomeric carbohydrate with a polymerization degree  $\approx 10$ , inulin is a moderately water soluble fructosyl-fructose-linked oligomeric carbohydrate with a polymerization degree  $>10$  that is resistant to human digestion (Zahn et al., 2010). Inulin forms a short spreadable gel network, when added at high concentrations, that acts as fat replacer in spreads and fillings, dairy products, desserts and dressings (Franck, 2007; Guven et al., 2005). In the case of adding inulin to muffins, only 50% of the fat could be replaced with similar crumb firmness as the full fat counterpart (Zahn et al., 2010).

### Protein-Derived Fat Replacers

The ability to mimic fat functionality by microparticulated proteins is attributed to their spherical shape and small size that are comparable in size to oil droplets found in food emulsions. There are numerous microparticulated protein based fat replacers that are derived from whey, egg, and zein proteins (Cheftel and Dumay, 1993). Often, the microparticulated protein is applied in combination with a stabilizing polysaccharide such as carboxymethylcellulose with zein protein and xanthan with egg white protein (Liu et al., 2016b). Specifically, microparticulated proteins effectively replace the sensory perception of oil droplets in liquid and semi-solid food matrices (Liu et al., 2016a). Microparticulated whey protein has numerous applications in dairy-based products where it has been shown to provide a smooth, creamy mouthfeel in low-fat applications such as low fat cheeses, yoghurts, ice creams (Cheftel and Dumay, 1993; Liu et al., 2016a, 2016b). In the case of microparticulated whey protein, the attributes of these ingredients are dependent on the size of the particles. Small particles, contribute to the creaminess, while large particles impart a roughness which suppresses creaminess (Liu et al., 2016a).

Microparticulated proteins induce a creamy oral sensation when they are dispersed into an aqueous phase because of the 'ball-bearing' lubrication properties of microparticulated whey proteins in liquid and semi-solid food matrices (Liu et al., 2016a). The 'ball-bearing' lubrication model suggests that creaminess arises when the microparticulated protein is dispersed in the aqueous phase allowing them to roll freely over one another under an applied shear. Although microparticulated proteins increase the creaminess by reducing the friction via the 'ball-bearing' model, the underlying mechanism differs considerably from how oil droplets impart creaminess. These differences may induce changes in sensory perception of creaminess.

### Lipid-Derived Fat Replacers

The most commonly thought of lipid-derived fat replacer is olestra, which is a mixture of hexa-, hepta- and octa-esters of sucrose. Six to eight long-chain fatty acids are covalently linked to sucrose by chemical transesterification. Gastric lipases and the bacterial lipases produced in the colon are incapable of cleaving the fatty acids from the sucrose ester and as such the fatty acids are not absorbed by the microvilli but instead are excreted in the stool (Jandacek, 1982). Since olestra is lipophilic, nondigestible, nonabsorbable, it can interfere with the absorption of other lipophilic components in the diet, consumed at the same time as olestra. An advantage of olestra over other fat replacers is that the physical properties can be tailored depending on the fatty acids esterified to sucrose. For example, if highly unsaturated fatty acids are esterified to sucrose then the resulting olestra will be fluid, conversely, if long chain saturated fatty acids are used then it will be solid (Ognean et al., 2006). Olestra has very similar organoleptic and thermal properties compared to triglycerides with similar fatty acids.

Caprenin is a triglyceride that contains glycerol esterified to two medium chain fatty acids (caprylic and capric) and a long chain fatty acid, behenic acid. The reduction in calories occurs because behenic acid is only partially absorbed, while capric and caprylic acids are readily metabolized and as such caprenin provides only 5 kcal/g (Akoh, 1998). Because of the fatty acid composition, the functional properties are similar to cocoa butter allowing it to be used in soft candy and confectionary coatings. Salatrim also relies on the fact that short chain fatty acids provide fewer calories per unit weight and that long chain fatty acid, stearic acid, is only partly



absorbed resulting in 55% fewer calories than traditional fats (Akoh, 1998). Salatrim is a generic name for triglycerides that contain at least one short chain fatty acid and at least one long chain fatty acid, typically stearic acid. The physical properties of salatrim can be manipulated by chaining the amounts of short chain and long chain fatty acids allowing the melting points and hardness to be manipulated. It is sold as Benefat 1. Its primary application is as a replacer for cocoa butter.

### Small Molecule Fat Replacers

Small molecule fat replacers are used to replace the hardstock fats, i.e. *trans* and saturated fats, while maintaining the concentration of unsaturated oils and the original physical properties of the fat blend. These liquid oil gels, termed oleogels, molecular gels and/or organogels, are similar to fats in that they can undergo the sol–gel transition numerous times simply by reheating and recooling the system (Weiss, 2014). Highly effective oleogelators form gels in vegetable oils, at concentrations as low as 0.5 wt% (Rogers, 2017). Oleogels self-assemble, in vegetable oils, via non-covalent interactions (i.e. hydrogen bonding,  $\pi$ - $\pi$  stacking, electrostatic and van der Waals interactions) forming fibrillar or platelet crystals (Patel and Dewettinck, 2016; Rogers et al., 2009). These intermolecular interactions favor 1-dimensional (1D) fibrillar growth leading to the formation of a 3 dimensional (3D) networks that is capable of entrapping the liquid oil phase. Few applications of oleogels in foods exist because it is difficult to identify new food grade, inexpensive gelators; however, research in this area is increasing exponentially. Although there is a plethora of reported small molecular gelators, here we will focus only on two systems – the waxes and the phytosterol/ $\gamma$ -oryzanol system.

Although numerous waxes have been studied as potential oleogelators to replace hardstock fats, candelilla wax (CDW), carnauba wax (CBW), rice bran wax (RBW) and beeswax (BW) are of the greatest interest as food-grade waxes for use as edible oleogels (Rogers et al., 2014). Oleogels comprised of wax and vegetable oils have varying fractions of n-alkanes, fatty alcohols, and fatty acids. Wax esters have a tendency to form platelet-like or needle-like crystals in edible oils at low concentrations that are very effective at gelling vegetable oil (Toro-Vazquez et al., 2013, 2010). CDW is obtained from the leaves of *Euphorbia cerifera* and comprises n-alkanes, between 29 and 33 carbons and structures safflower and canola oil at concentrations less than 2 wt% (Blake et al., 2014). CDW forms the most elastic gel (i.e., highest hardness value) while BW is the lowest (Lim et al., 2016).

$\beta$ -Sitosterol and  $\gamma$ -oryzanol are unable to gel vegetable oil when added individually, however they are able to self-assemble efficiently enough to entrain oil when combined at a 60:40 wt%  $\gamma$ -oryzanol/ $\beta$ -sitosterol ratio. At a 1:1 M ratio they self-assembled into hollow tubules, that have a diameter between 67 and 80 Å, are between 8 and 12 Å thick and have a length which can exceed 1000 Å, at concentrations as low as 2% (Bot et al., 2012). The ratio of  $\gamma$ -oryzanol to  $\beta$ -sitosterol and vegetable oil effects the self-assembled structures which in turn alters their ability to entrain liquid oil. For this system to gel it requires the molecules to form synergistic hydrogen bonding, they must contain a ring system, and alkyl residue (Bot and Agterof, 2006). Not only does  $\beta$ -sitosterol form effective gels with  $\gamma$ -oryzanol but so do other plant sterols such as ergosterol, stigmasterol, cholesterol, cholestenol (Bot et al., 2012). In sunflower oil, the gels are transparent and by changing the concentration, the mechanical and rheological properties can be fine-tuned to mimic saturated fats. These oleogels are food grade and phytosterols lower blood cholesterol levels making these attractive alternatives to colloidal fat crystal networks.

### Conclusions

The physical attributes that fats impart to foods are very diverse. In emulsions they provide enhanced viscosity, mouthfeel, and body to the food, while in solid fat matrices they provide an elastic network essential for the food structure. Depending on the type of food, which fat is being removed from, there are numerous fat replacers that can be used depending on the physical attributes that need replaced. Fat replacers that work well in emulsions, such as microparticulated whey proteins, will not work as a hardstock fat replacers, therefore it is important to identify a suitable fat replacer for the specific application or food that is being formulated. Although numerous fat replacers exist to create low-fat options, there are very few oleogelators that are allowed in foods that can effectively structure unsaturated oils. However, this is a rapidly growing area of research as there is a constant desire by manufactures to eliminate *trans* fats while also reducing the concentration of saturated fats and maintaining unsaturated oil concentration.

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# Flavor Enhancers and Modifiers

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## Introduction

Within the context of sensory science, flavor can be described as a combination of taste, odor and chemesthetic sensations. Generally speaking, there are five basic tastes, including sweetness, sourness, saltiness, bitterness and umami (recent and ongoing research indicates fat may also elicit taste; [Running et al., 2015](#)). Odor includes sensations experienced through the ortho- and retronasal routes, for example, vanilla and lemon aromas. Chemesthetic sensations activate the trigeminal nerve, such as the burn of capsaicin from hot peppers, or the coolness of menthol from mint. While the sensory perception from each of these sensory modalities may occur alone, they are more often experienced together during food and beverage consumption.

Flavor perception is dynamic and may be influenced, and thus, altered through the addition and/or removal of various ingredients. From a food product development standpoint, continuous pressure to remain market relevant is placed due to evolving consumer trends, including sugar, salt and fat reduction, clean labeling and incorporation of health-promoting, functional ingredients. Formulation strategies to satisfy these objectives can negatively impact flavor, and thus, the incorporation of ingredients that will optimize flavor to better align with consumer expectation and product liking are necessary. For example, sugar, salt and fat reduction may lead to a less flavorful product, while the addition of functional ingredients can introduce off-aromas and less desirable tastes (e.g., bitterness).

Flavor optimization during food product formulation may focus on the incorporation of flavor enhancers or flavor modifiers. Generally, enhancers are used to increase the overall palatability, richness, pleasantness and hedonics of savory foods ([Kemp and Beauchamp, 1994](#); [McCabe and Rolls, 2007](#)). Modifiers may be used to target the suppression or enhancement of basic tastes and odors within a food matrix, and thus, vary in their impact on overall flavor perception.

## Flavor Enhancers

Ready-to-eat meats, cheeses, sauces and condiments, ready meals and various snack items represent categories of savory foods that may be targeted for the application of flavor enhancing ingredients. In particular, new products or re-formulations that target lowered amounts of sodium in order to meet regulatory guidelines and consumer expectation may require the use of flavor enhancers to counterbalance the loss of sensory palatability from the removal or replacement of salt. Strategies include the use of umami-eliciting compounds that aim to elevate the overall palatability of savory foods.

Monosodium glutamate (MSG) elicits umami or savoriness, a basic taste sensation ([Zhao et al., 2003](#)). By itself, taste from MSG has not been characterized as 'delicious', nor does it provide flavor enhancing capacity ([Halpern, 2002](#)). However, when combined with additional compounds, including purine nucleotides - 5'-adenosyl monophosphate (AMP), inosine 5'-monophosphate (IMP), 5'-guanosine monophosphate (GMP) - and some amino acids, a flavor enhancing effect can result ([Halpern, 2002](#); [Chandrashekar et al., 2006](#)).

Enhancing capabilities for these compounds have been demonstrated in food model systems. For example, when paired with a congruent savory vegetable odor, the sensory impact of glutamate (MSG + inosine 5' monophosphate) has been found to elevate 'pleasantness' compared to glutamate or vegetable odor presented alone ([McCabe and Rolls, 2007](#)). Elevated pleasantness from glutamate + vegetable odor was also significantly higher compared to sodium chloride + vegetable odor, demonstrating the advantageous flavor enhancing capacity for glutamate over sodium chloride. In real food applications, the use of nucleotides and amino acids to enhance flavor has been demonstrated in reduced sodium processed meats ([dos Santos et al., 2014](#); [Campagnol et al., 2012](#)) and Cheddar cheese ([Khetra et al., 2016](#); [Grummer et al., 2012](#)).

Flavor enhancing compounds may successfully be used to elevate the palatability of various savory foods. However, the use of some ingredients may be less desirable if they are unfamiliar to consumers. A clean labeling approach - which may in part be defined as a back of the package ingredient list that is short and simple, containing 'kitchen cupboard ingredients' that are not 'chemical-sounding' ([Asioli et al., 2017](#)) - will target the use of umami-eliciting ingredients that may be perceived as more 'natural' by the consumer in order to better align with the product category. Here, umami-eliciting ingredients derived from more familiar sources may be applied to food formulations to enhance flavor. For example, the addition of mushrooms has been used to compensate for flavor loss and enhance overall flavor and umami taste in a reduced sodium ground beef taco blend ([Miller et al., 2014](#)). Modified soy sauce has been utilized to successfully increase the overall taste intensity, meat taste, and salty taste of reduced sodium frankfurters ([McGough et al., 2012](#)). In reduced sodium ham and turkey sausage, a milk protein and cultured milk based flavor enhancer was used to mitigate flavor loss ([Pietrasik and Gaudette, 2014, 2015](#)). Commercial yeast extracts have also been found to compensate for the potential flavor loss from salt removal in reduced sodium ready meals ([Mitchell et al., 2009](#)).

## Flavor Modifiers

In order to improve or optimize the sensory properties of foods and beverages, ingredients may be used to modify overall perceived flavor. Approaches include the use of ingredients to suppress or enhance basic taste and/or odor sensations within foods and beverages. Similar to the need for flavor enhancers in sodium reduced savory foods, flavor modifiers may be required in re-formulations where salt and sugar are reduced. While the addition of sodium chloride and sucrose are used, respectively, to deliver salty and sweet taste, their purpose in formulations may also be to suppress the bitterness elicited by other ingredients. Thus, sodium chloride and sucrose may have multiple functions within a food product regarding their direct or indirect impact on taste and odor sensations. For example, the bitterness of caffeine is decreased through the addition of sucrose, with increasing sucrose concentration leading to decreasing bitterness (Calviño et al., 1990). Sodium chloride can suppress the taste from bitter eliciting compounds, primarily due to the influence of the sodium cation (Breslin and Beauchamp, 1995); although the mechanism for this remains unclear (Gaudette and Pickering, 2011).

As mentioned previously, current food trends include the reduction of salt and sugar to satisfy health-conscious consumers. This remains challenging for industries that rely heavily on these ingredients (e.g., confectionary, meat, cheese, and bakery items), for they contribute to the functionality, safety and sensory properties of foods. Since salt and sugar reduction can negatively affect sensory properties, including decreased overall flavor intensity and increased intensity of less desirable tastes (bitterness, sourness), options for flavor modification are needed.

Flavor modifying strategies are more challenging for functional foods - where fortification with health-promoting ingredients elevates the nutritional profile of foods and beverages. For these products, functional ingredients (e.g., grape and green tea-derived polyphenols, glucosinolates, soy isoflavones) can negatively impact the flavor profile of foods by introducing off-flavors, including elevated bitterness, which may lead to decreased consumer acceptability. While bitterness suppression can be successful through the addition of sodium chloride and sucrose, this approach is not ideal in functional food formulations which are positioned for health conscious consumers. Thus, alternative approaches and ingredients should be considered. For example, the use of alternative sweeteners (e.g., agave, Rebaudioside A or stevia leaf, honey, monk fruit) may provide an option for some product formulations. However, due to the potential for the elicitation of off-flavors and bitterness, the successful use of some plant-based sweeteners for providing a pleasant aftertaste while masking the bitterness of other ingredients may be concentration dependent (Li et al., 2015).

The impact of odor on taste perception; a cross-modal, sensory interaction, is substantial (Delwiche, 2004; Prescott, 1999) and may provide a viable strategy towards flavor modification in salt and sugar reduced foods. Due to learned taste-odor associations experienced through everyday food and beverage consumption, congruent mixtures of tastes and odors can have an enhancing effect on taste intensity. For example, when compared to sucrose alone, the sweetness of sucrose can be elevated when paired with the addition of strawberry but not peanut butter odor (Frank and Byram, 1988). In simple aqueous solutions, the saltiness of sodium chloride can be enhanced through the addition of sardine but not carrot odor (Lawrence et al., 2009) - demonstrating the importance of taste-odor congruency on the impact of flavor. While odor-induced taste enhancing effects have been shown in simple matrices, their capacity in foods with more complex flavor profiles remains relatively unclear. Overall, the effect is likely dependent on the complexity of the food matrix, with increased complexity associated with a decreased effect.

## Other Considerations

Additional approaches towards flavor modification may target the use of ingredients that provide textural properties to foods and beverages. In particular, hydrocolloids used as thickeners or gelling agents can modify flavor by trapping compounds and thus, limiting interaction between tastants and odorants and sensory receptors. For example, several hydrocolloids have been demonstrated to reduce bitterness, including cellulose gum, low-viscosity carboxymethyl cellulose, and sodium alginate (Moskowitz and Arabie, 1970; Pangborn et al., 1973).

The binding of taste eliciting compounds is also of value for functional food formulations, especially towards bitterness suppression (Coupland and Hayes, 2014). Here, the use of cyclodextrins, polymers, surfactants, lipids and emulsions may provide an alternative strategy towards the incorporation of bitter tasting, health promoting functional ingredients into food and beverage formulations.

## Conclusion

The use of flavor enhancers and modifiers represents a valuable approach towards improving the overall sensory properties and consumer acceptability of foods and beverages. For simple matrices, it is possible that a single flavor enhancing or modifying strategy may be successful. However, it is likely that a multi-strategy approach that incorporates various concentrations of one or more ingredients is more impactful.

It is important to note that the impact of any particular flavor enhancing or modifying strategy on overall flavor perception is highly dependent on the presence of various within- (taste-taste) and cross-modal (taste-odor) sensory interactions. Suppressing

and additive effects on flavor will occur due to oral peripheral physiological interactions or a central cognitive effect. Therefore, it is important to understand that the sensory impact of enhancing and modifying ingredients within a matrix may not be straightforward.

In addition to various sensory interactions, the flavor response to an added ingredient is based on its dose - a relationship that is not always linear and easily predictable. Finally, chemical and/or physical interactions can occur within the food matrix, providing even more complexity towards the prediction of ingredient impact on overall flavor perception. Therefore, prior to the use of flavor enhancing and modifying ingredients in food and beverage formulations, testing at several concentrations should occur in order to understand, verify and optimize their impact on overall flavor.

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# Flavors (Bittering Agents, Astringent Flavors, Pungency, Menthol)

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## Introduction

Bitterness, astringency, pungency and menthol are primarily perceived in the buccal cavity. Whilst the former is regarded as a taste, the three latter are generally considered to be responsible for specific chemesthetic experiences, *ie* those sensory experiences that activate receptors not generally considered to fit the classical categories of aroma and taste, often known as somatosensation. As a group these taste qualities can have dramatic effects on the flavor perception of many foods and beverages, as well as other orally administered substances, such as pharmaceuticals and oral hygiene products. As such these tastes often need to be considered when these qualities are to be included in such products. The focus of this article is primarily on positive sensory responses so consideration of other related aspects, such as taste modulation and suppression are not discussed here.

The perception of bitterness, pungency and menthol requires interaction between the active compounds and their associated G-protein coupled receptors (Table 1). Additionally, certain pungent compounds, such as acrolein and ethanol, also interact with G-protein coupled olfactory receptors in the olfactory epithelium to give a retronasal sensory response (*ie* an “aroma”). These additional perceptions complicate the sensory appraisal of such compounds.

As we will discuss below, the mechanism of astringency perception is currently disputed, but to date there do not seem to be any astringency receptors identified. A commonly- but not universally-held view is that astringency perception is due to the non-covalent binding of astringent species with salivary proteins that results in the precipitation of the resulting complex. This chemistry is analogous to the initial stages of leather tanning and the formation of particulates in ageing red wines and beers.

## Chemical Entities and Their Occurrence

The range of compounds that can induce perceptions of bitterness, pungency and astringency is generally broad (Tables 2–4), whilst the flavor and cooling properties of menthol can also be substantially replicated in other menthol derivatives. Many of these compounds have commercial significance, either as pure substances or as part of food or beverage products. Whilst we recognize that some of the compounds discussed below have broad applications, for the purposes of this article we will not consider applications beyond the food and beverage sectors.

## Future Aspects

The scientific exploration of flavour perception is highly active at present, exemplified by the recent reports of putative oil- and starchy-related tastes reported since 2015. With the plethora of sensory stimuli and rapid developments in the physiological and sensory approaches to both chemesthetic and flavour perceptions, it is not beyond the bounds of expectation that more targeted design of chemical entities with specific bitter attributes will be more accessible. Where bitterness is undesirable, bitterness could be attenuated, either by tailoring the chemical entities themselves or by design of bitter antagonists or “masking agents”. Clarification of the mechanism(s) of astringency perception and its apparent association with bitterness will help to rationalize the sensory properties those foods and, especially, drinks, where astringency plays a key role in flavour perception, such as wood-aged drinks, wines and ciders.

## Bitterness

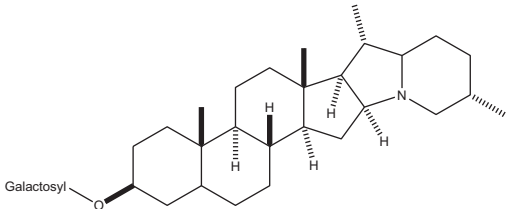
Humans can detect bitterness in many foods and beverages, including fruits, vegetables, drinks such as beer, cocoa, coffee and tea as well as in animal products such as dairy, fish and chicken. Generally bitterness is often considered to be an undesirable sensory quality, although this is not the case for certain foods and beverages. Additionally other substances, such as orally-administered pharmaceuticals can convey bitterness. Bitter compounds are structurally diverse and work carried out since the late 1990s has identified around 25 distinct G-protein-coupled bitterness receptors (Table 2). As of January 2018 the bitterness database BitterDB ([bitterdb.agri.huji.ac.il](http://bitterdb.agri.huji.ac.il)) listed more than 680 distinct bitter substances, either as pure compounds (*eg* quinine, 1) or as mixtures,

**Table 1** Receptors responsible for the perception of bitterness, astringency and menthol

<i>Taste quality</i>	<i>Receptors</i>	<i>Localization</i>	<i>Taste descriptors</i>
Bitterness	TAS2R (1,3-5,7-10, 13,14,16,38-50,60)	Oral taste receptor cells, larynx, upper esophagus	Bitterness, metallic
Pungency	TRPV1, TRPA1	Skin, mucous membranes	Hot, burning
Menthol	TRPM8	Skin, mucous membranes	Cooling, (pepper)mint



**Table 2** Example bitter compounds and their occurrences in nature

Bitter compound	TAS2R receptor(s)	Occurrence/sources
Alkaloids (1–3)	Include 4,10,39,46	Widespread in plant kingdom
Caffeine (3)	7,10,14,43,46	Many plants, including coffee beans, tea leaves and cacao pods
Saponins (4)	?	Various plants, including aubergine and maple
 <p>(α-solanine, 4)</p>		
Pyrazines (5–7)	38	Thermal degradation of free amino nitrogen
Hop-derived bitterness	1,40 (α-acids); 1,14 (iso-α-acids)	The cultivated hop, <i>Humulus lupulus</i>
Diketopiperazines (8,9)	?	Thermal degradation of free amino nitrogen
Bitter peptides	?	Thermal degradation of free amino nitrogen
PROP, PTC (10,11)	38	Synthetic
Sucrose octa-acetate	?	Synthetic
Glucosinolates	38	Cruciferous vegetables
Goitrin (12)	38	Cruciferous vegetables, canola
Cucurbitacins (13)	10	
Bitter tannins	Include 14,39	Widespread in the plant kingdom

**Table 3** Example pungency compounds and their occurrences in nature

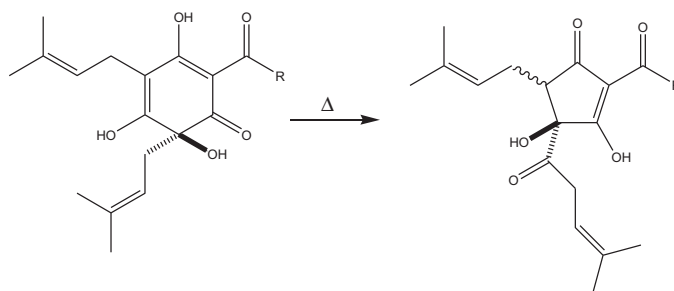
Pungency compound	Receptor(s)	Occurrence/sources
Ethanol	Nasal?	Adult beverages
Formaldehyde	Nasal?	Synthetic chemical
Acetaldehyde	Nasal?	Fermentation by-product
CO <sub>2</sub>	Nasal?	Carbonated beverages
Capsaicin (14) and derivatives	TRPV1	<i>Capsicum</i> species
Piperine (15)	TRPV1	Long, black and white peppers
Gingerol (16), shogaol (17) and analogues	TRPV1	Members of the Zingiberaceae (ginger) family
Acrolein, other lachrymators	TRPA1	Various, including thermal degradation
Nicotine	TRPA1	Tobacco
Allyl isothiocyanate	TRPA1, TRPV1	Mustard, radishes, horseradish, wasabi
Allicin	TRPA1, TRPV1	Raw garlic and onions
Syn-Propanethial S-oxide	TRPA1, TRPV1	Onions
Oleocanthal	TRPA1	Olive oil

**Table 4** Principal astringent compounds and their occurrences in nature

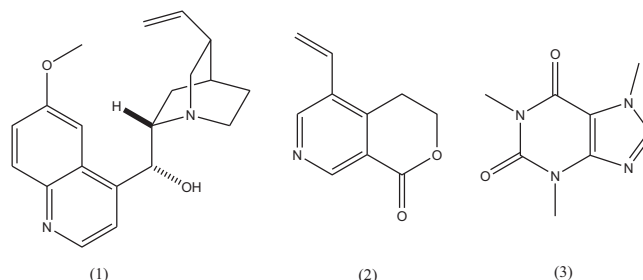
Astringent compound	Occurrence/sources
Tannins (18–20)	Widespread in the plant kingdom
Alums	Baking powders

such as fennel oil. The diversity of receptors and the structural diversity of bitter compounds has led to speculation that human perception of bitterness is in fact a defense mechanism to protect against toxic substances that also happen to be bitter, such as plant alkaloids. A corollary of this thesis is that human appreciation of bitterness is learned. Indeed Henry VIII of England attempted to ban bitter hops (“the pernicious weed”) because of their alleged ability to induce hangovers.

With the exception of the generally undesirable protein-derived bitterness, commercially sought bitter compounds are derived primarily from plant sources. Thus beer bitterness is derived from the a low-yielding isomerization reaction of the hop-borne



**Figure 1** Isomerization of hop-derived  $\alpha$ -acids into their bitter iso- $\alpha$ -acid counterparts. R = 2-methylpropyl-, 2-methylbutyl- and 3-methylbutyl-.



**Figure 2** Example alkaloid structures. (1) quinine, (2) gentianine, (3) caffeine.

$\alpha$ -acids (or humulones) to iso- $\alpha$ -acids (Fig. 1), traditionally brought about by a boiling stage during the beer production process, but now also commonly a reaction performed by hop product manufacturers outside of the brewery.

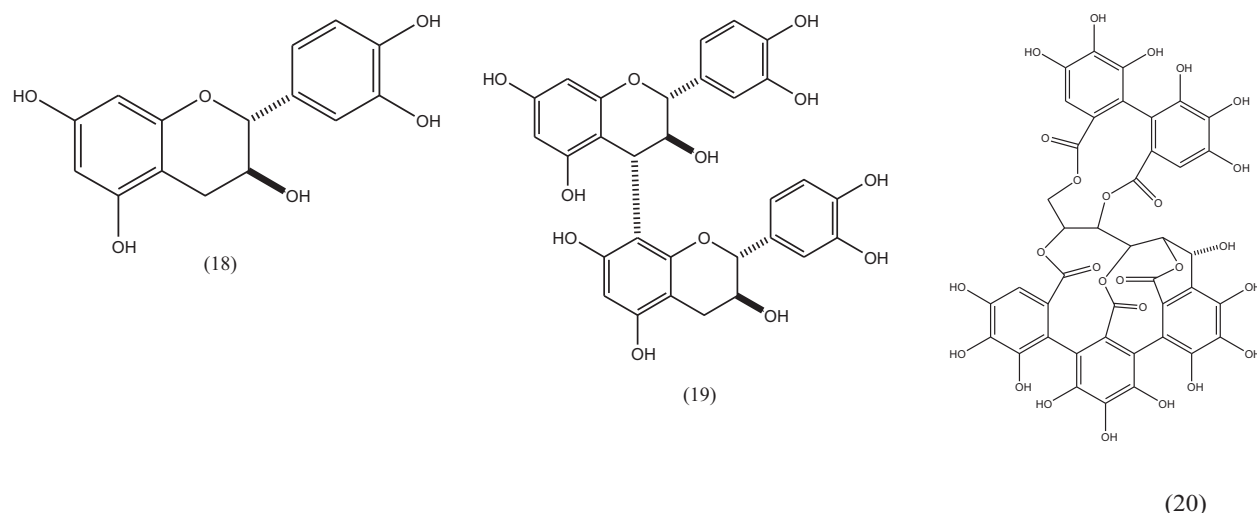
Alkaloids are nitrogen-containing compounds that are derived from plants and have significant effects on humans, specifically either as drugs (eg morphine) and toxins (eg strychnine; see Fig. 2 for some examples). Indeed, given the impact that alkaloids can have on humans, it is perhaps unsurprising that many are bitter, arguably a selection pressure for early humans as they sought to forage for foodstuffs that were both nutritious and safe. Today caffeine is the most commonly consumed alkaloid, with the top 50 caffeine-consuming countries ingesting 1.2–9.6 kg per capita per year. Being both water- and lipid-soluble it easily crosses the blood-brain barrier and acts as a stimulant in sufficiently high doses. Other alkaloids such as strychnine, atropine, brucine and curare are almost legendary in terms of their toxicity and are often described as bitter but of course have little use in the food and beverage industries.

### Pungency

Pungency is associated with the perception of spiciness and heat, based on the non-covalent binding of trigger molecules with the TRPV1 and/or the TRPA1 receptors (Table 3). A further term, piquancy, is often ascribed to a milder experience of pungency. As mentioned previously, pungency can also be attributed to an olfactory response, as in the case an acrid aroma. Indeed the trivial name for the pungent and lachrymatory propenal (acrolein), is a contraction of “acrid oil”, and is a common artefact of the thermal degradation of glycerol. Acetaldehyde and ethanol have also been associated with pungency. Clearly in these cases, pungency is also perceived *via* (retro)nasal detection by the olfactory epithelium, in addition to TRP-based mechanisms. While it is not clear what the mechanism of nasal perception of pungency is, presumably it is mediated at least to some extent by interaction with nociceptors.

The major natural sources of pungency are derived from the plant kingdom: the *Capsicum* genus (eg bell peppers), white/green/black peppers (*Piper nigrum*), *Allium* spp. (eg onion, garlic), tobacco and olive oil. Other sources of pungency can arise from thermal processing or from industrial activities such as carbohydrate fermentations to produce ethanol and related compounds such as acetaldehyde. Whilst the perception of pungency is often a positive sensory experience, the causative agents do find applications in other areas, especially healthcare and pharmaceuticals.

Industrially the principal capsaicinoid, capsaicin (14), is the most important pungency agent, with applications beyond the food industry, including pharmaceutical applications and as a repellent for animals and humans (“pepper spray”). Global production of the various *Capsicum* species is in excess of 30 million metric tons and is dominated by China. Other major sources of commercially-available pungent agents are black pepper and ginger. Black pepper is one of the most popular spices worldwide, with production volumes of around 500,000 metric tons per annum. Half of this production is in Vietnam and Indonesia, with India, Brazil and China also being major black pepper producing countries. The annual ginger harvest is more than 2.5 million metric tons, with India, China and Indonesia accounting for more than half of global production.



**Figure 3** Example tannin structures. (18) catechin (19) procyanidin B3 (20) castalagin.

The measurement of the pungency intensity of capsaicinoids and other pungent substances was traditionally based on sensory evaluation to derive the Scoville Heat Units (SHU's) of a sample. The methodology is based on ethanolic extraction of capsaicinoids and then dilution in a sucrose solution. The higher the dilution of the sample that gave a positive heat detection, the higher the number of SHU's. Pure capsaicin itself may register 15–16 million SHU's, whilst pepper spray is typically < 1 million. Some other naturally-occurring capsaicinoids (eg resiniferatoxin) can register as much as  $16 \times 10^9$  SHU's, three orders of magnitude more than pure capsaicin. By way of comparison, pure piperine (15) is estimated to register at 100,000 SHU's and 6-gingerol at around 60,000 SHU's. Today this sensory-based methodology has largely been superseded by high-performance liquid chromatography (HPLC) analysis, although this latter is still multiplied by a factor in an attempt to bring intensities on to a similar scale to the more traditional SHU's.

### Astringency

As mentioned earlier, no astringency receptors have yet been reported, although quite how astringency is perceived is, as yet, not fully resolved. The major astringent species in the diet are polyphenolic- or tannin-based, although alums and other inorganic salts (eg potassium permanganate, zinc oxide, silver nitrate) can also elicit the characteristic drying and mouth-puckering experience of astringency. For the purposes of this article we will focus exclusively on tannins. The term tannin is used here to denote the broad array of polyphenolic secondary metabolites of higher plants. We recognize the limitations of this definition but further elaboration is beyond the scope of this article (see Fig. 3 for example structures).

Studies *in vitro* have shown that astringent tannin species can reversibly bind to many proteins and, especially, to proteins with proline-rich domains (proline-rich proteins, PRP's). These interactions tend to be non-covalent, but result in precipitation of the protein-tannin adduct, an action that is, in principle, reversible. Irreversibility of complexation is thought to be due to the oxidation of tannins to, for instance, more reactive 1,2-quinones that can subsequently react to form covalent complexes with proteins.

The unambiguous perception of astringency is not always straight-forward, and can be erroneously conflated with bitterness, despite the apparent difference in perceptual mechanisms. There appears to be a complementarity between astringency and bitterness. It is interesting to speculate on the reasons for this. Certainly there will be some steric constraints on significant interactions between bitter TAS2R receptors and substances perceived as bitter, in comparison with the protein-binding model of astringency which can be expected to be much less dependent on steric factors. Work carried out on the sensory characteristics of tannins from apples in cider suggest that as molecular size increases, tannins tend to demonstrate more astringency and less bitterness.

In dietary terms, the occurrence of tannins is widespread. They are found in various woods, fruits, berries, nuts, herbs, spices, legumes and chocolate, as well as beverages such as tea, coffee, cocoa, beer, cider and wines. There are also detectable levels of tannins found in alcoholic beverages that are exposed to wood during their production process (eg whisky, brandy, wood-aged beers). Tannins also have uses beyond the diet, especially where favorable protein interactions are required. Thus oak tannins ("tannic acid") can effectively reduce the levels of potentially haze-forming proteins in beers, although much of the industrial use of tannins is to "tan" leather, which makes the leather more durable, less susceptible to degradation and even modifying its color.

## Menthol

Menthol or, more precisely (–)-menthol, L-menthol or (1R,2S,5S)-menthol (21) is a naturally-occurring cyclic monoterpene alcohol. Whilst there are eight possible stereoisomers (21) is the preferred one due to its low cooling threshold. Of compounds related to L-menthol, only L-menthone (22) and L-isomenthone (23) show more potent minty and cooling characteristics. Because of these characteristics L-menthol is considered, along with vanilla and citrus, to be one of the most important flavoring additives, with annual consumption probably in excess of 30,000 metric tons. The applications of menthol are diverse, and include: use to relieve sore throats, anti-itching, topical analgesics, decongestants, oral hygiene products, chewing gums and candies. One controversial use is as a tobacco additive, where some authors suggest that menthol can improve the sensory acceptability of cigarette smoke, arguably enhancing addiction.

Menthol occurs naturally almost always as the essentially pure L-menthol and is primarily sourced from wild mint (*Mentha arvensis*). It also occurs in peppermint oil, together with other cooling compounds such as menthone and menthyl acetate, and peppermint (*Mentha x piperita*), a hybrid of watermint and spearmint. Oil from spearmint itself also contains menthol, but is generally cultivated less extensively than peppermint or wild mint. Naturally-occurring mint oil is generally prepared by steam- or hydro-distillation. The leaves may be cured, allowing the mint plants to dry once cut to reduce their moisture content. In some cases the proportion of L-menthol exceeds 70% and can be crystallized out of mint oils and, if further purity is required, recrystallized from low boiling solvents.

The demand for L-menthol outstrips global supply, so various efforts have been made to produce it synthetically. Indeed Noyori won the 2001 Nobel Prize for chemistry based on his elegant asymmetric synthesis of L-menthol from the inexpensive monoterpene myrcene. Other approaches, such as the Haarmann-Reimer process, synthesize racemic menthol, and then separate the required stereoisomer by chiral resolution of the menthyl benzoate esters before hydrolysis of the ester. More recently, BASF have developed a number of synthetic routes to L-menthol, one of which requires the stereospecific production of (+)-R-citronellal, also an intermediate in the Noyori synthesis.

The cooling property of menthol is due to its ability to trigger the cold-sensitive TRPM8 (Transient Receptor Potential cation channel subfamily M member 8) receptor, also known as the Cold and Menthol Receptor 1 (CMR1). This channel is the main molecular transducer of cold somatosensation in humans. A range of menthol analogs have demonstrable cooling properties, many, but not all, based on the L-menthol skeleton.

# Galactomannans (Guar, Locust Bean, Fenugreek, Tara)

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## Introduction

Seed polysaccharides have attracted research attention due to a spectrum of diverse applications in food, pharmaceutical, and chemical industries. A class of seeds where the endosperm is present (i.e., endospermic seeds) are of industrial importance and include corn, wheat or rice. These polysaccharides are formed in the seed with ultimate goal to deliver carbohydrates to the developing seedling. The advantage of polysaccharides from endospermic seeds, as seen from industrial point of view, is that they are isolated easily in pure form and after suitable physical or chemical treatments are used as food or formulation ingredients. The major cell wall storage polysaccharides are most commonly divided into three polysaccharide groups, that is, mannans, xyloglucans, and galactans (Meier and Reid, 1982) with galactomannans falling in the mannan group. The distribution of galactomannans in the plant kingdom is limited and are mostly found in the endospermic seeds of four economically important plants that belong to the same family i.e., *Fabaceae* or *Leguminosae*. The focus of the present article are the galactomannans from guar (*Cyamopsis tetragonoloba*), carob or locust bean (*Ceratonia siliqua*), tara (*Tara spinosa*) and fenugreek (*Trigonella foenum-graecum*) that are the major sources of industrial galactomannans. As it will be discussed in the following sections, flour is obtained from the seeds of these plants, which is either used directly in food formulations or it is further processed to obtain special ingredients (Fig. 1).

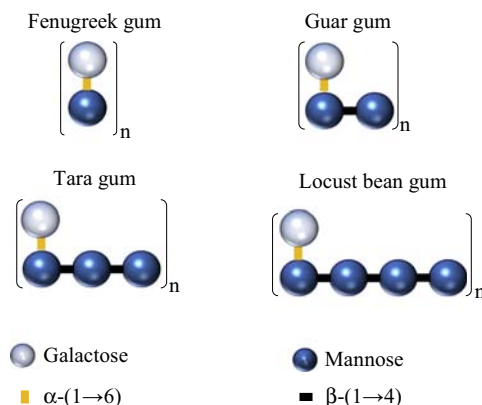
## Structure-Extraction

Galactomannans are heteropolysaccharides (i.e., they consist of more than one carbohydrate residue) composed of D-mannose, which is the residue that makes up the backbone of the chain, and D-galactose that forms single branches along the mannan chain. Mannose units are linked with each other via  $\beta$ -(1 $\rightarrow$ 4) glycosidic linkages whereas galactose is attached at the C-6 of mannose with  $\alpha$ -(1 $\rightarrow$ 6) bonds. Galactomannans are neutral polysaccharides, which means that they do not carry uronic acid residues or other charged groups (e.g., sulfo groups) on the backbone. They are distinguished from each other based on the mannose-to-galactose ratio (M/G), which is characteristic for each source. The ratio ranges approximately between 1:1, 2:1, 3:1 or 4:1 for fenugreek, guar, tara or locust bean gum, respectively (Figure 2) (Prajapati et al., 2013; Srivastava and Kapoor, 2005). For instance, an M/G ratio of 1:3 indicates that a galactose residue is found in every three mannose units. It should be noted that the experimentally determined ratios may vary anywhere between 1 and 5 depending on the source and extraction conditions.

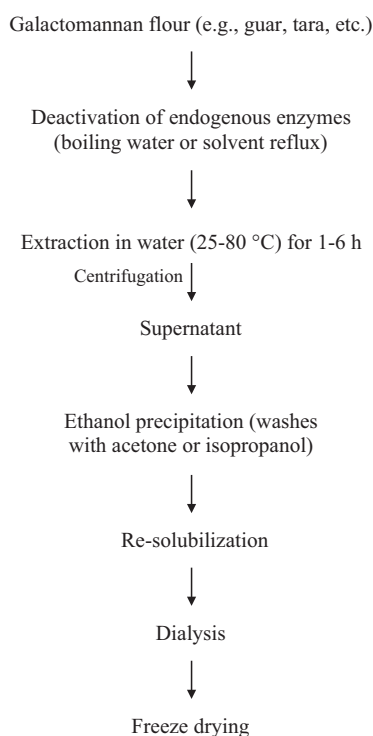
Generally, galactomannans are extracted from the endosperm of the seeds after milling, husk removal, and sieving steps. The flour (Fig. 1) usually contains impurities, most commonly protein and insoluble cellulosic polymers that are co-extracted during the milling process. Further purification usually involves dissolution in water at various temperatures followed by ethanol precipitation, dialysis and freeze drying (Fig. 3) (Sébastien et al., 2014; Salvalaggio et al., 2015). The first stage is usually a deactivation step of the endogenous enzymes that are naturally found in galactomannan flour and can potentially degrade the polysaccharide (e.g.,  $\beta$ -D-mannanases) during the extraction step. This step is carried out with thermal deactivation of enzymes in boiling water or



**Figure 1** Seeds and flours from fenugreek, guar, tara and carob. Reproduced with permission from Elsevier from Prajapati, V.D., Jani, G.K., Moradiya, N.G., et al., 2013. Galactomannan: a versatile biodegradable seed polysaccharide. *Int. J. Biol. Macromol.* 60, 83–92. Image of guar seeds is reproduced under the Attribution-Share Alike 2.0 Generic license (from Ton Rulkens, [Flickr.com](https://www.flickr.com/photos/tonrulkens/)).



**Figure 2** Idealised structures of repeating units of the four major galactomannans. Mannose residues are linked with  $\beta$ -(1 $\rightarrow$ 4) whereas galactose via  $\alpha$ -(1 $\rightarrow$ 6) glycosidic bonds.



**Figure 3** Generalised extraction strategy for galactomannan purification.

use of solvents (e.g., PET-ether or hexane reflux) that may also serve as a means for removal of polar contaminants (e.g., lipids). Extraction is then carried out for several hours after reduction of temperature to the required temperature that is usually higher for poorly soluble galactomannans (e.g., locust bean). A proteolytic enzyme may be added at this stage if protein contamination creates problems for the purity of the sample. After solubilisation, the polysaccharide is recovered with an ethanol precipitation step usually using twice as much volume of ethanol as that of the extraction medium. At this stage the polysaccharide is recovered but if further purity is required a dialysis or ultrafiltration step can be carried out. This step removes low molecular weight contaminants (depending on the molecular weight cut-off of the membrane that has been selected) that may have been co-precipitated. Contaminants may include, amino acids (if an enzymatic treatment was employed), monosaccharides, small peptides, and oligosaccharides. The final stage involves a freeze-drying step while further precipitation with alcohol and oven-drying should be preferably avoided, as it normally results in lower yields and potentially lower molecular weight.



## Physical Properties

Hydroxyl groups of mannose in carbons 2, 3 and of galactose in 3, 4 are in *cis*-configuration. These groups are responsible for extensive intra- and inter-chain hydrogen bond formation resulting in differences in solubility between galactomannans (Rinaudo, 2008). Galactose substitution provides steric hindrance to chain associations, thus improving solubility. As a result, highly substituted galactomannans such as those from fenugreek (Fig. 2) are highly soluble even in cold water (Mathur and Mathur, 2005). On the other end of the spectrum, locust bean gum has the least degree of substitution making the polysaccharide soluble only in hot water (Wielinga, 2009). As a result, the solubility follows the order (from more to least soluble): fenugreek > guar > tara > locust bean.

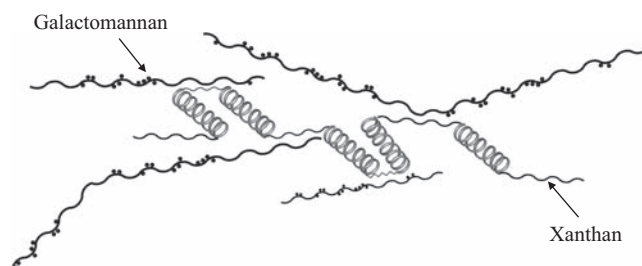
Fundamental and applied research interest for galactomannans stems primarily from their ability to enhance viscosity of aqueous media (Bresolin et al., 1997; Robinson et al., 1982). The solutions are Newtonian at low concentrations (<~0.5% w/v) whereas at higher (~1% w/v) they exhibit shear thinning behaviour. Due to particularly high viscosity build up galactomannans are rarely used at concentrations greater than 1% w/v in food applications. Complex foods contain several ingredients with remarkably disparate chemical structures that create challenges to formulations. For instance, protein-polysaccharide mixtures (e.g., ice cream mix or milkshakes) are incompatible at certain concentrations resulting in phase separation. This technological difficulty is commonly avoided by working at low polysaccharide concentrations normally in the range of 0.1%–0.6% w/v. It should be noted that because galactomannans do not carry charge, pH and ionic strength do not particularly influence the viscosity of their solutions. Additionally, to achieve maximum viscosity, dissolution should be carried out under specific conditions depending on the source. For instance, guar gum may generate highly viscous solutions after mild heating (20–40 °C) for ~2 h whereas locust bean gum with similar molecular characteristics (e.g., molecular weight) cannot be fully dispersed if it is not heated to at least 80 °C, and consequently, it will result in solutions of low viscosity (Srivastava and Kapoor, 2005; Barak and Mudgil, 2014; Brummer et al., 2003).

Galactomannans may also influence the rheology of the aqueous systems due to intermolecular synergistic chain associations with helix forming polysaccharides (Bresolin et al., 1997; Dea and Morrison, 1975), and a remarkable increase in viscosity (Pinheiro et al., 2011) or even gel formation (Barak and Mudgil, 2014; Miles et al., 1984; Dunstan et al., 2001; Lazaridou et al., 2001) may be achieved. The tendency of galactomannans to modulate the rheological characteristics depends on the degree of galactose substitution. Galactomannans with higher galactose content (e.g., fenugreek) show little tendency to form gels and generally exhibit lower viscosity than their counterparts with lower degree of substitution (e.g., locust bean gum) due to the steric hindrance posed by galactose residues (Mathur, 2012). Generally, unsubstituted regions in galactomannan backbone interact *via* multiple hydrogen interactions with certain polysaccharides (e.g., xanthan or  $\kappa$ -carrageenan) (Dea and Morrison, 1975). For example, locust bean gum interacts readily with xanthan leading up to formation of a stable three-dimensional structure. Although the exact mechanism of the synergism is still a matter of debate (Grisel et al., 2015) the most acceptable model is that interactions occur between disordered segments of xanthan chains and unsubstituted regions of galactomannan (Grisel et al., 2015; Cairns et al., 1986) (Fig. 4). Similar mechanisms of synergy exist between galactomannans and  $\kappa$ -carrageenan or agar.

Polysaccharides are frequently modified to meet certain industrial needs e.g., improve water solubility, enhance viscosity or modify the interfacial properties of the biopolymer. Physical modification usually involves changes to the raw material without affecting the chemical structure of the polysaccharide such as particle size reduction, instantinization or certain heat treatments (e.g., pregelatinisation in starches). In chemical modification, a functional group (e.g., methyl or carboxymethyl) is attached and the chemical structure changes along with the functional properties. Galactomannans are modified with reactions that include etherification, esterification, or crosslinking of hydroxyl groups (Warkar and Gupta, 2015; Savitha Prashanth et al., 2006). Galactomannan derivatives that are produced after modification have improved functional properties (e.g., improved solubility in water or enhanced hydrophobicity) (Maier et al., 1993; Zhang et al., 2005, 2007).

## Food Applications

Galactomannans are commonly used in the food industry, particularly those from guar and locust bean gum (Table 1). Tara and fenugreek gums are not widely used due to the availability and cost although they are steadily increasing their market share.



**Figure 4** Schematic diagram showing the predominant mechanism of interaction between xanthan and galactomannans. The interaction proceeds with formation of junction zone associations between the disordered segments of xanthan and the unsubstituted regions of galactomannan.

In most of the cases tara gum can be replaced by mixtures of locust bean gum and guar gum. Since the M/G ratio is in between that of guar and locust bean gum its physical properties are approximated by mixtures of guar and locust bean flours. Guar and locust bean gums are common ingredients in ice cream formulations where they modulate ice crystal formation and recrystallization, and prevent lactose crystallization, during frozen storage of the product. They also find applications in other dairy products such as cheese spreads to improve spreading or yoghurts where they are used in fat reduction to structure water and improve texture (Thombare et al., 2016). They are also used in salad dressings, sauces, soups and in a range of liquid formulations where they control viscosity, mouthfeel, and stability of the dispersions. For instance, in low fat mayonnaise they provide viscosity and prevent creaming of the emulsion. In sauces (e.g., salad dressings or stock condiments) they provide a way to control syneresis and phase separation of the solids which is particularly important considering the long shelf-life of these products. In processed meats (e.g., sausages, frankfurters etc.) they are used to bind meat pieces together, provide a uniform texture, increase yield through water management and prevent phase separation during heat treatments (Nussinovitch, 1997; Mudgil et al., 2014). Commercial galactomannans have been also shown to have surface active properties (Garti et al., 1997; Wu et al., 2009) that could be exploited in the encapsulation of flavour oils of beverages (Mikkonen et al., 2009). As mentioned earlier, the ability of locust bean gum to control rheology of aqueous media in synergism with other polysaccharides (e.g.,  $\kappa$ -carrageenan or xanthan) allows formation of gels with diverse rheological characteristics. This makes it possible to replace gelatin for certain applications (e.g., for religious or vegetarian diets) and permits manipulation of texture and stability in a range of products (e.g., bakery formulations, fruit based desserts etc.) (Gidley and Grant Reid, 2006). Additionally, galactomannans are also used in gluten-free bakery products where they improve volume and moisture content of the crumb, among other important technological characteristics (Anton and Artfield, 2008). Apart from the self-association due to hydrogen bond formation, galactomannans may interact with each other (inter-chain interactions) and alter the rheological properties of the food matrix, as a result of sub-zero temperatures. For instance, the freezing step in ice cream manufacturing results in formation of a freeze concentrated matrix where the effective concentration of all the soluble ingredients increases significantly. As a result of the increased concentration, cryogelation of galactomannans may occur with potentially detrimental results for the sensory characteristics of the product (Doyle et al., 2006; Lozinsky et al., 2000; Patmore et al., 2003).

Galactomannans also provide a sustainable source of biopolymers for edible film applications. These materials require tuning of their gas (mainly CO<sub>2</sub> and O<sub>2</sub>) and moisture permeability, mechanical, and optical properties. Generally, galactomannans with lower galactose content produce films with higher elongation at break and tensile strength thus presenting an opportunity to tune the film properties by varying the molecular structure of the biopolymer (Mikkonen et al., 2007). Galactomannan films are also used in conjunction with waxes, lipids, antimicrobials or antioxidants in order to modify their physical properties and extend their functionality to foods with a diverse range of physical properties (e.g., moisture or fat content) (Cerqueira et al., 2011). It should be, mentioned that in addition to the industrially relevant functional properties galactomannans may also exert *bio*-activity to a certain degree, as it has been reported to show immunomodulating and radical-scavenging activities (Liu et al., 2015). In addition, galactomannans that have been derivatised may have anticoagulant and antithrombotic properties or may be used as bioactives delivery-vehicles in drug and pharmaceutical formulations (Prabaharan, 2011).

## Conclusions

Galactomannans particularly those from guar and locust bean gum are widely utilized in the food industry and are of particular technological importance with those extracted from tara and fenugreek being less exploited at present. This is due to their suitable

**Table 1** Outline of food applications and functionality of galactomannans (Thombare et al., 2016; Mudgil et al., 2014; Gidley and Grant Reid, 2006)

<i>Applications</i>	<i>Functionality</i>
Dairy	Cream and milk desserts (e.g., milkshakes), yoghurts, ice creams (thickener, stabiliser), processed cheese (control syneresis and texture modification)
Bakery products	Icings, cake mixes, gluten-free (soften texture, improve loaf volume)
Gels	Fruit-based desserts (water structuring with the aid of other polysaccharides e.g., xanthan)
Beverages	Soft drinks (thickener, flavour oil encapsulation)
Seasonings	Sauces, salad dressings, mayonnaise and syrups (control of phase separation and texture, fat reduction)
Meat products	Sausages, frozen and tinned meats (consistency improvement, control syneresis)
Films	Biodegradable film formation for packaging. Inclusion of actives in the film, such as antibiotics, antioxidants, colour indicators.

functional properties, which include thickening of aqueous solutions, stabilizing and controlling phase separation, binding formulation ingredients to improve processing ability, and control sensory properties of the final product. The functional properties are tunable by controlling the galactose substitution of the chains and additionally by derivatisation of the polysaccharide to improve functionality (e.g., solubility or interfacial behaviour). As the use of galactomannans in food industry is well established future technologies should focus on the potential of these materials in biomedical, pharmaceutical and advanced nutrition applications with an overall aim to create advanced biomaterials with tailored functionality for food and non-food industries.

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## Gases and Vapors Used in Food

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### Overview

In the context of food, a vapor is a gaseous phase substance at a temperature where it can co-exist with its liquid or solid phase. The release of vapor from a liquid or solid does not necessarily involve boiling, i.e., through evaporation. On the other hand, a permanent gas does not form liquid or solid at typical atmospheric and temperature conditions. Many gases and vapours possess properties useful for food preservation, e.g., antimicrobial, anti-oxidative, anti-enzymatic and so on. Other gases and vapors are being exploited during processing to impart desirable physicochemical and sensorial characteristics in food products. In this section, selected gases and vapors commonly used in the food industry are discussed.

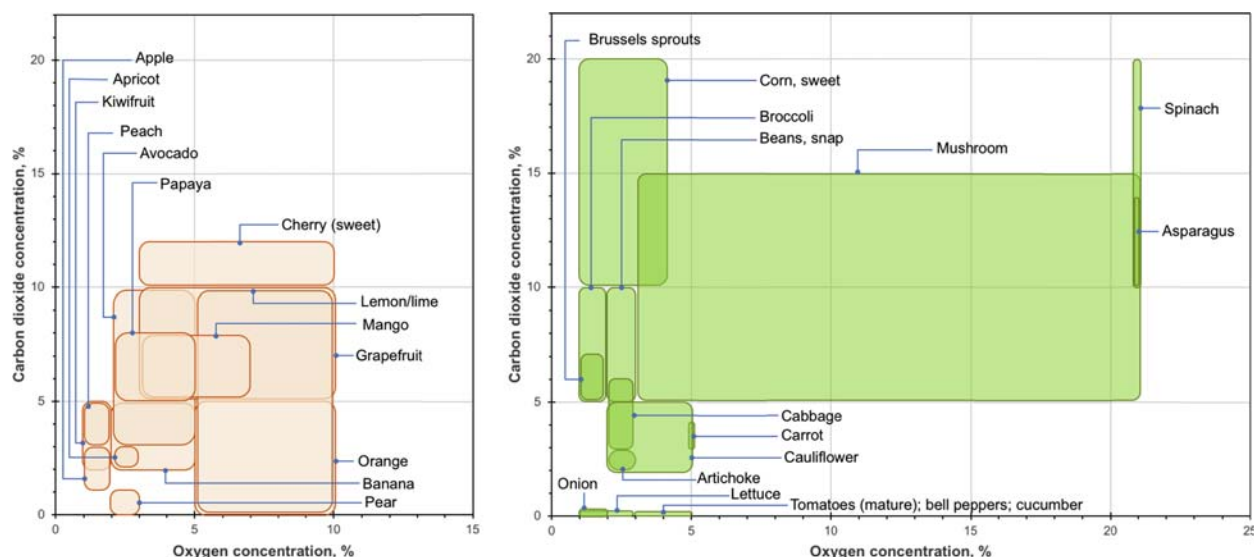
### Gases and Vapours for Modified and Controlled Atmosphere Applications

Modified atmosphere packaging (MAP) is common for protecting processed products from the deleterious ambient factors, in particular  $O_2$  and moisture, to prevent/delay deteriorative phenomena, such as rancidity, browning, microbial growth, loss of crispness, and so on. Typical MAP approach involves flushing the package headspace with an inert gas (e.g.,  $N_2$ , Ar and other noble gases) before package closure (Fellows, 2009; Robertson, 2013). To maintain the modified atmosphere, the use of package materials of adequate barrier properties is essential. Among the MAP gases,  $CO_2$  is the most important for extending the shelf-life of perishable products, due to its effectiveness against Gram-negative and aerobic bacteria. For example, the injection of  $CO_2$  in dairy products (e.g., cottage cheese, sour cream, ice-cream mix, and pasteurized milk) can result in up to 400% increase in shelf-life via improving the microbial stability (Singh et al., 2012). However, solubilization of  $CO_2$  in the aqueous phase of the product can produce carbonic acid which dissociates into bicarbonate and  $H^+$  ions, imparting acidity to the product. Also,  $CO_2$  is highly soluble in both water and oil. At  $0^\circ C$ , the Henry law constants for  $CO_2$  in water and oil are  $1.33 \times 10^6$  and  $2.3 \times 10^6$  Pa.m<sup>3</sup>/kmol, respectively. To put this in perspective, at atmospheric pressure, 1 L of water and oil can absorb approximately 1.7 and 1 L of  $CO_2$ , respectively (Lencki, 2005). Therefore, in MAP involving  $CO_2$ , its solubility in water and oil phases of the product should be taken into consideration. To prevent the unwanted package distortion,  $N_2$  is often used as a filler gas.

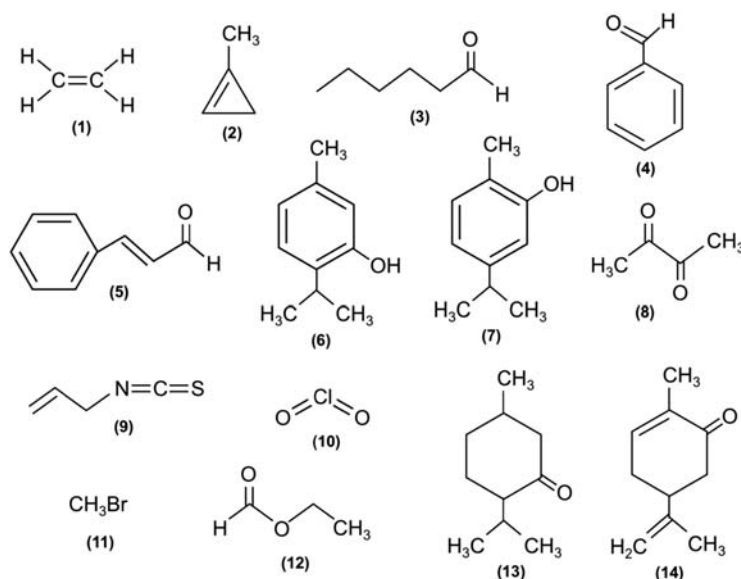
MAP of fresh perishable products (e.g., fruits, vegetables, meats) requires careful manipulation of headspace gas composition to prevent physiological damages of the live tissues, while suppressing the respiratory and microbial activities to extend product shelf-life. In red meats,  $O_2$  (35% to 80%  $O_2$ ; blended with 20%–60%  $CO_2$ , balanced with  $N_2$ ) is often used to maintain the myoglobin in oxygenated state (i.e., oxymyoglobin), inducing red coloration which is often being associated with freshness by consumers. For fruits and vegetables, concentration of  $O_2$  is typically reduced while  $CO_2$  elevated to tolerable levels to maximize storage stability (Fig. 1). The target  $O_2$  and  $CO_2$  concentrations can be established through the use of thermoplastic films with optimal permselectivity (i.e., ratio of  $CO_2$  permeability to  $O_2$  permeability) that matches the respiration rate of the live products (Mahajan et al., 2007; Robertson, 2013). Temperature abuse during transportation can result in dramatic increase in the respiration rate, creating anoxic condition which can be detrimental to the product. To compensate for the increase in respiration rate, side chain crystallizable polymers may be used to allow for proportional increases in  $O_2$  and  $CO_2$  transmission rates of the packaging structure. Other strategies for MAP of fresh produce include the use of plastic films perforated with holes (several micron in size) using a focused laser beam, and microporous film dispersed with porous fillers during the extrusion process (Clarke, 2011; Goswami and Mangaraj, 2011; Hussein et al., 2015; Mangaraj et al., 2009; Robertson, 2013; Zhang et al., 2016).

In general, the goal of controlled atmosphere storage (CAS) is to delay the senescence of fresh produce during storage through manipulating  $CO_2$  and  $O_2$  concentrations in the air. Here, fruits and vegetables are stored in large insulated and gas-tight rooms/containers equipped with sophisticated  $CO_2/O_2$ , humidity, and temperature control systems (Dilley, 2010; Thompson, 2010). During the ripening process, climacteric fruits (e.g., avocado, banana, nectarine, mango, pear, peach, apple) tend to produce ethylene vapor (1) – a naturally-occurring hormone that promotes the respiration and accelerates the development of aroma, color changes, and softening of tissues. On the other hand, non-climacteric fruits do not exhibit burst release of ethylene production during ripening, although exposing them to ethylene can increase their respiration and hasten the ripening process (Bapat et al., 2010). Scrubbing of ethylene in controlled storage atmosphere delays the ripening of fruits, which can be achieved via adsorption/absorption, high-temperature catalytic oxidation, photocatalytic oxidation, and chemical oxidation (Keller et al., 2013; Pathak et al., 2017). In addition to scrubbing, the food industry also applies various strategies to mitigate the effect of ethylene at a cellular level. Of particular importance is 1-methylcyclopropene (1-MCP) (2), which is a potent ethylene receptor blocker capable of delaying the ripening of fruits (Blankenship and Dole, 2003; Lee et al., 2006; Pepera et al., 2003; Watkins, 2006). Because climacteric fruits deteriorate rapidly once when the ripening process begins, they are often harvested at “commercially maturity” stage, i.e., hard green before ripening to provide adequate time for distribution. At the point of sales, the ripening of these fruits is then initiated in a temperature- and relative humidity-controlled ripening room injected with ethylene gas.

Senescence and wounding in fruits result in degradation of phospholipids in the cell membrane through the lipoxygenase pathway, leading to the production of hexanal (3) and other six carbon aldehydes important for defending the plant from microbial



**Figure 1** Optimal  $O_2$  and  $CO_2$  concentrations for MAP of selected fruits and vegetables. Plots are re-created based on the data from Fellows (2009).



**Figure 2** Selected volatile compounds used for food preservation.

proliferation and further tissue damages (Casey et al., 1999; Lanciotti et al., 1999; Patrignani et al., 2008). Besides being a potent antimicrobial, hexanal also elicits enzymatic inhibition properties to delay discoloration, softening, ripening, and generation of off-flavors in fruits (Paliyath and Murr, 2007; Paliyath et al., 2003). It inhibits the activity and expression of phospholipase-D, which is responsible for catalyzing the hydrolysis of phospholipids (e.g., phosphatidylethanolamine, phosphatidylcholine, and phosphatidylglycerol) into phosphatidic acid and their corresponding head-groups (Exton, 1997). Hexanal has been exploited to preserve the cell membrane, substantially delaying the ripening and senescence of fruits. Being a GRAS (generally regarded as safe) substance frequently uses as a flavour additive, hexanal can be very useful to reduce fruit spoilage during storage and distribution (Cheema et al., 2018; El-Kereamy et al., 2009; Paliyath and Murr, 2007; Paliyath et al., 1999; Paliyath et al., 2003; Tiwari and Paliyath, 2011).

### Antimicrobial Volatiles

The antimicrobial properties of hexanal are well documented in the literature, which are mainly due to its interaction with microbial cytoplasmic membrane, causing increased membrane permeability and cell death (Corbo et al., 2000; Gardini et al., 1997; Kubo et al., 2004; Kubo et al., 1999; Lanciotti et al., 2004; Lanciotti et al., 1999; Simons et al., 2000). Other aldehydes, many of which are naturally-occurring in spices and their essential oils, are also known to elicit antimicrobial activities useful for food preservation. For example, benzaldehyde (4), an aromatic aldehyde consisting a formyl substituted phenyl ring with a characteristic almond odour, is



a major constituent of the essential oil from the kernels of almonds (*Prunus amygdalus*) and other seeds (e.g., peaches, cherries, plums, and apricots) (Butzenlechner et al., 1989; Remaud et al., 1997; Sanchez-Perez et al., 2008). Its antimicrobial properties have been attributed to covalent attachment of the aldehyde's carbonyl group to the sulfhydryl groups of cysteine in microbial cell, disrupting active transport and oxidative phosphorylation in microbial cells (Hugo, 1967; Morris et al., 1984; Ramos-Nino et al., 1996; Ramos-Nino et al., 1998). Benzaldehyde is potent against pathogens responsible for fruit spoilage, such as *Bacillus subtilis*, *Serratia marcescens*, *Acinetobacter calcoaceticus*, *Erwinia carotovora*, *Escherichia coli*, *Flavobacterium suaveolens*, *Monilinia fructicola*, *Botrytis cinerea*, and *Tyrophagus putrescentiae* (Wilson et al., 1987; Deans and Ritchie, 1987; Sung et al., 2006).

Cinnamaldehyde (5) is extracted from the bark of *Cinnamomum zeylanicum* with an unique aroma of cinnamon spice (Burt, 2004). The aldehyde vapor exhibits broad-spectrum antimicrobial properties against mold, fungi, Gram-positive and -negative bacteria (López et al., 2005, 2007a,b; Rodríguez et al., 2008), including food-borne pathogens, such as *Bacillus cereus*, *B. subtilis*, *E. coli*, *Listeria monocytogenes* and *Campylobacter jejuni* (Tajkarimi et al., 2010). It interacts with microbial cell membrane to disperse the proton motive force, causing the leakage of small ions and the inhibition of glucose transport and glycolysis (Gill and Holley, 2004; Helander et al., 1998). Researchers have investigated the antimicrobial effects of cinnamaldehyde in various food products, such as *Salmonella tennessee* on peanut paste (Chen et al., 2015a), *E. coli* O157:H7 and *Salmonella typhi* in ground beef (Turgis et al., 2008), microflora in carp (Mahmoud et al., 2004), *Salmonella typhimurium*, *Staphylococcus aureus* and *Yersinia enterocolitica* in apple juice (Yuste and Fung, 2003), and spoilage microbes in melon juice (Mosqueda-Melgar et al., 2008). Solid matrices investigated as carriers for cinnamaldehyde include paper impregnated with paraffin (Echegoyen and Nerín, 2015), cast plastic films (Lopes et al., 2014; Qin et al., 2015), and edible films (Balaguer et al., 2013; Zhu et al., 2014).

Thymol (2-isopropyl-5-methylphenol) (6) is a major component in the essential oil of thyme (*Thymus capitatus*). The volatile can disrupt the phospholipid bilayers of the cell membranes, causing the leakage of cellular contents, as well as interacting with hydrophobic proteins in altering their structures (Chavan and Tupe, 2014; Nedorostova et al., 2009; Zheng et al., 2013). Researchers have shown that the application of thymol in MAP of sweet cherries and table grapes can reduce the growth of mesophilic aerobics, yeasts, and fungi during cold storage, in addition to decreasing weight loss, color changes, loss of firmness (Serrano et al., 2005; Valverde et al., 2005). In MAP of raw shrimp, thymol vapor has been shown to inhibit the growth of *Salmonella* spp., lowering the maximum growth rate by up to 71% and lag time by 100% (Zhou et al., 2013). An isomer of thymol, carvacrol (5-isopropyl-2-methylphenol) (7), found in the essential oil from oregano herb (*Origanum vulgare*) is another antimicrobial volatile potent against *Pseudomonas fluorescens*, *Erwinia amylovora*, and *Candida albicans* (Zheng et al., 2013). Carvacrol can delay the spoilage of table grapes, kiwifruits, and honeydew melon without substantially affecting their sensory properties (Martínez-Romero et al., 2007; Roller and Seedhar, 2002). Its mode of action is believed to be its interaction with cellular membrane proteins and periplasmic enzymes, disrupting the membrane proton motive force (Hyldgaard et al., 2012). Synergistic antimicrobial properties of thymol and carvacrol have been reported against a number of microorganisms. For examples, minimum inhibitory concentration (MIC) values of thymol and carvacrol against *P. fluorescens* inoculated in tryptic soy broth (incubated at 37 °C for 24 h) are reported to be 648 and 167 µg/mL, respectively, while the minimum bactericidal concentration (MBC) values are 1932 and 555 µg/mL, respectively. By combining both thymol and carvacrol, the MIC and MBC values decreased substantially to 78 and 156 µg/mL, respectively (Zheng et al., 2013). Similarly, other researchers reported synergistic antimicrobial effect of thymol and carvacrol against *Salmonella typhimurium* (Zhou et al., 2013) and *Listeria innocua* (García-García et al., 2011). In view of their strong flavour attributes, synergistic antimicrobial properties of thymol and carvacrol, as well as other potential essential oil volatiles, can be beneficial to minimize the possible undesirable sensory attributes via lowering the dosage required to exert the antimicrobial effects.

Diacetyl (2,3-butanedione) (8) is a metabolic by-product of lactic acid bacteria, such as the species from *Lactococcus*, *Leuconostoc*, *Lactobacillus* and *Pediococcus* (Šuškočić et al., 2010). It is naturally present in fruits, milk, dairy products, beer, wines, coffee, and other fermented food (Papagianni, 2012; Shibamoto, 2014). It is commonly used as a food additive to impart buttery flavor (Lanciotti et al., 2003). The dione has a wide antimicrobial spectrum against yeast, as well as Gram-positive and -negative bacteria due to the reaction of its dicarbonyl group (—CO—CO—) with arginine in enzymes, which weakens the microbial cells (Papagianni, 2012; Ray and Bhunia, 2014). It is more potent in acidic than in neutral pH conditions (Jay, 1982; Jay and Rivers, 1984). Jay (1982) reported antagonistic effects of several additives on the antimicrobial efficacy of diacetyl, that 1% (w/v) acetate exhibited the strongest inhibitory effect to diacetyl, followed by 5% glucose and 1% Tween 80. On the other hand, Lanciotti et al. (2003) reported that NaCl enhances the efficacy of diacetyl by increasing its vapor pressure through "salting out" effect. These factors should be taken in account when applying diacetyl in complex food systems.

Allyl isothiocyanate (AITC) (9) is a volatile compound produced by plants from the Cruciferae family (e.g., horseradish, mustard, cabbage) when their tissues are disrupted. In its natural state, AITC is glycosinolated as sinigrin. When the plant tissues are disrupted, the glucosinolate is hydrolyzed by the cell wall bound myrosinase, releasing AITC, along with D-glucose and sulfate ion (Mari et al., 1993). The antimicrobial properties of AITC have been well demonstrated in the literature (Delaquis and Mazza, 1995; Delaquis and Sholberg, 1997; Kim et al., 2002; Lin et al., 2000a,b; Nadarajah et al., 2005; Nielsen and Rios, 2000; Park et al., 2000). In vapor phase, its MIC values against bacteria, yeasts, and molds are reported to be 34–110, 13–37, and 16–62 ng/mL, respectively (Isshiki et al., 1992). Tsunoda reported that the toxic limits of AITC against five fungi on wood ranged from 3.8 to 118 ppm (Tsunoda, 2000). In view of its broad spectrum antimicrobial potency, AITC continues to gain research and development interests (Mari et al., 1993; Kim et al., 2002; Shofran et al., 2006; Winther and Nielsen, 2006; Shin et al., 2010; Wang et al., 2010; Ko et al., 2012; Ugolini et al., 2014; Dai and Lim, 2015; Chen et al., 2015b). Both synthetic and naturally-derived AITC are used for food preservation. In the latter approach, dried mustard seed meal powders have been used as a naturally source of AITC, the release of which is activated by water via the myrosinase-mediated hydrolysis of sinigrin (Dai and Lim, 2014, 2015).



Chlorine dioxide ( $\text{ClO}_2$ ) (10) vapor is a broad-spectrum oxidizing/antimicrobial agent potent against bacterial, viral and protozoan pathogens. Its efficacy is generally considered to be equivalent or stronger than chlorine, but less than that of ozone on a mass-dose basis (Erickson and Ortega, 2006; Gómez-López et al., 2009). The main mode of disinfection action can be attributed to its interaction with nucleic acid and/or peripheral cell structures, leading to a disruption of protein synthesis. The destruction of outer membrane proteins that alters the permeability of the cell membrane is also believed to be a possible mode of action (Aieta and Berg, 1986; Benarde et al., 1967; US EPA, 1999).  $\text{ClO}_2$  has been used for treating fresh produce (Garcia et al., 2003; Gil et al., 2009; Gómez-López et al., 2009; Sapers et al., 2003; Sy et al., 2005). It is often used as a sanitizing agent of packaging, food processing equipment, factory tools, potable water treatment, and so on.

## Fumigants

Methyl bromide (11), a very effective fumigant for quarantine purposes, has been used for decades during the pre-shipment treatment of horticultural products to destroy insect pests. However, its use as a fumigant has been prohibited in most countries due to its ozone-depleting properties (Benschoter, 1988; Fields and White, 2002; Tebbets et al., 1983). Various alternative fumigants have been explored. For example, ethyl formate (EF) (12), a volatile compound naturally present in many products (e.g., rice, beef, grapes, wine, beer, and cheese), is a FDA approved GRAS food-flavouring agent that exhibits potent fumigant activities against insects in various crops. EF at concentrations ranging from 0.8% to 4.7% are shown to be effective in inducing various degrees of mortality in flower thrip, two-spotted spider mite, mealybugs, and leafroller on strawberries and grapes (Simpson et al., 2007; Simpson et al., 2004). Ren and Mahon (2006) reported a 85 g/t for 4 h followed by the second 85 g/t treatment, which resulted in a high level of control of insects in wheat, split faba beans, and sorghum. Unlike other fumigants, EF degrades rapidly and do not pose long-term environmental residual concerns (Desmarchelier et al., 1999). The hydrolytic by-products formed, formic acid and ethanol, are both naturally-occurring and exhibit antimicrobial properties. Synergistic effects have also been reported when EF vapour is mixed with  $\text{CO}_2$  in compressed gas cylinder, at 16.7% wt. level, for the fumigation of fruits, vegetables, and grains. The product is commercially available as Vapormate® by Linde Group. EF also acts synergistically with AITC, menthone (13), carvone (14) and other essential oils in the destruction of pests (Dancevski et al., 2010; Lee et al., 2007; Ren et al., 2008; Ren et al., 2012; Simpson et al., 2007).

## Gases as Integral Product Constituents and Processing Aid

Gases are incorporated in food to impart desirable physicochemical and sensorial properties. Methods to incorporate gases into products are many, including injection, fermentation, whipping, missing, pressurization, steam generation, decompression, vacuum expansion, and so on. The gas incorporated can remain dissolved in the aqueous or lipid phases of the products if it is under-saturated. When supersaturated, the gas will nucleate and grow into bubbles and trapped within the product matrix or ascend to the product surface. The solubility of gases is strongly dependent on temperature and pressure, as well as product compositions.

In carbonated soft drinks (CSD), products are pressurized with  $\text{CO}_2$  gas to provide the characteristic effervescence and fizziness to the palate. In bottled/canned CSD, carbonation level is typically expressed in volume of  $\text{CO}_2$ , where one volume of  $\text{CO}_2$  produces about 1 atm of internal pressure at room temperature, equivalent to about 2 g/L of dissolved  $\text{CO}_2$ . Typical bottled CSD ranges from 1.5 vol for carbonated fruit juices, 4 vol for cola drinks, and up to 5 vol for club soda and ginger ale (Robertson, 2013). By contrast, carbonation in alcoholic beverages such as champagnes, sparkling wine, and beers are due to the  $\text{CO}_2$  produced during the fermentation of sugar by yeast. The level of carbonation in CSD are affected by beverage formulation; sugar reduces  $\text{CO}_2$  solubility in water, while free amino-acid and protein increase  $\text{CO}_2$  concentration (Descoins et al., 2006). The presence of surface-active agents can affect the hydrodynamics of bubbles. For example, the drag force of bubbles in beers as they ascend to the surface is greater than that exerted on bubbles in champagnes, due to higher adsorption of surfactant species (e.g., protein) onto the gas-liquid interface in beer. Also, because the dissolved  $\text{CO}_2$  content is three times lower in beer than champagne, the growth rate of beer bubbles is slower than that of champagne (Liger-Belair et al., 2000). The methods by which the carbonated beverages are poured into the glass and serving temperature will affect the magnitude of dissolved  $\text{CO}_2$  (Liger-Belair et al., 2010).

To reduce product acidity, stout beers are pressurized with a mixture of  $\text{CO}_2$  and  $\text{N}_2$  (instead of pure  $\text{CO}_2$ ) at approximately 0.80 and 3.00 bar (11.6 to 43.5 psi) partial pressures, respectively. The use of  $\text{N}_2$  here also results in smaller bubbles and creamier foam head texture than the use of  $\text{CO}_2$  alone, when the stout beer is poured into a glass (Lee et al., 2011; Vega-Martínez et al., 2017). Similarly, in nitrogen-infused cold brew coffee, also known as “nitro coffee”, coffee is pressurized with  $\text{N}_2$  (typically 30–50 psi) alone, rather than  $\text{CO}_2$ , to avoid the development excessive product acidity and to achieve fine bubbles in the foam head (Gerstner, 2011; Jarvis and Morrison, 2015). When these beverages undergo decompression (e.g., existing from a tap, uncapping a bottle), the dissolved  $\text{N}_2$  becomes over-saturated, forming small bubbles that rise to the top of the drinking glass to form a thick foam head. Concomitantly, a cascade of sinking bubbles appear near the glass wall due to the circulatory flow induced by the rising bubbles in the center of the beverage, as well as the geometry of the glass (Benilov et al., 2013). The infusion of  $\text{N}_2$  also imparts “sweetness” perception in cold brew coffee (Jarvis and Morrison, 2015).

Gases are incorporated in solid and semi-solid products to develop the desirable physical and sensory properties (Campbell and Mougeot, 1999). For example, ice-cream is an air foam in which the air cells are stabilizes by ice crystals. The smaller the size of ice crystals, the greater the proportion of air can be incorporated (Sofjan and Hartel, 2004). In aerated chocolate, air is infused into the molten chocolate mass, dissolving mainly in the lipid phase to provide the light and creamy mouth feel. To create a more creamier

texture, gases with low solubility (e.g., N<sub>2</sub> and Ar) are used instead to form smaller bubbles (Niranjan and Silva, 2008). In whipped cream, N<sub>2</sub>O is being introduced (often blended with N<sub>2</sub> and CO<sub>2</sub> to reduce cost) during mechanical agitation to give overrun properties better than the typical air-aerated cream (Adhikari et al., 2017). In pressurized aerosol whipped cream products, foaming is achieved by rapid expansion of the dissolved N<sub>2</sub>O as the cream emulsion exits the discharge valve of the aerosol can. The foam is stabilized by rapid adsorption of fat to the air cell interfaces, giving a typical overrun of 400% to 600% (Goff and Vega, 2007). Other gases explored in dairy manufacturing include H<sub>2</sub>, Ar, Xe and He (Adhikari et al., 2017), with limited commercial applications.

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# Gelatin

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## Introduction

Gelatin is derived from collagenous materials via thermal denaturation. Practically, gelatin is extracted with hot water at temperature higher than its mother collagen's thermal transition temperature. It has been widely applied in food, pharmaceutical, biomedical, cosmetic and photographic industries. The global demands of gelatin for food and non-food products have been increased. [Grand View Research \(2016\)](#) reported that the global gelatin market size was 412,700 tons in 2015 and the increasing demand for food and beverage and pharmaceutical applications, based on its excellent stabilizing characteristics and good binding features, is expected for the continuous market growth. Gelatin is produced from the collagenous materials, mainly from porcine and bovine skin and bone. Due to the their limitation as well as increasing demand of halal and kosher gelatins, alternative sources for gelatin production, such as fish, poultry, and other mammals (camel and goat), etc have gained more attention ([Abedinia et al., 2017](#); [Almeida and Lannes, 2013](#); [Al-Kahtani et al., 2017](#); [Mad-Ali et al., 2016](#)). The novel sources, e.g. amphibians have been also used for gelatin production ([Karnjanapratum and Benjakul, 2014](#)). Yield and properties of different gelatins are varying. Manufacturing processes involving pretreatment and extraction conditions, filtration and drying are considered to impact the final quality of gelatin. Owing to the varying molecular and functional properties, different gelatins are employed for the particular applications with different purposes, in which the full benefit can be gained.

## Sources

Most of raw materials for gelation production are from animal slaughtering or animal source food processing. The major raw materials of gelatin production are generally from bovine and porcine bone or skin, which are approximately 46% and 52% of the global demand, respectively ([Karim and Bhat, 2009](#)). Raw materials from other mammals such as goat skin and camel bone have been proved to be the promising sources for gelatin production ([Al-Kahtani et al., 2017](#); [Mad-Ali et al., 2016](#)).

Fish skin, bone and scale, etc., generated as the leftover from fish processing, are widely used for production of gelatin and its derivatives. Those raw materials are abundant and cheap ([Benjakul et al., 2012](#)). Moreover, gelatin can be also extracted from the other parts of fish, such as fish head and swim bladder ([Sinthusamran et al., 2016b](#); [Elavarasan et al., 2017](#)). Nevertheless, fish gelatin, especially from skin, has fishy odor, thereby limiting its applications. Fishy odor in fish gelatin could be diminished by several techniques, such as pretreatment, defatting and spray-drying under the appropriate condition ([Sae-Leaw et al., 2015](#), [2016](#)). In contrast, gelatin from fish scale has less fishy odor, but its yield is quite low ([Tu et al., 2015](#)).

Apart from porcine, bovine and fish collagenous materials, avian and amphibian processing leftover are also used for gelatin production. Chicken and duck feet and chicken and turkey heads obtained from slaughtering or dressing could be used as the alternative raw material for gelatin production without BSE transmission, but bird flu is still of concern. Gelling property of gelatin from these materials was comparable to that of mammalian counterparts ([Kuan et al., 2017](#); [Du et al., 2013](#)). However, their yield was quite low (7.01%–7.83%) when compared with those from other sources ([Abedinia et al., 2017](#); [Du et al., 2013](#); [Kuan et al., 2017](#)). Furthermore, frog skin, a byproduct from deskinning process, was also used for gelatin production ([Karnjanapratum et al., 2017](#)).

## Production

Gelatin production can be divided into 3 major steps, including pretreatments (non-collagenous material removal and swelling processes), extraction with water at high temperature and clarification/drying (hot air drying or spray drying or freeze-drying) ([Benjakul et al., 2012](#)). Evaporation, clarification, deodorization (only for fish gelatin) and sterilization can be implemented prior to drying as optional steps. This is dependent on the type of material and specification of final products. The removal of non-collagenous materials, including non-collagenous protein, fat and minerals, aims to increase extraction efficiency and purity of gelatin. Non-collagenous proteins are generally removed by alkaline solution such as sodium hydroxide, while fat and minerals are eliminated by non-polar solvent and EDTA/HCl, respectively. For swelling process, it is applied to disrupt noncovalent bond in collagen molecule, thus promoting the swelling of collagenous material. This can favor the subsequent extraction or solubilization of gelatin ([Stainby, 1987](#)). The swelling process is carried out by acidic or alkaline processes, based on the complexity of raw material. The acid and alkaline processes are used for material with low and high degree of crosslinking, respectively. The gelatin obtained by the acidic process is called type A gelatin, whereas the gelatin manufactured from the alkaline-treated raw material is named type B gelatin ([Karayannakidis and Zotos, 2016](#)). Acid introduced for acid pretreatment is generally organic acid, especially acetic acid, while the alkali used are sodium hydroxide or calcium hydroxide.

Extraction of gelatin is performed from pretreated material, in which hot water is used to destroy the hydrogen bonds stabilizing triple helix of mother collagen. During the transition of collagen to gelatin, non-covalent bonds are broken along with some

covalent inter and intramolecular bonds and a few peptide bonds are cleaved (Benjakul et al., 2012). Temperature has the profound impact on molecular property. Excessive heat negatively affects the gelation of gelatin by reducing the chain length of gelatin (Sinthusamran et al., 2016b). Thus, the appropriate temperature and time are recommended, in which the high quality gelatin can be manufactured (Sinthusamran et al., 2014). To augment the extraction efficacy, especially from animal bone or fish scale, demineralization should be implemented (Herpandi et al., 2011). Additionally, ultrasonication or pulsed electric field can be employed to increase the yield (Tu et al., 2015). These techniques can disrupt or loosen the matrix of those compact materials. As a consequence, gelatin can be more released from the starting material (Chemat et al., 2011).

Filtration and clarification are required to bring about the gelatin with desired clarity. Gelatin solution is treated with diatomaceous earth or activated carbon (Benjakul et al., 2012). Subsequently, the gelatin solution is subjected to drying. In general, drying method and condition used have the impact on characteristics and properties of resulting gelatin (Mad-Ali et al., 2016). Spray drying is a promising means to lower fishy odor of fish gelatin via evaporation of volatiles contributing to the offensive smell (Sae-Leaw et al., 2015).

## Characteristics of Gelatin

Characteristics of gelatin are influenced by both intrinsic and extrinsic factors. Those include isoelectric point, amino acid compositions and molecular weight distribution, etc. and are considered as the factors determining the functional properties and applications of gelatin.

### Isoelectric Point (pI)

According to pretreatment process (swelling step), gelatins possess different pIs. Type A gelatin has pI with the range of 6–9, while pI of type B gelatin ranges from 4.8 to 5.2 (Gelatin Manufacturers Institute of America, 2012). The higher pI of type A gelatin is due to the limited hydrolysis of the side chains of asparagine and glutamine, whereas the side chains of these amino acids are easily hydrolyzed to aspartic and glutamic acids, resulting in the lower pI of type B gelatin (Alfaro et al., 2015).

### Amino Acid Compositions

Amino acid compositions of gelatin vary with animal species, pretreatment and extraction processes. The amount of aspartic acid and glutamic acid in type B gelatin is higher than that of type A gelatin (Duconseille et al., 2015). Overall, gelatin shows the similar amino acid composition to collagen. Gelatin contains glycine (~33%), proline (~12%), alanine (~11%) and hydroxyproline (10%) as the major amino acids but has the low contents of histidine, methionine and tyrosine (Karayannakidis and Zotos, 2016). Proline and hydroxyproline, which are so called imino acid, play an essential role in gelling properties, especially rheological property and gel strength. Imino acid content of gelatin among animal species is different, depending on their normal habitat. Gelatins from tropical animal, both land or aquatic animals, had higher imino acid content (194–225 residues/1000 residues) than those from temperate animals (150–173 residues/1000 residues) (Abedinia et al., 2017; Karnjanapratum et al., 2017; Kittiphattanabawon et al., 2016; Benjakul et al., 2012).

### Molecular Weight Distribution

Gelatin is a heterogeneous polypeptide mixture of  $\alpha$ -chain (single chain),  $\beta$ -chain (two  $\alpha$ -chains covalently cross-linked) and  $\gamma$ -chain (three covalently cross-linked  $\alpha$ -chains) (Ramos et al., 2016). Hydrolysis of those components may occur as induced by the harsh extraction condition, in which peptides with molecular weight ranging from 15 to 400 kDa are formed. The adequate swelling and rupture of intra- and intermolecular bonds can favor the degradation of those components, particularly when extraction is performed at high temperature (Gómez-Guillén et al., 2002; Alfaro et al., 2015). The degradation of those components, especially  $\alpha$ -component, negatively affects gelling property of resulting gelatin. Therefore,  $\alpha$ -component should be maintained as much as possible (Muyonga et al., 2004; Gómez-Guillén et al., 2002). Generally, gelatin extracted at higher temperature with shorter chain length has the higher yield but lower gelling property, when compared to that obtained from milder process.

## Functionalities

Functional property of gelatin can be divided into two groups. The first group is the functionality associated with surface or interfacial behavior, such as the formation of emulsion, foam and film. The latter group is functionality related with gelling properties (Schrieber and Gareis, 2007). Gelatin can be used as gelling agent or provide elasticity, consistency and stability of food products. Gelatin can be also served as coating or edible/biodegradable packaging due to its film forming ability, where transmission of light and oxygen into agricultural produces or foods during storage can be prevented (Benjakul et al., 2012; Ramos et al., 2016).



## Gelling Properties

Gelling properties of gelatin, including gel strength and setting/melting temperatures as measured by texture analyzer and rheometer, respectively, are the indices determining its application. Gel strength or Bloom value, which is determined according to the standard method (BSI, 1975), is the most important property for grading the quality of gelatin (Alfaro et al., 2015; Gelatin Manufacturers Institute of America, 2012). Commercial gelatins have Bloom values from 50 to 300 g, classified as low Bloom (<150 g), medium Bloom (150–200 g) and high Bloom (>220 g) types (Meng and Cloutier, 2014). High Bloom gelatin generally requires lower amount of gelatin to reach the desired gel strength in the final product, when compared with low Bloom gelatin (Gelatin Manufacturers Institute of America, 2012; Schrieber and Gareis, 2007). Setting and melting temperatures are the temperature required for phase transition from liquid to solid and solid to liquid of gelatin, respectively. Gelatin gel is mainly stabilized by hydrogen bond. Hydrophobic and ionic interactions are also involved in gelation (Benjakul et al., 2012). The properties are governed by source, pretreatment, extraction conditions and endogenous protease present in raw material. Also, gelatin concentration and temperature and time for making gel affect the property of gelatin. Gelatin having more degradation of  $\alpha$ -,  $\beta$ - and  $\gamma$ -chains has poor gelling property (Kittiphattanabawon et al., 2016). Generally, fish gelatin, especially from cold water fish, had poorer gelling property than mammalian gelatin because it contains lower amount of imino acids (Hyp + Pro) (Karim and Bhat, 2009). Gelatin from some warm water fish had gelling property comparable to mammalian counterpart (Kittiphattanabawon et al., 2016; Sinthusamran et al., 2014). The property of fish gelatin depends on molecular weight distribution, which is governed by pretreatment and extraction conditions (Kittiphattanabawon et al., 2016; Niu et al., 2013).

The presence of endogenous protease in fish skin is associated with the decrease in gelling property. Endogenous serine protease, which is activated at 50–70 °C, is able to hydrolyze collagen molecules during extraction of gelatin from some fish species (Intarasirisawat et al., 2007; Ahmad et al., 2011).

To improve or strengthen gelatin gel, some substances or enzyme including protein crosslinkers (phenolic compounds, aldehydes, genipin, transglutaminase) are incorporated into fish gelatin during gel preparation, especially gelatin from cold water fish containing low imino acid content (Sinthusamran et al., 2016a; Benjakul et al., 2012). Hydrocolloids such as gellan or  $\kappa$ -carrageenan have been used for modification of gel property of fish gelatin (Sinthusamran et al., 2016a; Petcharat and Benjakul, 2017).

## Film Formation

Gelatin is a protein, which has been used to make the edible coating and film. Gelatin film generally has good mechanical property with an excellent oxygen barrier property, but still has high water absorbability. Gelatin based film is prone to swell, partially dissolve or disintegrate upon the contact with wet surface (Nilsuwan et al., 2016). To tackle the problem, gelatin based film has been modified by incorporation with different substances, such as phenolic compound as a protein crosslinker, etc. Hydrophobic plasticizer and the selected surfactant are employed to improve water resistance property via increasing hydrophobic characteristic of gelatin molecules (Nilsuwan et al., 2016; Ramos et al., 2016). Gelatin film from warm water fish had the comparable stress and elongation at break to bovine and porcine gelatin films (Muyonga et al., 2004). However, water vapor permeability of gelatin film from fish gelatin, especially from cold water fish, was lower than that from porcine and bovine because of much higher hydrophobicity of fish gelatin (Karim and Bhat, 2009). Bilayer films, emulsified film/fish gelatin film, has been developed to improve water vapor barrier property (Nilsuwan et al., 2017).

## Interfacial Properties

Gelatin is a surface-active agent, which is able to act as emulsifying and foaming agents in food and non-food applications. An emulsion is a dispersion or suspension of two immiscible liquids, while a foam is a gas phase dispersed in liquid (Hill, 1998). The hydrophobic domains or residues on the peptide chain are responsible for giving gelatin its emulsifying and foaming properties (Cole, 2000). Gelatin concentration has an impact on interfacial properties, but these properties are also governed by the source of gelatin, its molecular weight distribution and environment of emulsion (pH, temperature and salt) and foam preparation methods (Benjakul et al., 2012; Karayannakidis and Zotos, 2016). Increasing gelatin concentration results in the decrease in emulsifying ability but the increase in emulsion stability, foaming ability and stability (Nagarajan et al., 2012). Bovine gelatin had the better emulsifying ability and emulsion stability than duck feet gelatin. Gelatin from channel catfish had better emulsifying stability, but showed poorer emulsion stability (Kuan et al., 2017; Duan et al., 2018). Gelatin containing higher molecular weight peptides tends to have better emulsifying properties (Nagarajan et al., 2012). Modification of gelatin using oxidized tannic acid could enhance the stability of its emulsion (Aewsiri et al., 2010). In addition, gelatin modified with oxidized linoleic acid rendered the better emulsifying and foaming properties (Aewsiri et al., 2010).

## Applications

Gelatin has been widely used in both food and non-food products. For food applications, gelatin has been utilized as a food additive, such as stabilizer, thickener, gelling agent, film former, whipping agent, clarifying agent, etc. Also, gelatin can be employed in non-food applications, including the biomedical and pharmaceutical industries (hard and soft capsule manufacture, wound

dressings, adsorbent pads and health supplements), cosmetic industries (shampoos, conditioners, lipsticks and fingernail formulas) and the photographic industry (the unique combination of gelling agent and surface activity to suspend particles of silver chloride) (Persistence-Market-Research, 2017).

The unique characteristic of gelatin is the generation of melt-in-the-mouth characteristic and formation of thermo-reversible gel. Additionally, it has high solubility over a broad pH range (Gelatin Manufacturers Institute of America, 2012). The major applications of gelatin can be summarized into 5 groups, including confectionary and jelly desserts, dairy product, meat product, beverages and other applications, such as sauce, soup, frozen product, edible film and coating and hydrolyzed gelatin (Table 1). Gelatin from chicken feet with gel strength of 294 g was classified as high Bloom gelatin and was suggested to use for the production of gelatin gums, marshmallows, gelatin desserts and other products requiring high Bloom value (Almeida and Lannes, 2013). Low Bloom gelatin, obtained from cold water fish gelatin or gelatin extracted under improper condition, can be used in frozen or refrigerated products that are consumed after taking out from the fridge or defrosting to prevent syneresis (Karim and Bhat, 2009).

Confections such as gummy bears contain a relatively high percentage of gelatin. These candies dissolve more slowly, while smoothing the flavor. Gelatin is used in whipped confections such as marshmallows, where it can lower the surface tension of the syrup, stabilize the foam via the increased viscosity, set the foam via gelation, and prevent sugar crystallization (Gelatin Manufacturers Institute of America, 2012).

Gelatin is widely used in ice cream as a stabilizer by binding water, forming a gel network at low temperature and also decreasing ice crystallization (Duan et al., 2018). The addition of gelatin in yogurt can improve stability of yogurt by decreasing syneresis (Pancar et al., 2016). Addition of 0.4% (w/v) gelatin with Bloom value of 246 g in corn-milk yogurt gave the product with good acceptability. Syneresis of those gels can be reduced without causing the development of dense and compact structure (Supavititpatana et al., 2008).

Gelatin is used to gel aspics, head cheese, souse, chicken rolls, glazed and canned hams, and jellied meat products. Gelatin is able to absorb meat juice and acts as the binder. Normal usage level ranges from 1% to 5%, depending upon the type of meat, amount of broth, gelatin Bloom, and texture required in the final product (Gelatin Manufacturers Institute of America, 2012). Addition of cuttlefish gelatin in the octopus sausage could stabilize the emulsion and decrease cooking loss (Souissi et al., 2016). Also, the addition of 0.5% and 1.5% (w/w) porcine gelatin with Bloom value of 200 g in the pork sausage could decrease its cooking loss (Lee and Chin, 2016). In addition, low Bloom gelatin can be used as a stabilizer of emulsion produced at below room temperature, which allows the loading material encapsulated with the gelatin (Meng and Cloutier, 2014).

Gelatin can be used as a clarifying agent in beer and apple juice (Liu et al., 2009; Duan et al., 2018). Gelatin shows the binding ability with a floc, followed by binding the protein into jelly-like mass. Consequently, the aggregate can settle at the bottom of container, making the juice clear (Duan et al., 2018).

Gelatin in hydrolyzed form, which is commonly obtained from enzymatic hydrolysis, has gained an increasing attention from consumers. Gelatin hydrolysate, generally known as 'hydrolyzed collagen' has been fortified in several dietary foods and drinks. It

**Table 1** Application of gelatin with different Bloom values and functionalities in different foods

Product groups	Bloom value (g)	Product	Functionalities
Confectionaries	50–300	Gummy	Thickener
		Marshmallow	Whipping agent
		Nougats	Whipping agent
		Chiffons	Whipping agent
		Pudding	Thickener
		Frozen dessert	Protective colloid
Dairy products	150–250	Yogurt	Stabilizer
		Cream cheese	Stabilizer
		Whipped cream	Whipping agent, emulsifier
		Ice-cream	Protective colloid
		Cheese	Binding agent
Meat products	175–275	Meat roll	Binding agent
		Canned meat	Binding agent
		Sausage	Gel former, water holder
		Beer	Clarifying agent
Beverages	100–200	Wine	Clarifying agent
		Juice	Clarifying agent
		Power drink mix	Thickener
		Gravy, sauce, soup	Thickener
Others	–	Edible coating for fruit and meat	Film former
		Microencapsulation of color, flavor, oil, vitamin	Process aid/wall material

Modified from Gelatin Manufacturers Institute of America, 2012. Gelatin Handbook. Gelatin Manufacturers Institute of America, Inc, New York; Schrieber, R., Gareis, H., 2007. Gelatine Handbook. Wiley-VCH Verlag GmbH & Co. KGaA; Turner, W.A., 1988. Prepared foods. Dan Best, Chicago, USA.

has high digestibility and bioactivities, including antioxidant activity, ACE-inhibitory activity, human LDL-cholesterol oxidation inhibitory activity, dipeptidyl-peptidase IV (DPP-IV) inhibitory activity, antimicrobial activity, mineral binding capacity, the lipid-lowering effect and immunomodulatory activity (Gelatin Manufacturers Institute of America, 2012; Karayannakidis and Zotos, 2016). Those bioactivities depend on type and amount of enzyme used, imino acid composition and peptide sequence and size (Alemán et al., 2011). Hydrolyzed collagen can be used in several products with nutraceutical property such as protein drink and nutrition bars (Persistence-Market-Research, 2017). More importantly, the beneficial effects on skin nourishment have attracted the interest since it can induce the production of collagen as well as minimize the reduction of collagen via inhibiting metalloprotease. The daily ingestion of collagen hydrolysate on skin extracellular matrix proteins of male Wistar rats could increase their type I and IV collagen contents. Collagen hydrolysate uptake significantly decreased both proenzyme and active forms of matrix metalloproteinase 2 (MMP2) (Zague et al., 2011). Bone or joint health is also improved by intake of hydrolyzed collagen. From clinical studies in patients with degenerative hip or knee disease, administration of 10 g collagen hydrolysate daily for 2 months could reduce pain in patients with osteoarthritis of the knee or hip by increasing the synthesis of collagen in joint and cartilage as well as intake of collagen hydrolysate had a great effect in inhibiting bone collagen breakdown in osteoporosis patients (Moskowitz, 2000).

Apart from using gelatin as a food additive, gelatin can be used as an edible film and coating applied in meat and fishery products. Cold-smoked salmon wrapped with fish gelatin based film incorporated with 5.63% (w/w) olive leaf extract could reduce the growth of *L. monocytogenes* during storage at 23 °C, 58% RH for 6 days (Albertos et al., 2017). The application of gelatin based film and coating in pork meat and beef steak could reduce color changes. This was associated with an excellent oxygen barrier property of the film, leading to the retarded metmyoglobin formation (Davis and Lin, 2005; Cardoso et al., 2016).

In pharmaceutical and biomedical applications, gelatin is commonly used as an ingredient for wound dressing, drug delivery systems (soft and hard capsules), soft and hard-tissue engineering scaffolds and a cell-interactive coating or micro-carrier embedded in other biomaterials (Dubruel et al., 2007). Owing to gelling property, it can be used as a major ingredient for soft and hard capsule. Gelatin can melt in human digestive tract and the contained drugs can be released and function at the required target (Duconseille et al., 2015). Gelatin based scaffolds from various processing techniques exhibit excellent biocompatibility, biodegradability and porosity (Hoque et al., 2015). Gelatin has been used to produce biomedical materials, such as heart valve, cardiac tissue engineering, artificial skin, small intestine, liver, wound dressing, nerve regeneration, blood vessel, bone substitute, cartilage, contact lens, plasma substitutes, etc (Gorgieva and Kokol, 2011).

## Gelatin Speciation and Adulteration

Although gelatin is an ingredient commonly used in many applications, the major concern is drawn from some groups of consumers associated with religious practice and belief. The original materials and process used for gelatin must be approved or certified. For example, halal gelatin and its products need to be labeled on the package (Shabani et al., 2015). Additionally, some consumers have allergy to food containing mammalian and fish gelatin (Kuehn et al., 2009). Therefore, the adulteration of prohibited gelatin in the product is necessary to be determined. Many researchers have proposed the analytical methods for detection the presence and species origin of gelatins, such as chemical precipitation, Fourier transform infrared spectroscopy, NanoUPLC-ESI-Q-TOF-MS<sup>E</sup>, polymerase chain reaction (PCR), electrophoretic analysis, high-performance liquid chromatography, mass spectrometry, enzyme-linked immunosorbent assay (ELISA), etc (Cebi et al., 2016; Hidaka and Liu, 2003; Yilmaz et al., 2013; Venien and Leveux, 2005; Grundy et al., 2016; Hermanto and Fatimah, 2013; Shabani et al., 2015). These aforementioned methods have different advantages and limitations. To select any method for gelatin speciation and adulteration detection, specificity, sensitivity, accuracy, rapidity, simplicity and cost should be considered. Development of rapid method with precision to determine the origin of gelatin in food and pharmaceutical products is still required.

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## Hardstock Triglycerides

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### Glossary

**CBE** cocoa butter equivalent

**CBR** cocoa butter replacer

**CBS** cocoa butter substitute

**FDA** US Food and Drug Administration

**GRAS** Generally Recognized As Safe

**H** C<sub>16</sub> – C<sub>24</sub> saturated fatty acids

**K** C<sub>16</sub> – C<sub>22</sub> fatty acids

**O** oleic fatty acid

**M** C<sub>12</sub> – C<sub>14</sub> fatty acids

**N** saturated fatty acids with C<sub>8</sub> – C<sub>14</sub>

**P** palmitic fatty acid

**PHO** Partially Hydrogenated Oils

**PUFA** poly-unsaturated fatty acids

**S** stearic fatty acid

**S<sub>ps</sub>** palmitic or stearic fatty acid

**S<sub>t</sub>** saturated fatty acids

**SFC** solid fat content

**U** unsaturated C<sub>18</sub> fatty acids

For food products with a high fat content, such as butter, margarine and shortening, sensory attributes are largely dependent on the structure and properties of their underlying solid fat crystal networks. The solid fat, denoted as hardstock fat, serves to structure the fat phase and prevent coalescence of the liquid phase, which is either water droplets for water-in-oil emulsion products like margarine, or liquid oil for various shortening products.

Fats and oils are mainly triacylglycerides, which are esters of glycerol and three fatty acids. The behavior and functionality of hardstock fat is determined by their fatty acid composition, arrangement of the fatty acids on the glycerol backbone, and the polymorphic crystal forms of the triglycerides. Melting points of common fatty acids and triglycerides in food products can be found in some literature (O'Brien, 1998a; Larsson, 1986). The most common fatty acids in hardstock fats are lauric, palmitic, oleic, and stearic fatty acids, which form triglycerides and are solid at ambient temperature. Depending on the packing patterns of the hydrocarbon chains of the fatty acids, the crystal polymorphic form of triglycerides can be any of the  $\alpha$ ,  $\beta'$ , or  $\beta$  form with melting point increasing from  $\alpha$  to  $\beta'$ , to  $\beta$ . Some fats may have sub-polymorphic forms. The most well-studied fat having sub-polymorphic forms is cocoa butter, which has six polymorphic forms with different melting temperatures ranged from 16 °C for sub- $\alpha$  form, 21 °C for  $\alpha$  form, 25.5 °C and 27 °C for  $\beta_2'$  and  $\beta_1'$  separately, and 34 °C and 36 °C for  $\beta_2$  and  $\beta_1$  separately (Talbot, 2009).

The crystallization habit of hardstock fats or oils is determined by one or more of four characteristics: 1) palmitic fatty acid content, 2) distribution and position of palmitic and stearic fatty acids on the triglyceride molecule, 3) degree of hydrogenation, and 4) the degree of randomization (O'Brien, 1998b). In general, hardstock fats with a diverse triglyceride structure tend to form  $\beta'$  fat crystals and hardstock fats with limited number of triglyceride structure tend to form  $\beta$  fat crystals. For example, hardstock fats with high palmitic acid content normally form  $\beta'$  fat crystals, but lard tends to form  $\beta$  fat crystals even though it contains around 27% palmitic acid. It is because lard has a predominately asymmetric triglyceride structure with palmitic acid in the sn-2 position. Crystallization behavior of some common fats and oils is well studied and readily available (O'Brien, 1998b).

For food products structured with hardstock fat, a specific melting or SFC (solid fat content) profile is often desired according to the application. For example, the melting point of cocoa butter in chocolate should be around body temperature, so it can have a sharp melting and cooling sensation. All-purpose shortening has a flat SFC curve over a wide range of temperatures, so it can maintain its workability at different temperatures. To have the desired SFC curve, it is often necessary to modify the natural fats and oils through various techniques including hydrogenation, interestification, and fractionation.



## Hydrogenation

Hydrogenation has been used to produce hardstocks from liquid oils since the beginning of the 20th century. It involves addition of molecular hydrogen to the double bonds of unsaturated fatty acid in the presence of a catalyst such as Nickel, which results in decreased unsaturation of the fatty acids, and thus gives elevated melting point and increased oxidation stability of the fatty acids. Many of the hydrogenated oils can serve as hardstock fat. Depending on the conditions used, the starting oils, and the degree of saturation or isomerization, fats and oils with wide range of functionalities can be produced through the hydrogenation process. Hydrogenation can be either partial hydrogenation or full hydrogenation. While full hydrogenation converts the vegetable oils into fully saturated fats, vegetable oils being partially hydrogenated are partially saturated so the melting point increases to the point where solid fat is present at room temperature. The degree of hydrogenation of unsaturated oils controls the final consistency of the product. The partial hydrogenation gives edible oil companies a great flexibility to make fats and oils with a wide range of physical properties and functionalities. However, geometric isomerization during partial hydrogenation process produces trans-fat, which was demonstrated to have cardiovascular health risk, FDA has concluded that PHO (Partially Hydrogenated Oils) are not GRAS (Generally Recognized As Safe) and asked all food manufacturers to make sure their products do not contain PHO from June 18, 2018 (FDA, 2017).

Fully hydrogenated fats and oils do not contain trans-fat, thus do not have the same health risk, but average consumers cannot distinguish the difference between fully hydrogenated fats and oils and partially hydrogenated fats and oils, so a lot of food manufacturers try to avoid all hydrogenated fats and oils on their labels. It raised new challenges to the edible oil industry to manipulate the consistency and functionality of fats and oils through other measures.

## Fractionation

Fractionation is the process which separates a natural fat or oil into several fractions with different melting points. The high-melting fraction serves as hardstock fat and can be blended with other fats and oils to obtain desired consistency. There are two methods of fractionation: dry fractionation and solvent fractionation. Under dry fractionation the oil is heated and then cooled to a given temperature under agitation such that the partially crystallized mass can be filtered off under vacuum to give the high-melting fraction. The remaining liquid fraction gives the low-melting fraction. Each fraction of the oil can be blended with other fats and oils for different applications.

The most popular oil being fractionated is palm oil, which can be fractionated into at least four fractions: palm stearin, palm mid fraction, palm olein, and palm super olein. The main reason for palm oil fractionation is the demand for clear liquid oil (palm olein). The high-melting palm stearin fraction has been used to produce cocoa butter equivalents/replacers, and as a hardstock for margarine and other fat continuous products.

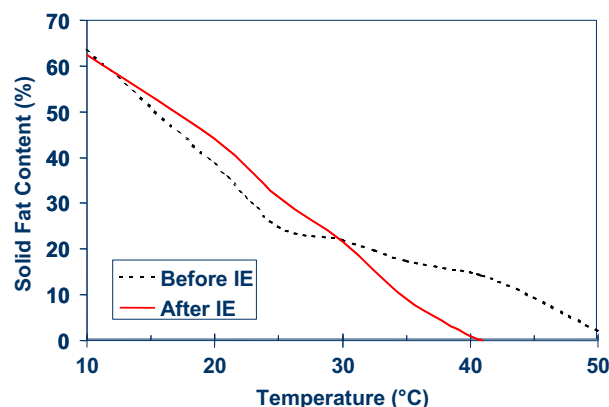
## Interestification

Interestification is the process by which fatty acids are rearranged on the glycerol fragment of the triacylglyceride molecule. Interestification can be either random interestification (chemically catalyzed) or selective interestification (enzyme catalyzed). The most commonly used chemical catalysts are sodium methylate and sodium ethylate. Alkaline catalyzed interestification produce new triacylglyceride molecules with fatty acids randomly distributed on the three available positions; while lipase mainly catalyze rearrangement of the fatty acids on sn-1 and sn-3 position, leaving the fatty acids on the sn-2 position unchanged. Interestification of a designed fat blend can reduce the solid fat levels at high temperature and make a hardstock fat better suited for certain applications. Fig. 1 (Wassell and Young, 2007). shows the SFC of a blend of 60% palm stearin and 40% palm kernel oil before and after interestification. The interestification process significantly lowered SFC of the mixture, especially the SFC above 30 °C, which improved the sensory sensation of the product. In combination with oil fractionation, interestification can modify the SFC curve of fats and oils and improve their functionality without the formation of trans fatty acid, thus make it an ideal alternative to hydrogenation process.

So far, the chemical structure and physical properties of hardstock fats including their melting point and polymorphic forms and the techniques to modify hardstock physical properties have been discussed. In the next two sessions, the application of hardstock fats in margarine and confectionery products will be discussed.

## Trans Free Hardstocks for Margarine

Margarine is water-in-oil emulsion, where finely dispersed water droplets and liquid oil are immobilized by the hardstock fat crystal network. The melting behavior of the hardstock used in margarine is extremely important to the quality and sensory perception of the product, because margarine must remain firm at refrigerating temperature, spreadable at room temperature (10–20 °C), and melt completely at body temperature (>35 °C). In addition to the requirement of its melting profile,  $\beta'$  form of the hardstock fat crystals is preferred in margarine since the needle-like shape and smaller crystal size of  $\beta'$  fat crystals give margarine smooth texture. PHO has been widely used for margarine production; however, the recognized health risk of trans fatty acid and saturated fatty acids has led to active research and development work on trans-free and lower-saturated fatty acid hardstock for margarine.



**Figure 1** A blend of 60% palm stearin/40% palm kernel oil before and after interesterification. Adapted from Wassell, P., Young, N.W.G., 2007. Food application of trans fatty acid substitutes. *Int. Journal Food Science Technology* 42, 503–517, with permission.

Margarine manufacturers have suggested that triglycerides with “H–U–H” structure form stable  $\beta'$ - crystals thus give smooth texture to margarine (Elliott et al., 1999; Floeter et al., 2004), where H is  $C_{16}$  –  $C_{24}$  saturated fatty acids, U is unsaturated  $C_{18}$  fatty acids. Hardstock triglycerides with “K2M” structure have been reported to give desired texture to margarine as well, where K is  $C_{16}$  –  $C_{22}$  fatty acids and M is  $C_{12}$  –  $C_{14}$  fatty acids (Sahasranamam, 2005).

In order to achieve the desired triglyceride composition and nutritional composition of the hardstock, margarine manufacturers either blend natural, unmodified vegetable oils, or modify the chemical structure of hardstock fats through hydrogenation, fractionation, and interestification of a mixture of fats and oils containing high level of mono- and PUFA (poly-unsaturated fatty acids). Palm oil fractions are the most used oil in margarine production, especially palm stearin has been widely used in blending and interestification with other liquid oils to produce trans-free and high PUFA margarine oils. For example, a typical trans-free fat blend for a table margarine may contain palm stearin, palm kernel olein, and sunflower seed oil in the ratio of 60:20:20 (Yusoff and Dian, 1995), or alternatively palm oil, palm olein and canola oil in the ratio of 57:23:20 (Yusoff et al., 1998). Thanks to its short chain lauric acid content, palm kernel oil is excellent for industrial type margarines due to its enhanced creaming properties and preferred fat crystallization properties (Yusoff and Dian, 1995). In addition to palm oil, some specialty oils such as Allanblackia oil and Pentadesma oil have been used in margarine production as well. Allanblackia oil and Pentadesma oil contains 60% - 80%, and 48% SOS (stearin-olein-stearin) triglycerides separately (Arellano et al., 2015; Floeter et al., 2006). The high level of high melting SOS triglyceride makes both oils perfect hardstock for margarine production, where both oils are either fractionated or use as a natural oil, and blended with other liquid oil. The high content of similar mixed fatty acids triglycerides such as SOS also increases the intersolubility of triglycerides and formation of mixed fat crystals, thus give smaller fat crystal size, uniformly dispersed fat crystal networks, enhanced emulsion stability and a smooth texture.

### Hardstock Fats in Confectionery

In confectionery industry Cocoa butter is one of the most important fats and oils thanks to its sharp melting profile around body temperature. Cocoa butter contains mainly three fatty acids – palmitic, stearic and oleic, which mostly in one of the three triglycerides – POP, POS, and SOS, where P is palmitic acid, O is oleic acid, and S is stearic acid. Because of its specific triglycerides composition, especially its high level of SOS triglycerides, cocoa butter can form six polymorphic forms with different melting temperature, where form V ( $\beta_2$ ) is the desired form for chocolate production.

In confectionery products, cocoa butter and cocoa butter alternatives are important hardstock fats providing structure to chocolate, compound coatings, confectionery fillings and other confectionery products and prevent or slow down oil migration from center filling to the coating. There are three basic types of cocoa butter alternative: CBE (cocoa butter equivalent), CBR (cocoa butter replacer), and CBS (cocoa butter substitute).

CBEs are based on lauric fats rich in lauric acid ( $C_{12:0}$ ) specifically palm kernel oil and coconut oil and has limited compatibility with cocoa butter. CBRs used to be produced from partially hydrogenated vegetable oils, but now it is mainly produced from fractions of palm oil. CBEs are vegetable fats composed of similar symmetrical  $S_tOS_t$  triglycerides as cocoa butter ( $S_t$  is saturated fatty acids and O is oleic fatty acid), and it behaves similarly as cocoa butter such as crystallization and tempering. A typical triglyceride composition of cocoa butter and some CBE component fats are available in literature (Talbot, 2015). CBEs can be produced from blending of those component fats according to applications. Various oil modification techniques are often used to produce cocoa butter alternatives from vegetable oils. For example, a cocoa butter – like fats were prepared from interestification of a blend of palm oil and fully hydrogenated soy bean oil in a ratio of 1.6:1 followed by fractional crystallization from acetone (Abigor et al., 2003).

In addition to providing structure, some hardstock triglycerides have excellent anti-blooming properties in confectionery products. It was found that blends of triglycerides comprising a fat A containing  $> 40\%$   $S_{ps}OS_{ps}$  ( $S_{ps} = C_{16:0}$  or  $C_{18:0}$ ; O =  $C_{18:1}$ )

and a fat B containing an ( $H_2N + N_2H$ ) fat, where N is saturated fatty acids with  $C_8 - C_{14}$ , displays excellent anti-blooming properties in chocolate compositions (Frederick et al., 1995). Blends of SOS-rich Vietnamese mango fat and Indian mango fat with cocoa butter at a ratio of 70/30 has shown to increase the heat resistance and anti-blooming properties of the chocolate made with the fat blends (Tran et al., 2015).

## Summary

Hardstock triglycerides are solid fat at room temperature, which provides structure to food products with high fat content. The crystallization and melting behavior of hardstock triglycerides is determined by their chemical structure and physical polymorphic forms. Various modification techniques including hydrogenation, fractionation and interestification were employed to produce hardstock triglycerides with desired properties, especially trans-free and low-saturated hardstock triglycerides.

Hardstock triglycerides with “H–U–H” structure was shown to give the desired  $\beta'$  crystal form and smooth texture to margarine. In contrast,  $\beta$  crystal form is often desired in confectionery applications, which is formed mainly by the symmetric  $S_1OS_1$  triglycerides.

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## Medium Chain Triacylglycerides

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### Glossary

**GRAS** abbreviation for “generally recognized as safe” by the American Food and Drug Administration (FDA) and it indicates that the substance is considered safe by experts

**Triacylglyceride** lipid molecule containing a glycerol as backbone with three esters bonds of hydrocarbons hydrophobic chains

**Phospholipids** lipid molecule consisting of a hydrophilic phosphate group and two hydrophobic fatty acids. The head and tail groups give this molecules an amphiphilic characteristic that is used to form bilayers

**Glycolipids** lipid molecule comprised of a hydrophobic lipid tail and one or more hydrophilic sugar groups linked by a glycosidic bond

### Nomenclature

**TAG** triacylglyceride

**DAG** diacylglycerides

**MAG** monoglyceride

**FA** fatty acid

**MCT** medium chain triacylglyceride

**LCT** long chain triacylglyceride

**GRAS** generally recognized as safe

**CB** cocoa butter

**MCFA** medium chain fatty acid

**LCFA** medium chain fatty acid

**HC** hydrocarbon chain

### Introduction

Edible fats and oils comprise one of the three major classes of foods, the others being carbohydrates and proteins. Edible fats and oils can be obtained from terrestrial or aquatic animals, from the seeds or leaves of many plants, from the pulp, or from the nut/stone of many fruits. Edible fats and oils are used in many products not only to make food palatable but also to make them functional.

The terms “fat” and “oil” are used according to the physical state of the material at room temperature. Fats are in a semi solid state, but the appearance is of a solid, while oils are in a liquid state. Chemically, fats and oils are mixtures comprised of more than 95% triacylglyceride (TAGs) molecules mixed with minor components. Triacylglycerides can also be found in the literature with the name triglycerides or triacylglycerols. These minor components can include phospholipids, glycolipids, free fatty acids, monoglycerides (MAGs) or diacylglycerides (DAGs) (Swern and Bailey, 1964). When dealing with edible fats and oils for the manufacture of food products, one tends to refer to the TAGs molecular composition, avoiding all other components as they represent less than 5%. More over, it is common to refer to the fatty acid (FA) composition instead of the TAG composition. TAGs molecules are esters of a glycerol molecule with three FAs. The FA is a hydrocarbon chain with a methyl (CH<sub>3</sub>) group at one end and a carboxylic acid (COOH) group at the other. The COOH is the one reacting with the alcohol group of the glycerol to produce the ester linkage. FAs are characterised by the hydrocarbon chain (HC) length (number of carbon atoms), the saturation (number of hydrogen atoms per carbon atom), the presence of *trans*- or *cis*-bonds (straight or bended hydrocarbon chain) and the type of bonds (single, double or triple bond) between carbon atoms (Small, 1986).

The melting point is a physical property commonly used to characterize FAs. The melting point is related to the way the molecules packed. The temperature at which FAs with short HC lengths melt are lower that those with large HC. Also, the more double bonds or the higher the number of bonds between two carbons (moving from single to triple bonds) the lower the temperature of the melting point.

Un-saturated FA *cis*-bond with a single, double or triple bonds create a FA that is non-linear. This non-linearity in the FA originates a loose packing of the molecules, hence low melting points. The loose packing configuration is due to weak van der Waals interactions since the molecules are not close to each other. Hence, the amount of energy needed in the solid–liquid transition (melt) is less than that required for well packed molecules, as is the case for *trans* or saturated TAGs.

Fig. 1 shows two FA, represented by using the expanded structural formula name, the condensed structural formula, the common name and the symbols by which they are commonly identified.

Table 1 expands on the symbols and names following the IUPAC convention (<http://www.sbcs.qmul.ac.uk/iupac/lipid/>) for only some cases. The reader is encouraged to see Akoh (2017) or Lehninger (1970) books for more complete tables.

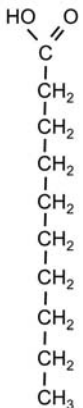
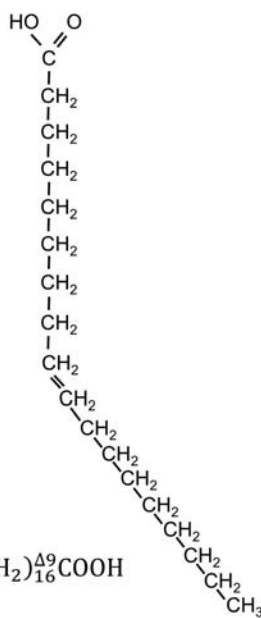
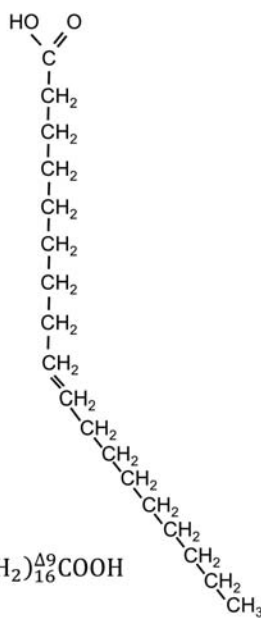
There is no “typical” TAG fat molecule in nature. The amount and type of TAGs that constitute a particular fat or oil varies not only with the cultivar or animal species, but also with the source of origin. For example, it is known that the environmental growing conditions notoriously affect the FA composition of cocoa butter, CB (Marty and Marangoni, 2009; Lehrina and Keeney, 1980; Chaiseri and Dimick, 1989). This “problem” of composition is not unique to CB, but other fats and oils obtained from animals or vegetables are also known to have different FA composition. Changes in the composition can lead to changes in some physical and chemical properties (Shukla, 1995; Schlichter-Aronhime and Garti, 1988; Foubert et al., 2004), hence, affecting the functionality of the product manufactured with that particular edible fat.

TAGs are named using the fatty acids that make them up. n-1, n-2 and n-3, or Sn-1, Sn-2 and Sn-3 are used to identify the FA position in the glycerol backbone. For example if stearic acid is in the Sn-1 position, oleic is in the Sn-2 position and stearic acid is in the Sn-3 position, then the TAG is referred as SOS and ways of naming it could be: stearic-oleic-stearic or 1,3-distearoyl-2-oleoylglycerol. One can anticipate that the number of possible combinations of similar or different FA that can be esterified to the glycerol back bone is very large. Fortunately, nature does not use all of those combinations. For example, unsaturated FA typically appear in the Sn-2 position in animal fats (Beppu et al., 2017), while oils originated from seed display a saturated FA in the Sn-2 position.

Examples of TAGs are given in Table 2, where the third column shows the FA position in relation to the backbone that make up that particular TAG.

Calling a TAG “saturated” or “unsaturated” is not a simple task because it contains 3 FA which can be saturated or unsaturated or a mix of them. For example calling PPP a saturated TAG is correct as it contains three fatty acids that are saturated, while OOO is an unsaturated TAG as it is made exclusively by unsaturated FA. The tendency has been to call a TAG molecule saturated if it contains 2 or 3 saturated FA and unsaturated if it contains 2 or 3 unsaturated FA.

FA analysis are easy to perform and the reader can find in the literature lists of FA compositions for many edible fats (Gunstone, 1970). On the other hand, the analysis to detect TAGs is expensive, as pure TAGs (usually synthesized ones) need to be used for

Expanded Structural formula		
		
	Condensed structural formula	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>8</sub> COOH
	Common name	Capric acid
	Symbol	8:0
		
	Condensed structural formula	CH <sub>3</sub> (CH <sub>2</sub> ) <sub>16</sub> <sup>Δ</sup> COOH
	Common name	Oleic acid
	Symbol	18:1

**Figure 1** Example of two fatty acids and their naming based on different conventions. The expanded structural formula is a visual way of seeing the number of carbon and hydrogen atoms, which gets simplified in the condensed structural formula. In the condensed structural formula, double bonds are indicated using the  $\Delta$  symbol. COOH identifies the carboxyl group at one end of the fatty acid and the CH<sub>3</sub> identifies the methyl group at the other end of the hydrocarbon chain. The symbolic name contains two numbers separated by “:”; the first number indicates the number of carbon atoms in the FA and the second number indicates how many double bonds are present. Hence, when the second number is “0”, it indicates that the FA is saturated, as there is no double bonds.

**Table 1** Name and symbols for some common fatty acids

<i>Symbol</i>	<i>Common acid name and abbreviation</i>	<i>IUPAC name</i>
<b>Most common saturated</b>		
4:0	Butyric - B	Butanoic acid
6:0	Caproic - C	Hexanoic acid
8:0	Caprylic - C	Octanoic acid
10:0	Capric - C	Decanoic acid
12:0	Lauric - L	Dodecanoic acid
14:0	Myristic - M	Tetradecanoic acid
16:0	Palmitic - P	Hexadecanoic acid
18:0	Stearic - S	Octadecanoic acid
20:0	Arachidic - A	Icosanoic acid
22:0	Behenic - B	Docosanoic acid
24:0	Lignoceric - Lig	Tetracosanoic acid
<b>Some MONO- unsaturated</b>		
10:1	Caproleic acid	dec-9-enoic acid
12:1 n-3	Lauroleic acid	(Z)-dodec-9-enoic acid
14:1 n-5	Myristoleic acid	(Z)-tetradec-9-enoic acid
16:1 n-7	Palmitoleic acid	(Z)-hexadec-9-enoic acid
18:1 n-9	Oleic acid	(Z)-octadec-9-enoic acid
<b>Some POLY- unsaturated</b>		
18:2 n-9,12	Linoleic acid (LA)	(9Z,12Z)-octadeca-9,12-dienoic acid
18:3 n-9,12,15	alpha-Linolenic acid (ALA)	(9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid
18:3 n-6,9,12	gamma-Linolenic acid (GLA)	(6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid

**Table 2** TAG examples using the abbreviations used in Table 1

<i>Symbol</i>	<i>Common name</i>	<i>Fatty acid position and name</i>
PPP	Tri-palmitin	Sn-1 palmitic acid Sn-2 palmitic acid Sn-3 palmitic acid
POS	1-palmitic, 2-oleic, 3-stearic	Sn-1 palmitic acid Sn-2 oleic acid Sn-3 stearic acid
LA,LA,S	1, 2-linoleic, 3-stearic	Sn-1 linoleic acid Sn-2 linoleic acid Sn-3 stearic acid
OOO	Tri-olein	Sn-1 oleic acid Sn-2 oleic acid Sn-3 oleic acid

standards. Instead, many researchers report only the percentage of each hydrocarbon chain present in the oil, without being specific about the Sn-position of the FA in the glycerol backbone.

Medium-chain triacylglyceride, MCT, are particular TAGs that contain between 6 and 12 carbons in the FA. MCT can be found in milk, coconut oil and palm kernel oil. MCT can also be found in structured lipids, the man-made lipids created by using enzymes, esterification or hydrogenation. On the other hand, LCT refers to those “long chain triacylglyceride” that contain more than 14 carbons in the FA, mainly 16 and 18 and up to 21. Because of the shorter hydrocarbon chain, MCTs are smaller in molecular weight than LCT with a lower smoke and melting point than the LCT (Gunstone, 1970; Lehninger, 1970). The saturated FA that make up MCT are caproic acid, caprylic acid, capric acid, and lauric acid.

## Metabolic Path Way

MCTs have several unique nutritional and physiological properties that started being used in 1950 for dietary treatment of malabsorption syndromes (Seaton et al., 1986). MCT are nowadays used for parenteral nutrition (Bach et al., 1989) when rapid energy supply is desired or for enteral nutrition when digestion, absorption or transport of LCT is impaired (Megremis, 1991; Timmermann, 1993). MCT have been shown to be a good source of nourishment for patients that displayed malabsorption



syndromes caused by rapid absorption (Seaton et al., 1986). Another successful use of these triglycerides has been for rapid calorie supply on postsurgical patients, who run into acute loss of energy (Winawer et al., 1996). In 1994, MCT were recognized as GRAS (Traul et al., 2000) and it is known that they are well tolerated when the amount consumed in one meal does not exceed 30 g (Jeukendrup and Aldred, 2004).

The usefulness of MCT resides in the way they are metabolized, absorbed and oxidise in the body. MCT hydrolysis, starts partially with enzymes in the mouth, but more importantly in the stomach (Fernando-Warnakulasuriya et al., 1981; Staggers et al., 1981; Hamosh et al., 1989) where the hydrolyses is faster and more complete than for LCT (Greenberger et al., 1966; Borgstrom and Patton, 1991), and the gastric transient time is faster (Harkins et al., 1964; Hunt and Knox, 1968; Hopman et al., 1984). It is also said that the absorption of MCT is more efficient (Webb et al., 1991; Harkins and Sarett, 1968; Roy et al., 1975; Lau et al., 1979; Jensen et al., 1986; Sulkers et al., 1992) than for LCT. The shorter medium chain fatty acids, MCFA, compared with the longer chain fatty acids, LCFA, pass directly into the bloodstream (Bach and Babayan, 1982). The portal system transports the MCFA to the liver for hepatic metabolism before they are sent to the heart. The metabolism for the longer triacylglycerides is different. The entrance of LCTs into the duodenum stimulates the enteric secretion of the hormone cholecystokinin (CCK) together with the pancreatic enzymes. Also, bile is secreted from the gallbladder to help emulsify the triglycerides into fat droplets (Isaacs et al., 1987; Symersky et al., 2002). The pancreatic lipase hydrolyzed the LCT into FA and either mono (MONO)-acylglycerides or di (DI)-acylglycerides. The FA, MONO or DI enter the enterocyte cells lining the intestine via passive diffusion or via a protein transporter. TAGs get re-assemble in the enterocyte from their fragments and are packed together with cholesterol and proteins to form the chylomicrons (Tso and Balint, 1986). The chylomicrons are excreted from the cells and collected by the lymph system and transported to the subclavian vein to reach the bloodstream. LCT are used either for energy or they are deposited into the adipose tissue.

### Natural Sources of MCT: Fatty Acid and TAG Profiles

Natural sources of MCT are palm kernel oil, coconut oil and milk fat. Some literature mention that MCT can be obtained from palm oil (the flesh of the palm fruit) but in its natural state, the amount of fatty acid with less than 12 carbon is 0.2%, an insignificant amount.

MCT can be produced either through enzymatic (with 1,3 specific or nonspecific enzyme), chemical methods or by fractionation.

The coconut fruit consists of an external husk, a white "flesh or meat" and a nut or stone. The flesh is dried to obtain what is called copra. To achieve this, the fruit is cut in half, the stone remove and the halved coconuts are exposed to the sun for drying, a process that facilitates removing the husk from the flesh. The "dry method" of obtaining coconut oil involves pressing the copra first, and solvent may also be used after the press to obtain more oil. If the oil is not refined, then the oil is called virgin. Typically the oil goes through a refining process for direct human consumption. The processed product is called "RBD coconut oil" to indicate that it has been refined, bleached and deodorized as any other oil for human consumption (Block and Barrera-Arellano, 2009).

Palm kernel oil is obtained from the kernel of the stone in the palm fruit. Similar to coconut, the palm fruit consists of a husk, yellow flesh, and a stone or nut. After harvesting the fruit, the fruit is separated into its flesh and the stone. The flesh is used to obtain palm oil. The stones are extremely hard, allowing for safe transport for processing far from the harvesting region. The dried-stones are cracked to remove the husk and expose the kernels. The kernels are pressed to obtain palm kernel oil and similar to coconut oil, solvent extraction may also be used. The refining process may also be employed to guarantee a product safe (free of pathogens, and physical contaminants) for human consumption.

Milk fat's MCTs are obtained from milk fat but in less quantities than from coconut or palm kernel oil as shown in Table 3.

All three edible oils listed in Table 3 contain a percentage of unsaturated fatty acids as well as palmitic and stearic acid. If the end product is to have only MCT, then those fats/oils must be fractionated or separated in order to obtain the desired MCT triacylglycerides (hydrocarbon chains containing between 6 and 12 carbons) from the rest.

**Table 3** shows the fatty acid profile for milk fat, palm kernel oil and coconut oil

<i>Fatty acid</i>	<i>Milk fat<sup>a</sup></i>	<i>Palm kernel oil<sup>b</sup> (PKO)</i>	<i>Coconut oil<sup>c</sup> (CNO)</i>
4:00	2.6–6.3		
6:00	07.–3.0	0.2–0.4	
8:00	0.4–1.6	3.2–4.7	4.6–10
10:00	1.4–3.2	2.9–3.5	5.0–8.0
12:00	2.4–3.7	45.4–49.8	45.1–53.2
14:00	0.3	15.4–17.2	16.8–10.2
16:00	29.4–39.4	7.9–9.3	7.5–10.2
18:00	12.6–21.1	1.9–2.3	2.0–4.0
Unsaturated	16–26.7	15–21	6–13

<sup>a</sup>Ransom-Painter et al., 1997.

<sup>b</sup>Ibrahim et al., 2003.

<sup>c</sup>Pantzaris and Basiron, 2002.

Fractionation is a process in which the desired TAGs (either high, medium or low melting point) are separated using waterbath or incubators at the different temperatures. By placing the material at particular temperatures, the desired fat-fraction can be removed. This method is used when dealing with coconut and palm kernel oil. In the case of milk fat, different methods can be used to obtain the MCTs: two-stage dry fractionation (Dimick et al., 1996), continuous counter-current supercritical carbon dioxide (Bhaskar et al., 1993) or vacuum filtration (Black, 1975). The goal is to separate TAGs with a short hydrocarbon chain length, hence with low melting point as compared to LCT.

One can see from Table 3 that coconut oil contains more saturated FA than palm kernel oil. More saturated FA can also be seen in the iodine value, which is in the range from 6 to 10 in coconut and 14 to 21 in PKO (Ibrahim et al., 2003). Contrary to those two materials, milk fat contains a good percentage of short chain fatty acids 4:0 which oxidize easily and which can confer a buttery flavor.

## Future Trends

There is a continuous demand of functional foods associated with health benefits. Edible fats are only one kind of these foods. The search for an ideal food that can prevent heart disease, diabetes or help in losing weight is on going.

For the last 30 years, nutritionists had been categorizing edible dietary fats as either “good” or “bad” according to their ability to increase or decrease the amount of low density lipoprotein (LDL) cholesterol or high density lipoprotein (HDL). It has been shown that having high levels of HDL cholesterol in the blood correlates well with a reduction in cardiovascular disease, hence the aim is to consume those fats or oils that can achieve this. In particular, increase amount of saturated fats by humans had been linked to increases in LDL level, while unsaturated fats have been linked with increasing the level of HDL. This has lead the World Health organization to advice the public into restricting the amount of saturated fats that are consumed. However Mensink and co workers (Mensink et al., 2003) showed that saturated fats neither raise nor lower the ratio of total cholesterol to HDL cholesterol. Mensink findings support that lauric acid, the saturated fat present in high percentage in palm kernel oil and coconut oil should not be categorized as a bad “fatty acid”.

The work mention in this section favours many positive physiological and nutritional aspects concerning MCT. These aspects have lead to MCT gaining popularity among high performance athletes as the MCT had been shown to be fast source of nourishment. MCTs are also being used in oils, sports drinks, energy bars, meal replacements and some special baby foods. As more is learned about the health benefits of MCTs, new applications of MCT will be developed.

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## Milk Proteins

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### Nomenclature

$\alpha$ -LA	Alpha-lactalbumin
AA	Amino acid
$\beta$ -LG	Beta-lactoglobulin
BSA	Bovine serum albumin
Ca	Calcium
CCP	Colloidal calcium phosphate
EtOH-HCl	Ethanol-hydrochloric acid
GMP	Glycomacropeptide
LF	Lactoferrin
MW	Molecular weight
pI	Isoelectric point
SH	Sulfhydryl group
(S-S)	Disulfide bond

### Introduction

Milk is a complex biological fluid secreted by the females of all mammalian species. It is produced to meet the nutritional requirements of the neonate such as delivering the energy, essential amino acids (AA) and micro-nutrients required for adequate growth and development. Depending on the mammalian species, the new-born will have very different nutritional requirements leading to large interspecies differences in gross composition and yield of milk (**Table 1**) (Fox et al., 2015b). Bovine milk (especially from the species *Bos taurus*) is the predominant raw material for dairy products which accounts for ~ 84% of total global milk production and has been subject to extensive characterization (Thompson et al., 2009). Bovine milk proteins and their physical, chemical, functional and nutritional properties will be the focus of this review.

Bovine milk contains ~ 3.4% protein which was initially believed to be in the form of one protein only. Between 1883 and 1885, the Swedish scientist Hammersten (1883) showed that milk proteins could be divided into two groups, casein proteins and serum (whey) proteins, by adjusting the pH of bovine milk to the isoelectric point (pI) of caseins (pH 4.6). At this pH, the casein proteins were found to precipitate from milk while the whey protein fraction remained soluble. Later, studies would establish that both casein and whey protein fractions were in turn composed of a number of different proteins.

The concentrations of these two fractions in the milk of any particular mammalian species again differ, and are presumed to be tailored to the nutritional and physiological requirements of the young (**Table 1**). The whey protein:casein ratio of bovine milk is ~ 20:80 (Jensen, 1995).

### Casein Proteins

Caseins are defined as milk proteins that precipitate from raw skim milk when the pH is adjusted to pH 4.6 (i.e. pI of casein) at temperatures greater than 10 °C. At temperatures less than 10 °C, aggregation of caseins does occur but the aggregates are fine enough to remain in suspension (Fox et al., 2015b).

### Fractionation and Heterogeneity of Caseins

The heterogeneous nature of caseins was first described by Linderstrøm-Lang (1925) who fractionated isoelectric/acid casein using an ethanol-hydrochloric acid (EtOH-HCl) extraction process (O'Mahony and Fox, 2013). The four proteins that were identified from isoelectric casein are:  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -casein, which account for ~38%, ~10%, ~35% and ~15% of total casein, respectively. Variations occur between different casein proteins such as molecular weight (MW) and differences in AA profile (**Table 2**). More subtle variations (e.g. single AA substitutions) may occur in each of the individual proteins also yielding different forms of the same protein, which is termed micro-heterogeneity. All caseins are phosphorylated, i.e., have phosphate groups attached to side chains of serine, threonine and tyrosine (Klump and Kriegelstein, 2002), while  $\alpha_{s1}$ - and  $\alpha_{s2}$ -casein are more highly phosphorylated than  $\beta$ - and  $\kappa$ -casein (typically 8, 11, 5 and 1 phosphate groups, respectively (**Table 2**) (Eigel et al., 1984). The degree of

**Table 1** Composition of milks of selected mammalian species

Species	Total solids (%)	Protein (%)			Fat (%)	Lactose (%)	Ash (%)
		Total	Casein	Whey			
Cow	12.7	3.4	2.8	0.6	3.7	4.8	0.7
Human	12.2	1.0	0.4	0.6	3.8	7.0	0.2
Sheep	19.3	5.5	4.6	0.9	7.4	4.8	1.0
Goat	12.3	2.9	2.5	0.4	4.5	4.1	0.8
Horse	11.2	2.5	1.3	1.2	1.9	6.2	0.5
Pig	18.8	4.8	2.8	2.0	6.8	5.5	n.a.
Donkey	11.7	2.0	1.0	1.0	1.4	7.4	0.5
Grey Seal	67.7	11.2	n.a.	n.a.	53.1	0.7	n.a.
Polar Bear	47.6	10.9	7.1	3.8	33.1	0.3	1.4

n.a. = Not available.

Adapted from Fox et al. (2015b).

**Table 2** Properties of individual casein proteins

Property	Caseins			
	$\alpha_{s1}$ -	$\alpha_{s2}$ -	$\beta$ -	$\kappa$ -
<b>Molecular weight (kDa)</b>	~23.5	~25.5	~24.0	~19.0
<b>Residues/molecules</b>				
Amino acids	199	207	209	169
Proline	17	10	35	20
Cysteine	0	2	0	2
Phosphate	8–9	10–13	4–5	1–3
Carbohydrate	0	0	0	0–4
<b>Hydrophobicity (kJ/residue)</b>	4.9	4.7	5.6	5.1
<b>Charged residues/molecule</b>	34	36	23	21

Adapted from Fox et al. (2015b).

phosphorylation of each casein protein is commonly included in the abbreviated name of the casein protein, e.g.  $\alpha_{s1}$ -8P,  $\alpha_{s2}$ -11P,  $\beta$ -5P and  $\kappa$ -1P.

Additionally,  $\kappa$ -casein is a glycosylated protein, meaning that carbohydrate moieties (e.g., N-acetylneuraminic acid, galactose and N-acetylgalactosamine) are attached to the C-terminal end of the  $\kappa$ -casein molecule. The extent of glycosylation of  $\kappa$ -casein that occurs can vary; 0–4 glycosides may be present on any given  $\kappa$ -casein protein, yielding 9 different possible molecular forms of  $\kappa$ -casein (Dziuba and Minkiewicz, 1996).

## Properties of Caseins

### Structure and Heat Stability

Caseins are relatively small molecules (MW ranging from 19–25 kDa) that lack high levels of secondary ( $\alpha$ -helices and  $\beta$ -turns) or tertiary protein structures. This is somewhat due to high concentrations of the amino acid proline in the proteins: 17, 10, 35 and 20 proline residues are present in  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -casein, respectively (Swaigood, 1982). Proline inhibits the formation of  $\alpha$ -helices and  $\beta$ -turns due the presence of a cyclic amine in its side chain (Morgan and Rubenstein, 2013). As secondary and tertiary structures are not prevalent in the structural analysis of caseins they are considered to have a non-ordered and open structure (Holt and Sawyer, 1993; Holt et al., 2013).

Protein denaturation occurs when a protein loses its native secondary or tertiary structure, strongly influencing the biological and technological functionality of that protein. As caseins have a low level of secondary or tertiary structure, high temperature treatments have little effect on the caseins, as evidenced by the fact that bovine milk can be heated at 140 °C for ~20–30 min without gelation occurring (Singh and Latham, 1993). Such high heat treatments will, however, have other effects on caseins, such as dephosphorylation of AA (Fox et al., 2015a).

### Hydrophobicity and Calcium Sensitivity

All casein proteins have a high concentration (35–45%) of hydrophobic AAs such as valine, leucine, isoleucine, phenylalanine, tyrosine and proline (Swaisgood, 1982). In structured proteins, hydrophobic AAs are generally buried within the protein's tertiary structure; however, as caseins have an open and unordered structure these AAs remain exposed, which leads to caseins being regarded as having a high surface hydrophobicity (Creamer et al., 1982; O'Mahony and Fox, 2013). Hydrophobic bonds readily form between the hydrophobic regions of caseins leading to caseins having a strong tendency to self-associate (Horne, 1998).

Hydrophobic amino acids are associated with bitterness, meaning that the hydrolysis of casein molecules has the potential to yield bitter hydrolysates, which is problematic in some applications such as cheese production (Lemieux and Simard, 1992). Caseins can act as amphipathic molecules which adsorb readily at air–water and oil–water interfaces in order to reduce interfacial tension, thereby stabilizing emulsions and foams (Dickinson, 1989).

All caseins (excluding  $\kappa$ -casein) are insoluble in the presence of calcium (Ca) which is naturally present at high concentrations in bovine milk (~30 mM; Gaucheron, 2005), it would therefore be expected that caseins would precipitate out of milk due to their insolubility and Ca-sensitivity.  $\kappa$ -casein, which is Ca-insensitive, and soluble, at all Ca concentrations found in dairy products acts to stabilise the other, Ca-sensitive, caseins from precipitation in milk by combining with both Ca and the Ca-sensitive caseins to form large colloidal structures termed casein micelles (Müller-Buschbaum et al., 2007; O'Mahony and Fox, 2013). Although  $\kappa$ -casein only accounts for ~ 15% of total casein protein in milk, it can stabilise up to 10 times its own weight of the Ca-sensitive caseins *via* the formation of the casein micelle (Fox and Brodtkorb, 2008).

### Casein Micelle

Holt et al. (2013) summarized the three main biological functions of the casein micelle, based on its structure and stability. It ensures that very high levels of Ca and phosphate can be secreted from the mammary gland and transported to the neonate by ensuring that these minerals are colloiddally stable in milk (prevents calcified build-up in mammary glands). The micelle also provides a means of safe excretion of the potentially fibrilligenic caseins (may build up in fibril structures causing blockages) through the mammary gland. Finally, the casein micelle also allows caseins to be retained in the stomach of neonates for a length of time required for sufficient proteolysis of the caseins into smaller peptides which can then be more readily absorbed in the small intestine.

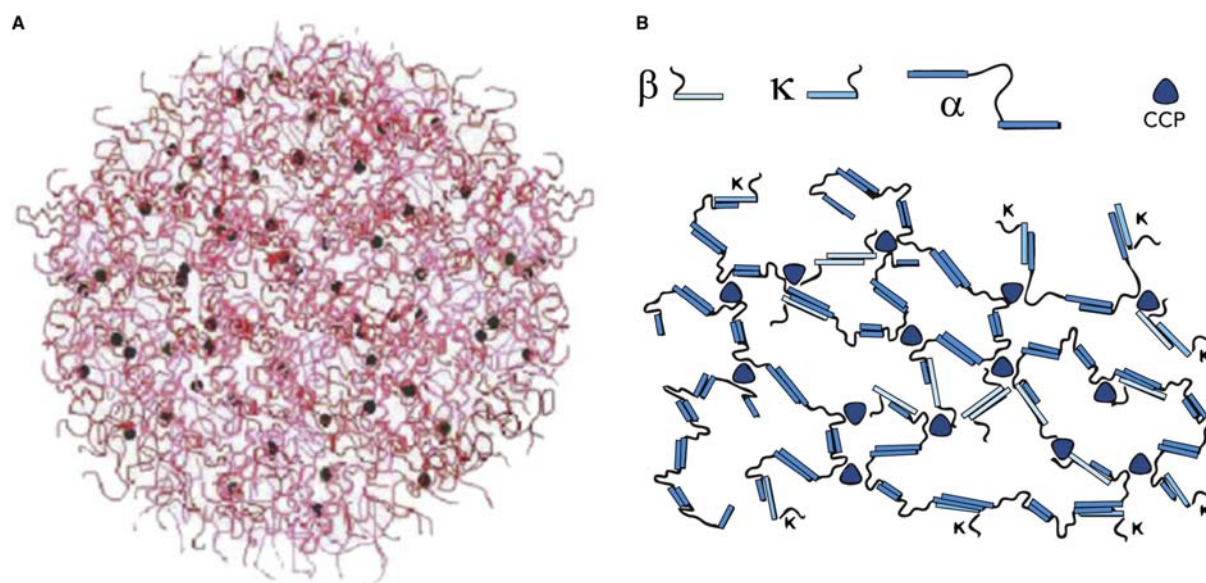
The structure of the casein micelle has been widely debated over recent years (De Kruif and Holt, 2003; Horne, 2006, 2008; Farrell et al. 2006; Fox and Brodtkorb, 2008; Dalgleish, 2011; Dalgleish and Corredig, 2012) and different models for the structure of the casein micelle have been suggested and will be discussed briefly below. It is widely agreed that casein micelles are large, spherical (diameter = 50–500 nm) (de Kruif, 1998; O'Mahony and Fox, 2013) and highly hydrated (3.5 kg H<sub>2</sub>O per kg of protein) (Jeurnink and de Kruif, 1993) structures containing ~ 70,000 individual protein molecules. The dry matter of the casein micelle is 94% protein and 6% low MW species, referred to as colloidal calcium phosphate (CCP). This CCP is composed mainly of Ca (~1,400,00 Ca<sup>2+</sup>) and phosphorus (~1,000,000 PO<sub>4</sub><sup>3-</sup>) with small amounts of magnesium, citrate and other species (O'Mahony and Fox, 2013). The external surface structure of casein micelles is rich in  $\kappa$ -casein where it acts to stabilise the micellar structure and protect against destabilization or aggregation of casein micelles (Dalgleish et al., 1989). The glycosylated, hydrophilic C-terminal of the  $\kappa$ -casein molecule (termed macropeptide) can be found protruding ~ 7 nm out of the casein micelle into the aqueous phase of milk. This creates a hairy  $\kappa$ -casein layer surrounding the casein micelle, responsible for micellar stabilization *via* steric and electrostatic repulsion (zeta potential of ~ -20 mV at pH 6.7) (Müller-Buschbaum et al. 2007; O'Regan et al., 2009).

In comparison, the arrangement of  $\alpha$ - and  $\beta$ -casein and CCP within the internal structure of the casein micelle has yielded greater debate in the various models proposed in recent years. The simplest model was proposed initially by Waugh and Von Hippel (1956) and suggested that the  $\alpha$ - and  $\beta$ -caseins are surrounded by a layer of  $\kappa$ -casein. In the intervening period to now, two main models have been developed detailing the internal structure of the casein micelle: the submicelle and the nanocluster model.

The submicelle model was firstly proposed by Schmidt (1982) and suggests that the casein micelle is composed of smaller submicelles fused together by CCP. This model has not generated widespread support as it relies on the hypothesis that there are two forms of submicelle units present in a casein micelle - a  $\kappa$ -casein-rich and a  $\kappa$ -casein-depleted submicelle. The  $\kappa$ -casein-rich submicelles are located at the surface of the micelle, with the  $\kappa$ -casein-depleted submicelles in the internal structure of the casein micelle. Research has never confirmed that these different submicelles actually exist. Following this, the nanocluster model was introduced by Holt (1992, 1998). This model depicts the internal structure of casein micelles as a tangled web of  $\alpha$ - and  $\beta$ -caseins linked by hydrophobic interactions. This casein web contains nanoclusters of CCP (radius of 2.3 nm) which act to stabilize the casein web structure. Dalgleish (1998, 2011) has presented an updated nanocluster model which incorporates large pores within the casein micelle and a more sparse distribution of  $\kappa$ -casein in the  $\kappa$ -casein layer on the surface of the casein micelle.

These pores are large enough to allow (I) easy access to the protruding  $\kappa$ -casein for proteolytic enzymes (important for digestibility and destabilization of caseins) (Diaz et al., 1996) and denatured whey proteins (formation of disulfide linkages between denatured  $\beta$ -lactoglobulin ( $\beta$ -LG) and  $\kappa$ -casein) (Singh and Creamer, 1991; Anema and Li, 2003) and (II) movement of individual proteins in and out of the casein micelle structure (e.g.  $\beta$ -casein leaving and re-entering the micelle on heating and cooling) (Creamer et al., 1977). Both the submicelle and nanocluster models retain the key feature that CCP, which is





**Figure 1** (a) Holt's nanocluster model, depicting an internal protein matrix of  $\alpha$ - and  $\beta$ -casein which contains nanocluster like particles of CCP (●) with steric stabilization provided by the protruding  $\kappa$ -casein at the micelle surface, from [De Kruif et al. \(2012\)](#). (b) Dual-binding model proposed by [Horne \(1998\)](#), depicting the hydrophobic bonding occurring between  $\alpha$ -,  $\beta$ - and  $\kappa$ -casein, as well as the CCP nanocluster bridging.

located within the casein micelle, plays a vital role in stabilizing the Ca-sensitive casein present in the interior of the casein micelle ([Fig. 1](#)).

## Whey Proteins

Whey proteins were traditionally separated from caseins *via* isoelectric precipitation of the caseins by adjusting the pH of milk to 4.6. The whey proteins remained in the soluble phase at this pH, producing a whey stream termed acid whey ([O'Mahony and Fox, 2013](#)). Another common practice for the separation of the whey from the caseins is through the addition of an enzyme preparation such as rennet (primary enzyme chymosin) to milk, as is done during cheese making, which causes the destabilization (cleaving of  $\kappa$ -casein at the micelle surface), aggregation and precipitation of casein micelles (the curd), leaving the whey proteins and glycomacropeptide (GMP) (C-terminal of  $\kappa$ -casein which is cleaved off by chymosin) in the soluble phase. Whey produced *via* enzymatic coagulation of milk is termed sweet whey and contains GMP.

In industrial and lab scale applications, the separation and further enrichment of whey proteins from caseins is routinely achieved using unit operations such as chromatography (e.g., ion exchange chromatography) membrane filtration and centrifugation which exploit the physico-chemical differences (such as molecular weight and charge) between whey proteins and caseins to achieve separation. The form of whey produced from these operations is sometimes termed technical whey.

## Heterogeneity and Fractionation of Whey

Work carried out throughout the 20th century by [Palmer \(1933\)](#), [Sorensen and Sorensen \(1939\)](#), [Gordon and Semmet \(1953\)](#) and [Polis et al. \(1950\)](#) showed that many different whey proteins are present in bovine milk and established that the major proteins of bovine whey are:

- $\beta$ -LG-; (~50% of total whey protein in bovine milk)
- $\alpha$ -Lactalbumin ( $\alpha$ -LA); (~20% of total whey protein in bovine milk)
- Immunoglobulins (Ig); (~10% of total whey protein in bovine milk)
- Bovine serum albumin (BSA); (<10% of total whey protein in bovine milk)
- Lactoferrin (LF); (~1% of total whey protein in bovine milk)

Whey proteins differ in terms of their amino acid composition, size and physicochemical properties (e.g., charge and surface hydrophobicity); however, for the purpose of this review, whey proteins will be discussed as a collective.

## Properties of Whey Proteins

### Structure and Heat Stability

Whey proteins are highly structured with high levels of secondary and tertiary structure.  $\beta$ -LG, which is the predominant whey protein in bovine milk, is highly structured with more than half of its structure existing in the form of  $\alpha$ -helices (15% of  $\beta$ -LG structure) or  $\beta$ -sheets (50% of  $\beta$ -LG structure) ultimately forming a tightly packed globular structure (Whitney, 1988; Kinsella and Morr, 1984a; Creamer et al., 1983), held together strongly by internal disulfide (S-S) bridges. This highly ordered globular structure is common for most of the major whey proteins, and thus whey proteins are very heat-labile as they are susceptible to loss of structure during processing treatments common in the dairy industry (e.g., high heat treatments, and high-pressure homogenization).

On exposure to temperatures greater than 65 °C, whey proteins tend to unfold from their native structure, exposing AAs such as Cysteine (which contains a free sulfhydryl (–SH) group). The now exposed free–SH group is readily accessible and highly reactive, allowing other denatured whey proteins and proteins containing exposed–SH groups (e.g.,  $\kappa$ -casein) to interact and aggregate via the formation of new S–S bonds (Kinsella and Morr, 1984a; Mulvihill and Donovan, 1987; Kinsella and Whitehead, 1989; Singh and Creamer, 1991; Wijayanti et al., 2014).

### Charge and Hydrophobicity

As seen in Table 3, whey proteins contain high levels of polar and charged AAs. Kinsella and Whitehead (1989) stated that proteins with high levels of polar and/or charged AAs, such as whey proteins, interact strongly with water due to their highly hydrophilic nature. Whey proteins, due to their hydrophilic nature are associated with good solubility characteristics and proteins in whey protein isolate have been shown (Pelegrine and Gasparetto, 2005) to be highly soluble over a wide range of pH (Table 4). Due to their heat sensitivity, the solubility of whey proteins is very temperature dependent, as influenced by denaturation and aggregation.

Due to their charge and hydrophilic nature, whey proteins (unlike the caseins) display a very limited tendency to self-associate and are less sensitive to changes in pH and ionic strength when compared to caseins (O'Regan et al., 2009).

## Functionality of Milk Proteins

The functional properties of proteins are defined as physical and chemical properties that influence the behavior of food systems during processing, storage and consumption. Milk proteins have a wide range of functional properties that lend to their application in numerous food systems (Kinsella and Melachouris, 1976; Kinsella and Morr, 1984a). For the purpose of this article, the functional properties, solubility, gelation and surface activity will be of focus for both casein and whey proteins.

**Table 3** Amino acid composition of the major whey proteins in bovine milk

AA	$\beta$ -LG	$\alpha$ -LA	BSA	LF
Aspartate	11	9	41	53
Asparagine	5	12	13	44
Threonine	8	7	34	52
Serine	7	7	28	50
Glutamate	16	8	59	30
Glutamine	9	5	20	68
Proline	8	2	28	44
Glycine	3	6	15	74
Alanine	14	3	46	98
Cysteine	5	8	35	38
Valine	10	6	36	66
Methionine	4	1	4	4
Isoleucine	10	8	14	26
Leucine	22	13	61	106
Tyrosine	4	4	19	34
Phenylalanine	4	4	27	43
Tryptophan	2	4	2	17
Lysine	15	12	59	78
Histidine	2	3	17	14
Arginine	3	1	23	58
Total	162	123	581	987

Adapted from Kinsella and Whitehead (1989).

**Table 4** Protein solubility values of whey protein isolate solutions<sup>a</sup> over a pH and temperature range (Pelegrine and Gasparetto, 2005)

Temperature (°C)	pH	Protein solubility (g/100 g)
40	3.50	87.1 ± 0.03
	4.50	81.8 ± 0.12
	5.65	86.3 ± 0.02
	6.80	87.7 ± 0.02
	7.80	92.8 ± 0.49
43	3.50	87.7 ± 0.28
	4.50	78.8 ± 0.41
	5.65	89.0 ± 0.88
	6.80	83.9 ± 0.26
	7.80	88.9 ± 0.35
50	3.50	87.1 ± 0.06
	4.50	72.2 ± 0.26
	5.65	89.6 ± 0.05
	6.80	74.6 ± 0.01
	7.80	88.6 ± 0.32
57	3.50	82.0 ± 0.20
	4.50	64.9 ± 0.16
	5.65	87.6 ± 0.50
	6.80	75.6 ± 0.08
	7.80	85.2 ± 0.32
60	3.50	80.7 ± 0.18
	4.50	62.4 ± 0.06
	5.65	92.4 ± 0.10
	6.80	68.2 ± 0.15
	7.80	87.8 ± 0.05

<sup>a</sup>Whey protein solutions were made by adding ~ 0.5 g of whey protein isolate (94.3% protein) to a volumetric flask and making to 50 mL volume.

## Solubility

The solubility of a protein is defined as the amount of protein that goes into solution or colloidal dispersion under specified conditions (temperature and ionic strength) and does not sediment out by defined centrifugal force (Morr et al., 1985; IDF, 1995). The solubility of a protein is regarded as the most important functional property as it is a prerequisite for many other functional properties and acts as a useful index of overall protein functionality. If a protein has good solubility, its potential applications are naturally expanded (Kinsella and Morr, 1984a; Zayas, 1997c). The solubility of a protein is ultimately decided by a combination of (I) the balance of intermolecular hydrophobic and electrostatic interactions, which themselves, are controlled by surface hydrophobicity and (II) the state (native or denatured) of the protein under specific environmental conditions of temperature, pH and ionic strength (O'Regan et al., 2009).

## Gelation

Gelation refers to the ability of proteins to form a gel under practical conditions during processing or storage of a food product (Zayas, 1997b) and is a very important functional attribute in many food systems, e.g., yoghurt and cheese production. A gel is defined as a system containing a relatively small proportion of solid in a relatively large proportion of liquid, yet the system has mechanical rigidity, thus a protein gel is composed of a three dimensional network of proteins entrapping a large amount of water (Kinsella and Whitehead, 1989). A protein gel is formed when protein molecules are altered in a way which allows unfolding (denaturation) to occur, yielding polypeptide regions which are capable of forming various interactions (protein–protein and protein–water), resulting in a three-dimensional, cross-linked network (O'Regan et al., 2009). The alteration of milk proteins to form gels is most commonly achieved *via* thermal denaturation or enzymatic coagulation.

## Surface Active Properties

Molecules with surface active properties are used routinely in food processing to stabilize emulsions (oil dispersed in water, O/W, or water dispersed in oil, W/O) and foams (air dispersed in a liquid phase) in formulated foods (e.g., mayonnaise, cake batter). In order for proteins to act as surface active agents they must be amphipathic, meaning they contain both hydrophilic and hydrophobic regions (Kinsella and Morr, 1984a). Milk proteins adsorb readily at the interface and surface of emulsions and foams, respectively, and act to reduce interfacial and surface tension between the hydrophobic and hydrophilic phases, in turn stabilizing emulsions and foams (Table 5).

**Table 5** Functionality and applications of milk protein ingredients

	Solubility		Gelation		Surface Activity	
	Casein	Whey	Casein	Whey	Casein	Whey
<b>Description</b> (Kinsella and Melachouris, 1976; Kinsella and Morr, 1984b; Zayas, 1997a; O'Regan et al., 2009)	<p>-Soluble to high conc. at pH values outside of pH 4–5 (casein pI = 4.6).</p> <p>-Caseins' heat stability allows them to remain soluble after heat treatment.</p> <p>-Solubility improved by use of calcium chelators (e.g., citrates).</p>	<p>-Soluble over entire pH range encountered in food applications (at low ionic strength).</p> <p>-Solubility decreases at high ionic strength due to salting out effect (de Wit and van Kessel, 1996).</p> <p>-Thermal denaturation and aggregation causes a reduction/loss in solubility.</p>	<p>-Casein gels are formed from milk by acid and enzymatic coagulation</p> <p>-Rennet gelation occurs via proteolysis of the <math>\kappa</math>-casein layer of the casein micelle leading to micelle destabilization and aggregation.</p> <p>-Acid gelation of casein occurs on adjustment of pH to 4.6 (pI of caseins) and produces gels that are prone to syneresis (whey expulsion).</p>	<p>-Due to their heat labile nature, whey proteins have excellent thermal gelation properties.</p> <p>-The characteristics (hardness, elasticity and turbidity) of the gels formed is dependent on the solution environmental conditions: mainly pH and ionic strength.</p>	<p>Both types of milk proteins are known to have high surface-activity properties leading to their use as emulsion and foam stabilizers in food applications.</p> <p>Caseins, due to their open structure, allow for a large surface coverage at the interface/surface of an emulsion or foam.</p> <p>The emulsion properties of both casein and whey proteins are improved via conjunction with polysaccharides as forming a polysaccharide portion on the interface protruding outwards into the emulsion continuous phase, increasing steric stabilization of the emulsified droplets (Drapala et al., 2016).</p>	<p>Whey proteins, in their native globular state, adsorb in thicker films at the interface/surface of emulsions or foams. Denatured whey protein aggregates are associated with enhanced surface activity.</p>

	Solubility		Gelation		Surface Activity	
	Casein	Whey	Casein	Whey	Casein	Whey
<b>Applications &amp; Ingredients</b> (O'Regan et al., 2009)	<u>Beverages</u> Caseins used in nutritional beverages which require high heat treatments for protein fortification -Sodium caseinate	<u>Beverages</u> Whey proteins are used in the production of protein beverages due to their solubility over a wide pH range. –WPC, WPI	<u>Dairy Products</u> The formation of a casein gel is a core step in the production of cheese. Different cheese varieties are produced depending on the gelation mechanism (enzymatic vs acid)	<u>Dairy Products</u> Some cheese varieties such as ricotta are produced by forming a whey protein coagulum/curd using heat and acid gelation.	<u>Beverages</u> Caseins, due to their stability, are used as emulsifiers and stabilizers in cream liqueurs which is a challenging environment. -Sodium caseinate	<u>Beverages</u> Whey protein solubility at low pH lends to their use as emulsifiers in protein fortified fruit juices or soft drinks which contain volatile oil flavor compounds. –WPC, WPC
	<u>Dairy Products</u> Caseins and caseinates are commonly used in the production of analog cheese where one of their main functions is to bind water and emulsify oil. -Rennet casein, acid casein	<u>Bakery Products</u> Due to their solubility, whey proteins are incorporated into baked goods to retain water and to provide additional functionality. –WPC	Both caseins and whey proteins are utilized in the production of yoghurts. This not only improves the nutritional value of the yogurt but also allows increased yield, viscosity and gel strength (reduced syneresis)		<u>Dairy Products</u> In the production of table spreads, a water in oil emulsion is created. Emulsifiers are used to stabilise this emulsion but due to the very high level of oil in the dispersed phase, casein ingredients are used to further provide stabilization to the emulsion. -Sodium caseinate	<u>Bakery Products</u> Whey protein ingredients are used as a replacement for egg proteins in many bakery applications as both very effectively stabilise foams (e.g. meringues). –WPC, WPI
	<u>Meat Products</u> Caseins act to bind large amounts of water, assisting in fat emulsification and improving the final texture in the production of processed meat. -Sodium caseinate	<u>Confectionary Products</u> Protein-rich bars are produced with whey protein ingredients to increase their level of protein and to control texture via their water binding capabilities (Hogan et al., 2012) -Hydrolyzed WPC	Casein gelation occurs and is vital in the production of kefir which is a fermented dairy beverage. As fermentation occurs the pH of the kefir drops which causes acid induced gelation leading to an increase in the beverage viscosity.	<u>Textured Products</u> Whey proteins are used in the production of surimi due to their gelation properties, as a replacement for beef plasma protein or potato starch (Hsu and Kolbe, 1996). –WPC		<u>Meat Products</u> Fat used in processed meat product production is pre-emulsified with whey proteins to allow for the formation a strong fat containing gel network during cooking. –WPC

## Nutritional Properties of Milk Proteins

### Delivery of Amino Acids

The main nutritional function of bovine milk proteins is to act as the principal source of nitrogen and amino acids which are required for growth and maintenance of protein synthesis (Tipton and Wolfe, 2001). Amino acids are classified as essential or non-essential, with Rose et al. (1948) defining essential amino acids as those which cannot be synthesized in sufficient amounts by the body to maintain growth or nitrogen balance. Milk proteins have a high biological value as all essential amino acids (Histidine, lysine, phenylalanine, leucine, threonine, valine, tryptophan, methionine and isoleucine) are present at relatively high levels in the major milk proteins ( $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ -,  $\kappa$ -CN,  $\beta$ -LG and  $\alpha$ -LA) (Table 3). Whey proteins have higher levels of essential AAs (Hambraeus and Lönnerdal, 2003) while caseins, in addition to delivering AAs, play a vital role in delivery of calcium *via* the CCP in casein micelles.

### Biologically-Active Proteins and Peptides

Biologically-active compounds are those that affect biological processes, beyond the nutritional value, in a way which has an impact on body function. For milk proteins, the proteins themselves, as well as the peptides formed from their proteolytic digestion, may be biologically active. For the purpose of this review the effect that milk proteins and peptides have on the immune, cardiovascular and nervous systems will be discussed.

Milk proteins and peptides that have a beneficial effect on the immune system are termed as either immunomodulatory or antimicrobial. Immunoglobulins (whey proteins) are an example of immunomodulatory proteins, and are in fact a family of proteins with varied structure and functions; however, their structures and functions critically revolve around their ability to identify and bind specific antigens presented by bacteria and viruses which aids host protection (Schroeder et al., 2010). During proteolytic breakdown of LF in the stomach by pepsin, an antimicrobial peptide, lactoferricin, is formed which targets Gram-negative bacteria and binds to their cell wall. Once attached to the cell wall, lactoferricin causes the release of lipopolysaccharides, which irreversibly damages the cell wall, leading to further morphological changes to the structure and function of the Gram-negative bacteria (Bellamy et al., 1992; Appelmek et al., 1994; Tomita et al., 2002).

Antithrombotic milk peptides have a positive effect on the human cardiovascular system. These peptides are mainly formed during the enzymatic break down of  $\kappa$ -casein and have been shown to interrupt the formation of thrombi which, when formed, can block veins, arteries or the chambers in the heart (Clare and Swaisgood, 2000; Mills et al., 2011).

The nervous system of newborn infants has been shown to be affected by opioid peptides from milk, which obtain their name due to pharmacological similarities to opium. The major opioid peptides of milk are formed during the proteolytic digestion of  $\beta$ -casein and are called  $\beta$ -casomorphins, which are thought to be biologically potent (Teschemacher et al., 1997; Clare and Swaisgood, 2000).  $\beta$ -casomorphins have only been found in the intestinal tract and blood plasma of newborn infants and have yet to be identified in children or adults. The specific activity of  $\beta$ -casomorphins is somewhat unclear, while it has been shown to be associated with stimulating food intake and increasing the output of insulin in infants (Xu, 1998; Mills et al., 2011).

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# Bioavailability of Minerals (Ca, Mg, Zn, K, Mn, Se) in Food Products

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## Introduction to Minerals

Minerals as essential micronutrients are required for maintaining normal metabolic functions in human body. These chemical elements are classified as either major or trace elements, which are needed in larger or fewer amounts for the body, respectively. Some examples of major minerals are calcium (Ca), magnesium (Mg), and potassium (K), while some trace minerals are manganese (Mn), selenium (Se), and zinc (Zn). All the aforementioned minerals exist in specific food sources; moreover, they are vital for the health of bones, teeth, and structural parts of enzymes, which are explained briefly in [Table 1](#). Despite all the key roles of minerals in the body, they have to be consumed on a regular basis with a balanced diet. Therefore, having enough information about recommended dietary allowance (RDA) for these minerals is vital, which is provided in [Table 1](#) ([Lukaski, 2004](#)). Human requirements for essential minerals vary from a few micrograms per day up to about 1 g per day. Ca with an average RDA of 1200 mg per day has the highest RDA among minerals, and it needs to be consumed to be beneficial for health and bones ([Heaney, 1993](#)). Whereas Se with an average RDA of 30 µg per day for an adolescent has the lowest RDA, in which this Se level is essential for growth and development of a human body. However, consuming the minerals greater than the maximum tolerable intake leads to the toxicity symptoms. On the other hand, consuming less than the recommended intake can lead to the specific deficiencies ([Motarjemi, 2013](#)). The various side effects of toxicity and deficiencies for different minerals in our body are also shown in [Table 1](#).

## Bioavailability of Minerals

The total mineral content of foods is not a useful indicator of its available amounts in the body through absorption, since only a certain quantity is bioavailable ([Jafari and McClements, 2017](#)). The bioavailability of major and trace minerals is defined as the proportion of the ingested minerals, which is absorbed and available for metabolic functions. The bioavailability of minerals is varied from less than 1% to more than 90%, and it is a key factor to food effectiveness. The reasons that influence mineral bioavailability are the chemical form of the minerals in foods, chelating agents, redox activity of food components, mineral–mineral interactions, and physiological state of consumers ([Miller, 2007](#)). As a main rule, the mineral bioavailability in the body is associated with digestion, release from food matrix, absorption rate of the minerals by intestinal cells, and its transport to body cells ([Fairweather-Tait, 1993](#)).

## Bioavailability Enhancers

Organic acids, including ascorbic, citric, and lactic acids, can enhance the mineral bioavailability. Their effect is dependent on the food composition, type, and concentration of mineral and the concentration of organic acids. They can form soluble chelates with minerals, which protect the minerals from precipitation and bind to other inhibitors ([Miller, 2007](#); [Suh et al., 2003](#)).

## Bioavailability Inhibitors

Phytates are the main storage form of plant seeds known as potent inhibitors and chelators, limiting the bioavailability of particularly divalent and trivalent minerals ([Hurrell, 2004](#)). High levels of phytates are mainly found in cereals, legumes, roots, and vegetables ([Sandberg, 2002](#)). Polyphenolic compounds also decrease the mineral bioavailability, which exist in foods such as tea, coffee, and many vegetable and fruit extracts ([Graham et al., 2001](#); [Yeung et al., 2003](#); [Zijp et al., 2000](#)).

## Bioavailability of Major Minerals (Ca, K, and Mg)

The concentration of Ca in the food products and the existence of inhibitors and enhancers of Ca absorption determine the absorption of Ca from foods ([Weaver and Plawecki, 1994](#)). The prime dietary inhibitors of Ca absorption are oxalate and phytate; therefore, the poor Ca bioavailability is dedicated to the spinach and pinto beans due to their high concentrations of oxalate and phytate, respectively. Whereas dairy products are classified in food groups with the high bioavailability of Ca.

Potassium is generally absorbed well within the body up to 90% because of its high water solubility ([Agarwal et al., 1994](#)). Therefore, there is not a lot of information about its absorption from different foods.

The efficiency of Mg absorption varies largely depending on Mg intake, from 25% to 75% in high- and low-intake groups, respectively ([Schwartz et al., 1984](#); [Seelig, 1982](#); [Spencer et al., 1980](#)). High intakes of fiber result in lower Mg absorption because of the inhibitory effect of phytate associated with fiber. However, the high concentration of Mg in phytate and cellulose-rich products increases Mg intake and compensates the decrease in its absorption ([Kelsay et al., 1979](#)).

**Table 1** Food sources, functions, recommended dietary allowance (RDA) (Joint FAO/WHO, 2005), deficiency, and excessive intake effects of minerals

<i>Minerals</i>	<i>Food sources</i>	<i>Main functions</i>	<i>RDA (per day)</i>	<i>Deficiency effects</i>	<i>Excessive intake effects</i>
<b>Major minerals</b>					
Calcium (Ca)	Milk and dairy products, fortified juices, tofu, kale, broccoli, legumes	Essential for healthy bones and teeth, skeletal growth and development, nerve transmission, insulin release, blood coagulation	Men 1000 (mg) Women 1000 Children 500–700 Pregnant women 1200	Increased colon cancer risk, hypertension, osteoporosis and growth arrest	Hypercalcemia (nausea, kidney stones, constipation) and in extreme conditions coma and death
Magnesium (Mg)	Milk and dairy product, seafoods, nuts and seeds, legumes, leafy and green vegetables, unrefined cereal grains, chocolate,	Proper maintenance and growth of bones, cofactor for numerous enzyme, plays a vital role in proper functioning of nerves and muscles	Men 260 (mg) Women 220 Children 60–100 Pregnant women 220	Muscle cramps, weakness, nausea, tremors, anxiety, high blood pressure, and respiratory disorders	Rarely occurs, intestinal distress: diarrhea, cramping, nausea
Potassium (K)	Meats, fish (salmon), milk, yogurt, fresh and dried fruits and vegetables, whole grains, legumes	Nerve transmission, proper fluid balance, muscle contraction, maintains normal blood pressure, and waste elimination	No RDA	Gastrointestinal disorders, depression, and confusion	Rarely occurs
<b>Trace minerals</b>					
Manganese (Mn)	Seafoods, fish, hazelnut, seed (pumpkin), beans, tea (black, brewed), spinach, brown rice, raisin, almonds, tofu	Bone development, a part of many enzymes, important for the normal functioning of the brain and proper activity of nervous system, useful for postmenopausal women	Men 2.3 (mg) Women 1.8 Children 1.2–1.9 Pregnant women 2	Hypocholesterolemia, dermatitis, and weight loss	Extrapyramidal, amidal movement disorders, and hallucinations
Selenium (Se)	Skim milk, salmon, shrimp, chicken, beef, pork, and crab meat, Brazil nuts, brown rice, wheat bread	A component of special proteins in the body called antioxidant enzymes; stimulation of the immune system involved in conversion of thyroxine; protecting the organism from various viruses	Men 34 (μg) Women 26 Children 17–21 Pregnant women 28	Myalgias, cardiomyopathies, increased risk for some cancers	Nausea, diarrhea, and alteration in mental status and loss of hair and nails
Zinc (Zn)	Milk and dairy products, red meat, poultry, shellfish, oyster, dried beans, nuts, whole grains, soy food	A cofactor in metalloenzymes, a regulation of gene expression, growth and development of human body	Men 12 (mg) Women 10 Children 5–8 Pregnant women 12	Growth retardation, poor wound healing, delayed sexual maturation, impaired immune response	Inhibition of Cu and Fe absorption, impaired immune response

### Bioavailability of Trace Minerals (Mn, Se, and Zn)

The absorption of Mn is generally less than 5%. However, the Mn bioavailability has not been studied widely in humans. Ca, fiber, and phytate make an insoluble complex with Mn, leading to an increase in the requirement of Mn and decrease the available soluble fraction of Mn for absorption (Keen and Zidenberg-Cherr, 2003).

The Se absorption is fairly high, around 80%, but it depends on the existence of other trace minerals and proteins. It has been recognized that the total bioavailability of Se is around 14% of the original content in the food (Tsuji et al., 2016).

Zn is found in many diets. However, its bioavailability varies widely. Animal-based foods have more Zn bioavailability than grain-based products. This reason is all because of phytic acid that is found in whole grains and legumes; it binds to Zn and decreases Zn absorption from dietary sources. Thus, people with vegetarian diets need 50% more dietary Zn than nonvegetarians because of the large amounts of phytates consumption (Bel-Serrat et al., 2014).

### Effect of Food Processing on Bioavailability of Minerals

During food processing and storage, minerals are subjected to light; moisture; oxygen; various processing techniques including heating, soaking, grinding, germination, fermentation; and so on. These conditions can substantially influence the mineral bioavailability and degradation of special trace minerals by deactivating enzymes that degrade inhibitors or by generating insoluble mineral compounds. However, these treatments can also increase the mineral bioavailability and diffusion through separation, partitioning, or destroying inhibitors and transforming food components into complex ligands for metal ions (Gibson et al., 2006; Watzke, 1998).

Table 2 shows some bright and dark sides of different processing operations on the mineral bioavailability of food products.

### Fortification and Enrichment of Food Products With Minerals

Despite an ideal condition of food diversity and availability in many parts of the world, lots of people in low-income countries suffer from mineral deficiencies. Among varieties of methods for controlling mineral malnutrition, fortification is an advantageous strategy and long-term solution, which can satisfy peoples' nutritional needs in both developing and developed countries. FAO and WHO define food fortification as enrichment or addition of one or more nutrients to foods, even if it is naturally present in that food, with the aim of preventing or correcting nutrient deficiencies in a population. To make a plan for food fortification, some conditions should be considered: the whole process needs to be cost-effective and safe. Moreover, the selected food vehicles depend on their affordability and availability in all season; therefore, the foods are usually chosen from staple foods in which the specified amount of them are regularly consumed in the local diet. The minerals and their available fortified foods are shown in Table 3 (Fathima et al., 2017). Furthermore, the whole process of fortification should have no adverse effects on the sensory properties of the final products, with an increase in absorption and bioavailability of minerals, also with positive effects on consumers' healthiness (Gharibzahedi and Jafari, 2017a). With these aims and conditions, there are some suitable fortification methods, including mineral biofortification, direct addition of minerals to processed foods, and nanoencapsulation.

#### Mineral Biofortification

Biofortification is a breeding process of specific crops such as rice, wheat, beans, and other cereals and legumes, with the aim of enhancing both their mineral density and bioavailability. Mineral biofortification is implemented at the growing phase of agricultural crops (Dwivedi et al., 2012). Many researchers have focused on biofortification of Zn, Ca, and Se, since they are considered as limiting minerals in plant-based diets (Kim et al., 2006; Kutman et al., 2010; Morris et al., 2008; Park et al., 2005; Poblaciones and Rengel, 2016; Ram et al., 2016; Ramos et al., 2010; Thavarajah et al., 2015).

#### Direct Addition of Minerals

Direct addition of minerals prior to food consumption is another fortification method. One of the main benefits is that the minerals can be protected from different degradation factors during food processing, since they can be sprayed on the finished products.

Ca, as one of the essential major minerals, has lots of salutary properties, and its deficiency can lead to many diseases that are shown in Table 1. Therefore, it is vital to fortify foods with this mineral to improve its absorption and availability. However, fortification with major minerals may cause undesirable interactions in food structures such as coagulation and precipitation, which can lead to the chalkiness and bitter taste in the finished products (Singh et al., 2007). For choosing suitable Ca sources in foods, a variety of properties such as Ca level, solvability, bioavailability, and sensory characters of the whole product needs to be considered. Some Ca sources for milk and dairy products are calcium carbonate ( $\text{CaCO}_3$ ), calcium phosphate ( $\text{Ca}(\text{PO}_4)_2$ ), calcium lactate ( $\text{C}_6\text{H}_{10}\text{CaO}_6$ ), and so on (Münchbach and Gerstner, 2010).

Regarding trace minerals, some foods are fortified with sodium selenite ( $\text{Na}_2\text{SeO}_3$  or  $\text{Na}_2\text{O}_3\text{Se}$ ) and sodium selenate ( $\text{Na}_2\text{O}_4\text{Se}$ ), to increase Se bioavailability and reduce its deficiencies (Van Dael et al., 2001). Zn deficiency is one of the most common shortages in the world. Since beef, poultry, and fish are the best sources of Zn, population with predominantly plant-based diet is faced with

**Table 2** Impact of different processing techniques on the bioavailability of minerals

<i>Food processing techniques</i>	<i>Food products</i>	<i>Influence on mineral content</i>	<i>References</i>
Autoclaving	Lentils grown in Egypt	Significant reduction in calcium (Ca), potassium (K), magnesium (Mg), and copper (Cu) contents	Hefnawy (2011)
Baking	Fortified whole wheat flour	Decrease in manganese (Mn), Cu, and zinc (Zn) contents	Akhtar et al. (2010)
Baking	Bread from selenium (Se)-enriched whole meal/white flour	Without any significant reduction in selenomethionine content after bread processing and production	Hart et al. (2011)
Blanching	Spinach leaves	Increasing HCl extractability of Zn and Ca	Yadav and Sehgal (1995)
Cooking	Mineral enriched carrots	Improving the contents of major and trace minerals	Biezanowska-Kopeć et al. (2016)
Canning	Different processed foods	Complex destruction	Ranhotra and Bock (1988)
Debittering	Lupin seeds	High decrease in minerals and 71.4% phytic acid removal	Ertaş and Bilgiçli (2014)
Dehydration	Cabernet and Merlot grapes	Increasing the contents of major and trace minerals	Panceri et al. (2013)
Drying	Egg white	Maillard reaction, denaturation of binding proteins	Leahey and Thompson (1989)
Extrusion	Extruded foods	Inactivation of phytase enzyme	Mercier (1993)
Fermentation	Leavened bread	Decrease of phytate content	Gibson et al. (1998)
Fermentation	Milk	Increase Zn and Ca bioavailability in iron-fortified dairy products	Drago and Valencia (2002)
Freezing	Leafy vegetables	High retention of mineral constituents	Lisiewska et al. (2009)
Germination	African yam bean	Decrease of phytate content	Ene-Obong and Obizoba (1996)
Grilling	African catfish	Increase of mineral contents	Ersoy and Özeren (2009)
Microwave cooking	Lentils grown in Egypt	Lesser decrease of minerals than other preparation methods	Hefnawy (2011)
Microwave cooking	Cereals and green leafy vegetables	A considerable decrease in the bioavailability of Se	Khanam and Platel (2016)
Pressure cooking	Cereals and green leafy vegetables	A considerable decrease in the Se content	Khanam and Platel (2016)
Radiation	Cucumber	High mineral sensitivity of minerals to different doses of irradiation	Rahman et al. (2015)
Soaking	Brown rice	A significant decrease in Zn content	Albarracín et al. (2013)
Soaking	Maize flour	Activate endogenous phytase, reduce phytate levels, and improve zinc protoporphyrin	Manary et al. (2002)
Sonication	Apple juice	Increase in K, Mg, Cu, and Zn	Abid et al. (2014)
Refrigerated storage	Fruits and vegetables	Decrease in Ca, Mn, Zn, and Cu during storage	Bouzari et al. (2015)

**Table 3** Minerals and their associated fortified foods

<i>Minerals</i>	<i>Available fortified foods</i>
Calcium	Fortified orange juice, fortified milk and soy milk, fortified cereals (raisin bran, corn flakes), enriched breads, grains, and waffles
Magnesium	No fortified foods available
Potassium	No fortified foods available
Manganese	Cereals
Selenium	Noodles, milk, meat, cereals, flour, salt, sports drinks, and baked products
Zinc	Cereals, wheat flour, maize meal, corn/soy blend, and wheat/soy blend

Zn shortages with lots of side effects. Therefore, fortification of cereal staple foods with Zn could benefit this population (Badii et al., 2012). Some common Zn types for food fortification are zinc oxide (ZnO), zinc sulfate (ZnSO<sub>4</sub>), zinc acetate (C<sub>4</sub>H<sub>6</sub>O<sub>4</sub>Zn), and zinc chloride (ZnCl<sub>2</sub>) (Kahraman and Ustunol, 2012).

### Nano/Microencapsulation of Minerals

Nano/microencapsulation is a novel technology that involves coating a bioactive compound into a matrix at different capsule sizes. Encapsulation improves the retention time of the minerals in the food products and leads to the controlled release of minerals within the body. In general, nanoencapsulation can be a new delivery system for minerals and compensate many weaknesses of the direct addition method, since it can cover and mask the undesirable flavor of minerals and increase their bioavailability, water solubility, and physicochemical stability (Katouzian and Jafari, 2016; Pegg and Shahidi, 1999).

In recent years, nanoencapsulation of Ca, Zn, and Mg has attracted lots of attention. The encapsulated form of Ca, Mg, and Zn has been used in soy milk, dairy products, and additive supplements, respectively (Bonnet et al., 2009; Hirotsuka et al., 1984; Öner et al., 1988; Saeidy et al., 2014). Despite some efforts about fortification of bakery products with minerals, nanoencapsulation strategy can be implemented more and more for different minerals to incorporate them in various food products and widen the variety of fortified foods for people's consumption (Gharibzadeh and Jafari, 2017b).

### Conclusion

This article provides a useful and synopsis information about some minerals including Ca, Mg, and K as major minerals and Mn, Se, and Zn as trace ones, their bioavailability, and fortification strategies. Since bioavailability and absorption of minerals are roughly depending on dietary enhancers, inhibitors, and different processing techniques, there is a wide range of efficiency in mineral absorption. To overcome these weaknesses and have balanced and adequate mineral consumption, different fortification strategies have been conducted. However, because of significant differences between sensory properties of fortified foods with direct addition of minerals and original ones, some new technologies have been appeared. These technologies can encapsulate the minerals and protect them from different conditions until suitable release. Mineral encapsulation gives assurance to the food industries for improving their food production, packaging, shelf life, and bioavailability without changes in nutritional and sensory quality of their products. However, there is still a wide scope of demands on research about efficient fortification and encapsulation methods to have the highest bioavailability for minerals and to cure lots of mineral insufficiency all over the world.

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# Monoglycerides: Categories, Structures, Properties, Preparations, and Applications in the Food Industry

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## Introduction

Monoglycerides (MGs), diglycerides (DGs), and their derivatives account for about 70% of the total world production of emulsifiers and are widely used in food, detergent, plasticizer, cosmetic and pharmaceutical formulations, thus they are considered as the most important group of emulsifiers (Krog, 1997). In food science and nutrition, MGs have been investigated from at least four aspects: molecular structures and physical properties, preparation methods, functional properties, and applications in the food industry. Different types of MGs have different molecular structures and physical properties. There are mainly two kinds of preparation methods of MGs: chemical synthesis and enzymatic synthesis. Additionally, MGs have many functional properties, e.g., antimicrobial activity, emulsibility, and self-assembly in aqueous systems, and thus have various applications in the food industry. Although there were numerous independent studies on MGs, very limited information about an overall summarization was known. A specific review of MGs is necessary for a current understanding of the structures, properties, production, and industrial applications of MGs.

## Categories, Structures and Physical Properties of Monoglycerides

MGs can be divided into two categories: 1-monoglyceride (also named  $\alpha$ -monoglyceride) and 2-monoglyceride (also named  $\beta$ -monoglyceride) according to the position of the acyl group in MGs (Fig. 1) (Susi et al., 1961). Under different temperature conditions, the saturated 1-monoglyceride usually has four different polymorphic forms: sub  $\alpha$ ,  $\alpha$ ,  $\beta$ , and  $\beta'$  (Lutton, 1971; Vereecken et al., 2009). MGs in  $\alpha$  form are unstable and have active chemical property, which contributes to their emulsification of shortening into the water phase, as well as aids in the incorporation of air into the fat phase (Goldstein et al., 2012; Heertje et al., 1998). MGs crystallize directly in the  $\alpha$  polymorph, and they transition from  $\alpha$  polymorph to sub  $\alpha$  polymorph after being cooled at 35–50 °C (Vereecken et al., 2009). However,  $\alpha$  and sub  $\alpha$  polymorphic forms can transform into a more stable  $\beta$  polymorph during longtime storage at ambient temperatures, making it widely used in the design of firm food products such as fat spreads (Heertje et al., 1998; Vereecken et al., 2009). The  $\beta'$  polymorph is only formed during quick crystallization by using specific solvents, and it cannot be observed by differential scanning calorimetry (DSC) (Hagemann, 1988). On the other hand, 2-monoglyceride is only in one form (the  $\beta$  polymorph) due to its symmetric molecular structure (Susi et al., 1961; Vereecken et al., 2009).

MGs can also be divided into saturated MGs (e.g., glyceryl monostearate and glyceryl monopalmitin) and unsaturated MGs (e.g., glyceryl monooleate and glyceryl linoleate) depending on the degree of saturation of fatty acids attached to the glycerol (Alfutimie et al., 2014). Saturated MGs have greater ability to initiate fat crystallization and better emulsifying ability than unsaturated MGs, while unsaturated MGs have better health functional properties and are better at displacing protein from the interface than saturated MGs (Barford et al., 1991; Davies et al., 2001). Additionally, unlike saturated 1-monoglycerides, unsaturated 1-monoglycerides can only exist in the  $\beta$  polymorphic form (Vereecken et al., 2009).

According to the chain length of fatty acids attached to the glycerol, MGs can also be divided into short-chain MGs containing fatty acids with less than eight carbon atoms, medium-chain MGs containing fatty acids with eight to twelve carbon atoms, and long-chain MGs containing fatty acids with more than twelve carbon atoms. Long-chain MGs (e.g., monopalmitin, glyceryl monostearate, glyceryl monooleate, and glyceryl behenate) only have emulsifying ability, while medium-chain MGs (e.g., glyceryl monocaprylate, monocaprin, and monolaurin) have not only emulsifying ability but also antibacterial activity (Petschow et al., 1996).

Table 1 shows the structures and physical properties of some common MGs in the food industry. As the chain length of fatty acids increase, the melting point of MGs also increases and the physical appearance of MGs gradually harden (from an oily fluid into a hardened fat and then a waxy solid). Conversely, the hydrophilic property of MGs is inversely proportional to the number of carbon atoms of fatty acids. However, all kinds of MGs have strong lipophilic properties.

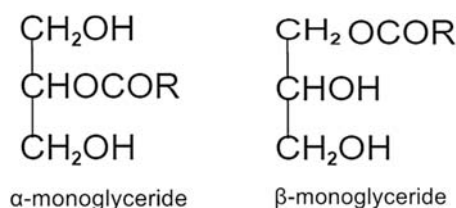


Figure 1 The structures of  $\alpha$ -monoglyceride and  $\beta$ -monoglyceride.

**Table 1** Structures and physical properties of some common monoglycerides

Name	Structural formula	Chain length	Degree of saturation	Physical appearance	Melting point (°C)	Hydrophilic property
Glyceryl Monocaprylate	$\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2-\text{OOC}-(\text{CH}_2)_6-\text{CH}_3$	C <sub>8</sub>	Saturated	Viscous liquid	40–42	Slightly dissolve in water, and dissolve in ethanol
Monocaprin	$\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2-\text{OOC}-(\text{CH}_2)_8-\text{CH}_3$	C <sub>10</sub>	Saturated	Fatty solid	48–55	Insoluble in water, but dissolve in ethanol
Monolaurin	$\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2-\text{OOC}-(\text{CH}_2)_{10}-\text{CH}_3$	C <sub>12</sub>	Saturated	Fatty solid	52–62	Insoluble in water, but dissolve in ethanol
Monopalmitin	$\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2-\text{OOC}-(\text{CH}_2)_{14}-\text{CH}_3$	C <sub>16</sub>	Saturated	Fatty solid	65–68	Insoluble in water, but dissolve in ethanol
Glyceryl Monostearate	$\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2-\text{OOC}-(\text{CH}_2)_{16}-\text{CH}_3$	C <sub>18</sub>	Saturated	Waxy solid	62–64	Insoluble in water, but dissolve in ethanol
Glyceryl Behenate	$\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2-\text{OOC}-(\text{CH}_2)_{20}-\text{CH}_3$	C <sub>22</sub>	Saturated	Waxy solid	65–77	Insoluble in water and ethanol
Glyceryl Monooleate	$\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2-\text{OOC}-(\text{CH}_2)_7-\text{CH}=\text{CH}-(\text{CH}_2)_7-\text{CH}_3$	C <sub>18</sub>	Unsaturated	Liquid	35	Insoluble in water, but dissolve in ethanol
Glyceryl Linoleate	$\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2-\text{OOC}-(\text{CH}_2)_7-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-(\text{CH}_2)_4-\text{CH}_3$	C <sub>18</sub>	Unsaturated	Liquid	–	Insoluble in water

### Preparation Methods of Monoglycerides

The direct esterification of glycerol with fatty acids, the glycerolysis (also named the interesterification), and the hydrolysis of triglycerides (TGs) are the three most important processes for the preparation of MGs (Pouilloux et al., 1999). According to the type of catalyst used in these processes, preparation methods of MGs fall into two main categories: chemical synthesis (using homogeneous acid, basic, or metal oxide catalysts) and enzymatic synthesis (using lipase catalysts). However, the final product is usually a mixture of MGs, DGs, and TGs as well as free glycerol, fatty acids, and catalysts (Kuhrt et al., 1950). MGs are usually a minor constituent in the mixture, but only they have emulsifying ability (Kuhrt et al., 1950). Thus, preparation methods are constantly being improved, and the separation and purification technologies have been used to produce MGs with high purity (Fregolente et al., 2007; Kuhrt et al., 1950).

#### Chemical Synthesis of Monoglycerides

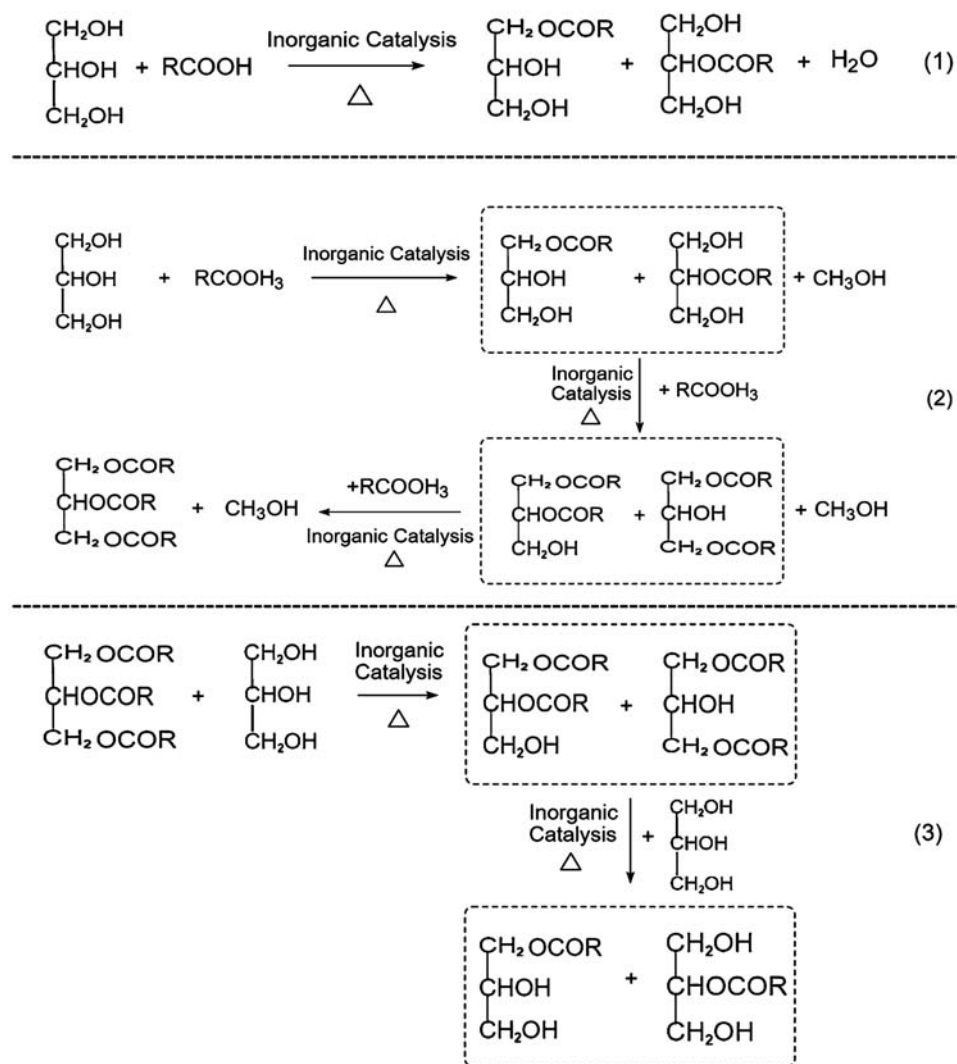
Fig. 2 presents the schemes of the esterification and the glycerolysis of chemical synthesis of MGs. The direct esterification of glycerol with fatty acids has been known since the mid-19th century (Kuhrt et al., 1950). An acid catalyst (e.g.,  $\text{H}_2\text{SO}_4$  and  $\text{H}_3\text{PO}_4$ ) or a base catalyst (e.g., NaOH) is required for the esterification (Corma et al., 2005). However, this process was considered uneconomic because of rising prices of fatty acids, which made the glycerolysis preferred. Additionally, the glycerolysis is less corrosive, and has a higher miscibility of reactants and lower level of acidic contaminants in the final products than the esterification (Siri-nguan and Ngamcharussrivichai, 2016). In the glycerolysis, glycerol is transesterified with fatty acid methyl esters or triglycerides (the major components in vegetable oils and animal fat). In addition, a base catalyst (e.g., KOH, NaOH, and  $\text{Ca}(\text{OH})_2$ ) is required for this process (Corma et al., 2005).

The main advantage of chemical synthesis is the short reaction period (2–3 h) (Siri-nguan and Ngamcharussrivichai, 2016). However, this reaction is carried out at relatively high temperatures (220–250 °C), which involves high-energy consumption. In addition, high temperature and the use of homogeneous catalysts usually lead to undesirable side reactions and produce dark colored by-products with an undesirable flavor, and thus results in low monoglycerides selectivity (40%–50%) with poor quality (Wee et al., 2013). Thus, separation and purification technology is required to purify MGs from the mixture, which is complicated and energy consuming (Wee et al., 2013).

In recent years, researchers used severe novel heterogeneous acidic catalysts (e.g., Keggin type heteropoly acid and silicotungstic acid) or solid metal oxides (e.g., MgO and ZnO) impregnated on support materials (e.g., molecular sieves, activated carbon, silicon dioxide, clay, titanium, and zeolites) as catalysts to produce MGs (Hoo and Abdullah, 2016; Yu et al., 2003). This process has many advantages: increased surface area of catalysts, high monoglyceride selectivity, environmental friendliness, ease of catalyst separation and hence inexpensive product purification processes (Hoo and Abdullah, 2016; Simsek et al., 2015). However, it is more time consuming than using acid or base catalysts (Ferretti et al., 2010). Additionally, protected glycerol (e.g., 1,2-O-isopropylidene glycerol) was also used to instead glycerol to produce highly pure MGs (Yu et al., 2003).

#### Enzymatic Synthesis of Monoglycerides

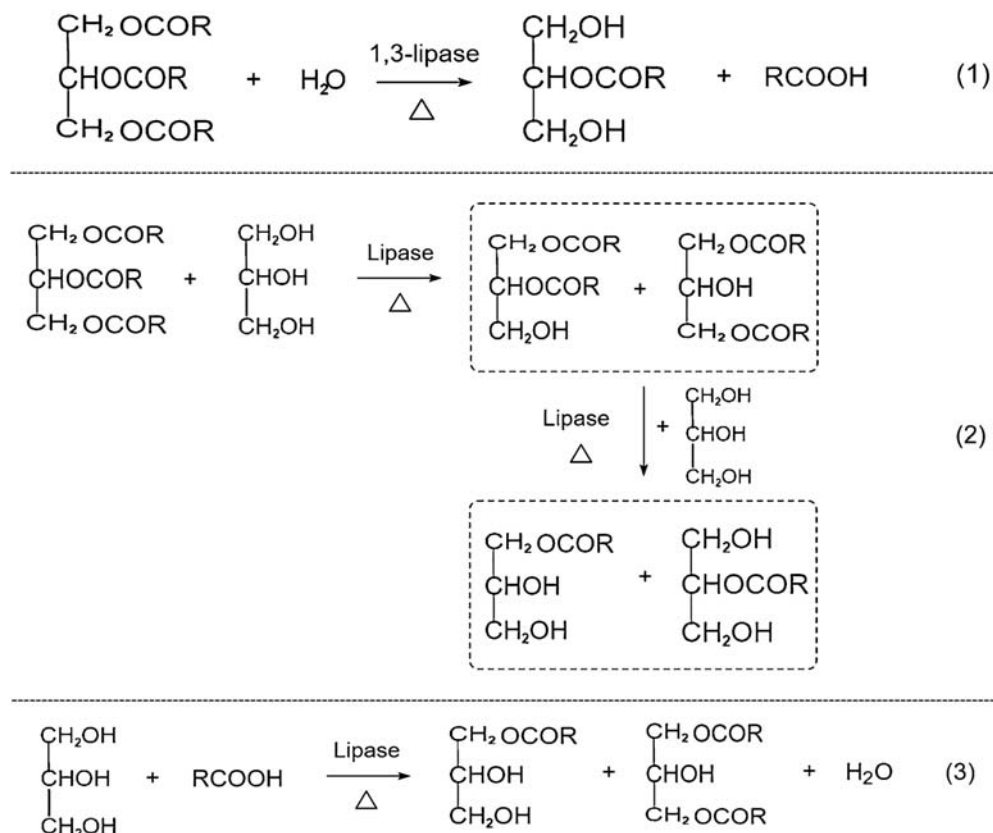
Over the last decades, enzymatic synthesis of MGs has received increasing attention. Lipase-catalyzed selective hydrolysis of triglycerides, glycerolysis, and direct esterification of glycerol with fatty acids are the three most important enzymatic processes for



**Figure 2** Chemical methods for preparation of monoglycerides: (1) direct esterification of glycerol with fatty acids (Pouilloux et al., 1999), (2) glycerolysis of fatty acid methyl ester with glycerol (Ferretti et al., 2012), and (3) glycerolysis of triglycerides with glycerol (Corma et al., 1998).

the preparation of MGs (Fig. 3) (Zhao et al., 2011). In the process of hydrolysis, TGs are converted to 2-monoglycerides in a selective manner by 1,3-regiospecific lipases (Holmberg and Osterberg, 1988). One problem of this process is the low yield of MGs, because 2-monoglycerides usually convert to 1-monoglycerides by acyl migration that can be further hydrolyzed by 1,3-regiospecific lipases to glycerol and fatty acids. Thus, most studies focused on the enzymatic glycerolysis and esterification in different reaction systems, including organic solvent systems (Cetina et al., 2011), solvent-free systems (Zhao et al., 2011), supercritical carbon dioxide systems (Rezaei and Temelli, 2000), and reversed micelle systems (Fan et al., 2010) with immobilized enzyme or free enzyme. In the processes of esterification and glycerolysis, organic solvents are usually used to form aqueous-organic two-phase systems or microemulsion, and thus improve mutual miscibility of the glycerol-fatty acids-lipases mixture or the glycerol-oil-lipases mixture (Yahya et al., 1998). However, the use of organic solvents may lead to pollution, produce various undesirable effects on enzyme molecules, and bring additional separation steps for solvent removal. Thus, solvent-free systems and supercritical carbon dioxide systems can be used to avoid these problems (Esmelindro et al., 2008). However, in the solvent-free systems, the concentrations and viscosity of the reactants are high, thus affecting the yield of MGs. And in supercritical carbon dioxide systems, the non-polarity of carbon dioxide can lead to improper dissolution of both hydrophobic and hydrophilic compounds (Esmelindro et al., 2008). This problem can be solved by reverse micelles, which are nanometer-sized aqueous or polar dispersion in a polar media in which the interface is controlled by surfactants/cosurfactants (Hayes and Gulari, 1991). In this technology, the lipases are encapsulated into reverse micelles, which provide large degree of interface area. However, organic solvents are also necessary in this technology.

The reaction temperature of enzymatic synthesis has a big influence on the yield of MGs. Many studies suggested that enzymatic synthesis must be carried out below a critical temperature ( $T_c$ ), which depends on the type of fat and is related to the melting point



**Figure 3** The enzymatic methods for preparation of monoglycerides: (1) lipase-catalyzed selective hydrolysis of triglycerides (Holmberg and Osterberg, 1988), (2) glycerolysis of triglycerides with glycerol (Wongsakul et al., 2003), and (3) direct esterification of glycerol with fatty acids (Multzsch et al., 1994).

of the fat (Mcneill et al., 1992). At a higher temperature than  $T_c$ , the yield of MGs is approximately 30%, while at a lower temperature than  $T_c$ , the yield of MGs can reach up to > 70% (Mcneill et al., 1990).

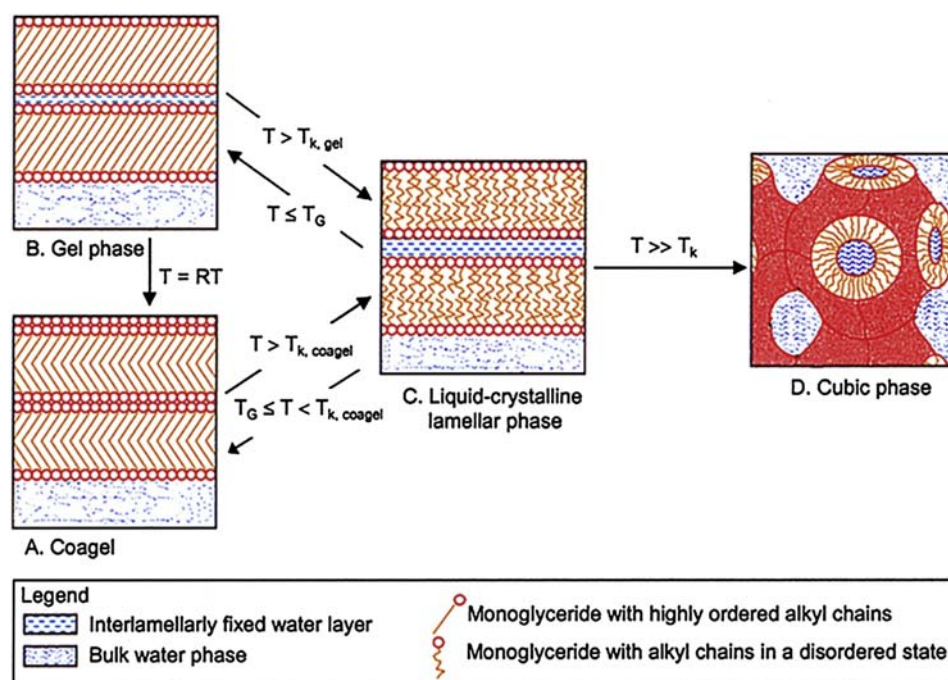
Compared to chemical synthesis, the advantages of enzymatic synthesis are milder reaction conditions, higher yields, lower energy consumption, more pure products, and stronger positional specificity (Freitas et al., 2010; Lee et al., 2004), however drawbacks also exist. The enzymatic processes are time consuming and complex (Lin et al., 1999). Another disadvantage is that it is difficult to separate the enzyme from the products (Avander et al., 1992). Immobilized lipases can be used to solve this problem, but they are uneconomic (Elfman-Börjesson and Häröd, 1999). Additionally, although enzymatic synthesis has been shown to be quite successful in producing long-chain MGs, it may not be applicable to the preparation of short-chain MGs because of the high acidity of short-chain fatty acids (Lee et al., 2004).

## Functional Properties of Monoglycerides

### Self-assemble in Water or Organic Liquids

MGs are insoluble in water but can swell and self-assemble into four liquid crystal phases: the coagel (the  $\beta$ -gel), gel phase (the  $\alpha$ -gel), liquid-crystalline lamellar phase ( $L_\alpha$ ), and cubic phase, depending on water content and temperature (Alberola et al., 2006). At room temperature, when a small amount of water was added to MGs (usually less than 5%, w/w), MGs-water system is in the form of isotropic fluid with a relatively low viscosity in coexist with the gel phase (Chen and Terentjev, 2011; Sagalowicz et al., 2006). With the increase in water content, the liquid-crystalline lamellar phase (approximately 10%, w/w) and cubic phase (approximately 15%, w/w) are formed (Sagalowicz et al., 2006). The phase transition of MGs in water are also dependent on the temperature for the formation of gel phase ( $T_G$ ) and the Krafft temperature of the system ( $T_K$ ), which is defined as the melting temperature of an amphiphilic component (Alberola et al., 2006; Luzzati et al., 2010) (Fig. 4). Above the  $T_K$ , layers between the polar head group of crystalline MGs are penetrated by water molecules, thus forming the liquid-crystalline lamellar phase in the presence anionic co-surfactant. With further increases of temperature (above 80 °C), the cubic phase is formed (Chupin et al., 2001). Below the  $T_G$ , the liquid-crystalline lamellar phase converts into the gel phase. The gel phase is not thermodynamically stable, and subsequently transforms into the coagel (Van Duynhoven et al., 2005). When the temperature of the system is between





**Figure 4** The effect of the temperature for the formation of gel phase ( $T_G$ ) and the Krafft temperature of the system ( $T_K$ ) on monoglyceride phase transitions in water (Alberola et al., 2007).

$T_G$  and  $T_K$ , the liquid-crystalline lamellar phase can also slowly convert into the coagel (Alberola et al., 2006). In this changing process, water is fully expelled from the crystal lattice.

The liquid-crystalline lamellar phase and the cubic phase are considered promising crystal phases for food applications. The liquid-crystalline lamellar phase can form a fat-like gel network, and thus encapsulating large amounts of food ingredients (Calligaris et al., 2013; Wang and Marangoni, 2015). The cubic phase is usually highly viscous and can also be used to the preparation of controlled release systems due to its adhesive property (Stonewall et al., 2017).

In organic liquids, MGs can also self-assemble themselves to form organogels, which are thermoreversible, anhydrous, and viscoelastic materials with a three-dimensional supramolecular network (Da Pieve et al., 2010). When MGs are mixed with organic liquids, inverse bilayers are formed and subsequently grow and organize into lamellar platelet microstructures and eventually convert into a continuous three-dimensional network via capillary forces containing the liquid (Chen et al., 2009; Chen and Terentjev, 2009). MGs organogels can be used as organogelators for unsaturated oils with plastic structures (Zheng et al., 2016), oil migration inhibitors (Hughes et al., 2009), and vehicle for the delivery of lipophilic bioactive compounds in the food industry (Da Pieve et al., 2010).

### Emulsifying Property

MGs are very common emulsifiers in the food industry, due to their hydrophilic head (the hydroxy of glycerol) and hydrophobic tail (the tail of fatty acid). At the air–water interface, GMs can form closely packed Langmuir monolayers (Pantoja-Romero et al., 2016). At the oil–water interface, crystallized GMs self-assemble to lamellae to form walls (hydrogel) surrounding oil droplets (Manzocco et al., 2012). However, MGs are low polar emulsifiers and have weak solubility in water, thus co-emulsifiers (e.g., fatty acids, and sodium salts of fatty acids) are usually used to increase their dispersibility in water (Hughes et al., 2009).

The molecular structure of MGs influences their chain packing and emulsifying stability at the air–water or oil–water interface (Shen et al., 2008). Many researchers indicated that emulsions or foams stabilized by unsaturated MGs or short-chain MGs are less stable due to the desorption and collapse than those stabilized by saturated MGs or long-chain MGs (Rodríguez Patino et al., 1999). Additionally, saturated MGs are better initiators of fat crystallization than saturated MGs (Zhang and Goff, 2005). Saturated MGs can accelerate fat crystallization by inducing heterogeneous nucleation, which is essential for the production of edible fat products with desirable melting and crystallization properties (Daels et al., 2015). On the other hand, unsaturated MGs are considered better destabilizing agents of the protein stabilized-emulsion than saturated MGs, and promote partial coalescence of fat globules, which contributes to the formation of a fat network (Zhang and Goff, 2005).

Mixing MGs with other types of emulsifiers (e.g., sodium caseinate and  $\beta$  lactoglobulin) is a good method to improve mutual emulsifying property (Cho and Cornec, 1999; Doxastakis and Sherman, 1984). Additionally, concentration and particle size of each emulsifier have significant effects on the structures of mixtures. For instance, after mixing MGs (low molecular weight) with proteins (high molecular weight), the adsorption of proteins at air–water interface was enhanced with in a shorter time possibly due to the

interaction between proteins and MGs. However, after a long time, a densely packed monolayer formed by MGs completely displaces proteins, possibly because of the surface pressure and exclusion effects (Cho and Cornec, 1999).

### Antimicrobial Activity and Antivirus Effect

Medium-chain MGs (especially monolaurin) are regarded as safe and “natural” antimicrobial preservatives (Branen and Davidson, 2004). They have significant inhibitory effects on fungi (e.g., *Aspergillus niger*, *Saccharomyces cerevisiae*, and *Penicillium italicum*) (Altieri et al., 2007; Nikolovska, 2012), Gram-positive spoilage bacteria (e.g., *Staphylococcus* and *Lactobacillus* species) (Blaszyk and Holley, 1998; Fu et al., 2009) and pathogenic bacteria (e.g., *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*) (Kabara, 2010; Stecchini et al., 1996). The mechanism is that the hydrophobic tail (the tail of fatty acid) of MGs can attach to cell membranes and puncture them, thereby altering membrane permeability and disrupting the cell metabolic activity (Altieri et al., 2007). However, Gram-negative bacteria are resistant to MGs because Gram-negative bacterial cell walls are protected by an outer membrane (Branen and Davidson, 2004; Proctor and Cunningham, 1988). Combining MGs with other antimicrobials (e.g., nisin (Branen and Davidson, 2004), herbs (Hamed et al., 2014), lactoperoxidase (McLay et al., 2002), and ethylenediamine-tetraacetic acid (Sadiq et al., 2016)) can increase their antimicrobial effects and even inactivate Gram-negative bacteria.

In addition, MGs have been found to possess antiviral activity. For instance, monocaprin has been shown to be effective against enveloped viruses, such as vesicular stomatitis virus, herpes simplex virus and human immunodeficiency virus type 1 (HIV-1) (Schlievert et al., 2008). In addition, unsaturated MGs (e.g., glyceryl monooleate) were found to be extremely potent inactivators of enveloped viruses (Sands et al., 1979). Future research may focus on the clinical use of these MGs as virucidal agents after addressing the technical challenges, such as the poor solubility in aqueous buffer solutions, and the sensitivity to the concentration (e.g., critical micelle concentration) and environmental factors (Jackman et al., 2016).

### Applications of Monoglycerides in the Food Industry

MGs are mainly used in bread, accounting for approximately 40% of all MGs used in the food industry (Van de Walle et al., 2008). There are two main advantages for using MGs in bread. First, MGs can be used with oil to reduce the overall fat and saturated/trans fat intake and so reduce the health and economic burden of overweight, obesity and their clinical sequelae (Calligaris et al., 2013). In addition, MGs possess the capacity to favor oil spreading over flour and the anti-stale properties based on their ability to complex with the major starch components, amylose and amylopectin (Calligaris et al., 2013; Sawa et al., 2009). Thus, MGs can improve dough machining properties, enhance slicing performance and achieve superior bread quality (e.g., increased bread volume and bread crumb softness, and reduced bread stale) (Inoue et al., 1996). Generally, the chain length of MGs can influence their anti-stale performance in bread. Longer chain MGs (e.g., glyceryl monomyristate, monopalmitin, and glyceryl monostearate) perform better than monolaurin with shorter chain length and glyceryl behenate with a very long chain length (Sawa et al., 2009).

Approximately 20% of all MGs are used in sponge cakes and cakes (Van de Walle et al., 2008). Saturated MGs can be used to reduce the overall fat content in cakes and increase bread volume probably due to the interaction between MGs and proteins and their increased foaming properties (Kuhrt and Welch, 1950; Manzocco et al., 2012). However, the use of unsaturated MGs in sponge cakes leads to a less voluminous and harder structure (Vereecken et al., 2010). In addition, MGs have some adverse effects on the quality of cakes, such as increased water-loss during reheating or storage, crunchier crust, higher acrylamide content and higher tendency to going stale, probably because of the development of a peculiar system morphology promoted by hydrogel incorporation in the food matrix (Cloke et al., 1984; Manzocco et al., 2012).

MGs can also be used as dough conditioners and amylose complexing agents in noodles (Charutigon et al., 2008). Many studies indicated that noodles prepared with MGs showed induced stickiness, higher cooking time, lower cooked weight and lower cooking loss (Charutigon et al., 2008; Kaur et al., 2005). The presence of MGs in noodles probably leads to the restricted supply of water to the starch granules and thus prevents the swelling of starch granules to their full extent, and thereby increasing cooking time and reducing cooking weight (Kaur et al., 2005). In addition, the complexes formed by the interaction between MGs and the amylose may result in lower cooking losses and longer cooking time of noodles (Kaur et al., 2005; Moorthy, 1985).

Saturated MGs and unsaturated MGs have different effects at different stages of ice cream preparation. In the first step, the preparation of a protein-stabilized oil-in-water emulsion, although MGs are not required to aid emulsification, they are still added and function as follows (Davies et al., 2000; Govin and Leeder, 2010). During the low temperature aging step, the surface tension between the fat and the water phases decreases due to MGs crystallization, and protein molecules are displaced by MGs due to their lower molecular weight, which make the fat droplets more susceptible to subsequent destabilization (Davies et al., 2000). However, the competitive adsorption of unsaturated MGs against proteins is better than saturated MGs, which makes unsaturated MGs better destabilizing agents (Barford et al., 2010; Johansson and Bergenstahl, 1992). During the concomitant freezing/whipping step, partial coalescence or fat destabilization contributes to the formation of a fat network, and crystallization of emulsified oil occurs in this step also (Méndez-Velasco and Goff, 2012). However, saturated MGs are better initiators of fat crystallization than unsaturated MGs (Davies et al., 2000).

Furthermore, MGs are also used in cookies (Goldstein and Seetharaman, 2011), margarines (Vereecken et al., 2010), cheese (Bunka et al., 2007), and sausages (Kouzounis et al., 2017). The function of MGs in cookies is similar to that in bread (Goldstein and Seetharaman, 2011). On the other hand, MGs act as emulsifiers and structural stabilizers in margarines, cheese and sausages,

due to their good emulsifying performance and the property of self-assembling to create a gel with “fat-like” properties (Alfutimie et al., 2015; Kouzounis et al., 2017).

## Conclusions

We can conclude that the structures of MGs (e.g., chain length and degree of saturation of fatty acids attached to the glycerol) have a big effect on their physical (e.g., the polymorphism, melting point, physical appearance, and hydrophobicity) and chemical (e.g., the self-assemble, emulsifying property, antimicrobial activity, and antivirus effect) properties, and thus their applications in the food industry. We also found that the esterification and the glycerolysis used in both chemical and enzymatic processes are the two most common methods for the preparation of MGs. However, many problems associated with both chemical and enzymatic processes remain to be solved. In addition, there is a need for further studies elucidating the interactions between MGs and other polymers (e.g., proteins, fat, and starch), properties of these complexes and their applications in the food industry.

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## Muscle Proteins

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### Introduction

Muscle proteins are an important part of the diet in many cultures, as they make up the major proportion (other than water) of lean meat. These proteins provide most of the texture, color and nutritive value of meat and most meat products. In life, muscle proteins provide motive power to the animal. Following slaughter, muscle tissue forms meat.

Meat and meat products are today regarded in the developed world as a luxury, and in many Western countries meat consumption is on the decline due to both the price and the environmental cost. But strong demand for meat and meat products is expected to continue because of strong meat-eating traditions in many cultures, a general recognition of meat as one of the best sources of high quality bioaccessible protein and iron, and a general preference for the textures and flavors of meat-containing dishes. Further to this, meat plays an important part in many developing countries, particularly meat from pigs and chickens that can convert waste materials, and from ruminants that can convert plant materials that are inedible to humans, into high quality food.

### Structure of Muscle

Skeletal muscles consist of multinucleated muscle fibers made up of bundles of elongated myofibrils in a parallel configuration (Fig. 1) (Gault, 1992; Strasburg et al., 2008). Depending on animal species, muscle size and anatomical location, muscle fibers differ in length (a few millimeters to 30 cm), diameter (10  $\mu$ m to 100  $\mu$ m), and orientation (parallel or at a specific angle to the length). Most of the skeletal muscles are composed of muscle fiber type I, IIA, IIX and IIB (Pette and Staron, 2000; Schiaffino and Reggiani, 2011; Spangenburg and Booth, 2003). These muscle fiber types are characterized based on their contraction speed (slow-twitch or fast-twitch) and preferred metabolic pathway for glycogen degradation (glycolytic or oxidative). Different muscle types vary in ATPase activity, which reflects their contractile characteristics as well as enzyme activities involved in their metabolic pathways. Type I fibers are slow-twitch, red, with oxidative metabolism; type IIA fibers are fast-twitch, red, with intermediate (oxidative glycolytic) metabolism; whereas type IIB and IIX fibers are fast-twitch, white, with glycolytic metabolism (Lefevre et al., 1999; Peter et al., 1972).

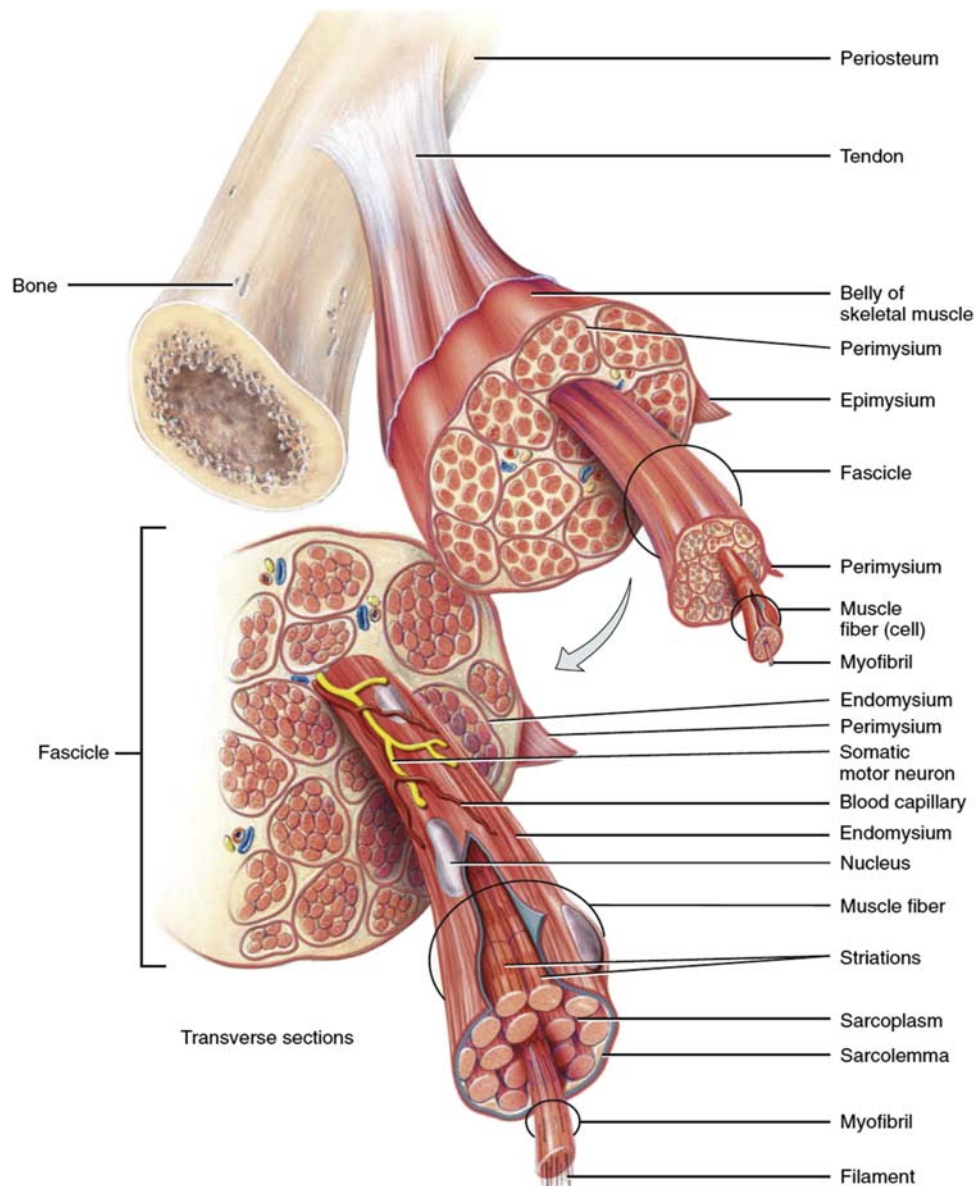
A muscle fiber is ensheathed by a fine connective tissue layer, the endomysium, which contains blood capillaries and nerves. Each muscle fiber is composed of a few dozen myofibrils and is enclosed by the muscle plasma membrane, called the sarcolemma, which is made up of a phospholipid bilayer with embedded proteins, glycoproteins and glycolipids. The myofibrils are bathed in sarcoplasm containing several nuclei, sarcoplasmic reticulum, Golgi apparatus, mitochondria, lysosomes, glycogen granules, enzymes and other soluble constituents, that are vital for muscle function. The sarcoplasmic reticulum acts as a calcium ion reservoir for muscle contraction. Bundles of muscle fibers are organized into fascicles that are encased in another layer of connective tissue containing larger blood vessels and nerves called the perimysium. Several fascicles are assembled into a whole muscle by an outer layer of connective tissue known as the epimysium, which extends into tendons to join the muscles and bones together.

Unlike those of terrestrial animals, the muscles of fish are much shorter and are segmented into myotomes by a fine connective tissue layer called the myocommata, or myosepta, as depicted in Fig. 2 (Johnston, 2001; Venugopal and Shahidi, 1996). Each myotome consists of muscle fibers organized in parallel to the length axis of the fish body. Fish muscle fibers are enclosed by the sarcolemma. However, the connective tissue of the extracellular matrix in fish fuses with myocommata at the myotome-myocommata junction, in contrast to terrestrial animal muscles, that are tapered into a tendon.

Regardless of the animal species, myofibrils in muscle fibers consist of longitudinal myofilaments comprising thick and thin filaments (Fig. 3) (Strasburg et al., 2008; Tortora and Derrickson, 2013). Thick filaments are made up of myosin molecules and several cytoskeletal proteins such as titin, while thin filaments are composed of actin, tropomyosin and troponin. Alternating light isotropic (I-band) and dark anisotropic (A-band) bands are seen on myofibrils under a polarized light microscope. At ultrastructural scale, each I-band is separated into two by a dark and narrow band called the Z-disk. The region between two Z-disks is known as a sarcomere, which is the repeating longitudinal contractile unit of the myofibril. A sarcomere comprises an I-band consisting purely of thin filaments, an A-band containing alternatively overlapping thin and thick filaments, an H-zone that is in the center region of the A-band, where the thin filaments are absent, and an M-line that is in the middle of the H-zone. When a muscle contracts, the Z-disks shift closer together due to shortening of the I-bands, and the length of the sarcomere is decreased (Gault, 1992).

Skeletal muscle proteins can be classified into myofibrillar (50%–60%), sarcoplasmic (30%) and stromal (10%–20%) proteins, based on their solubility at varying salt concentrations (Strasburg et al., 2008).





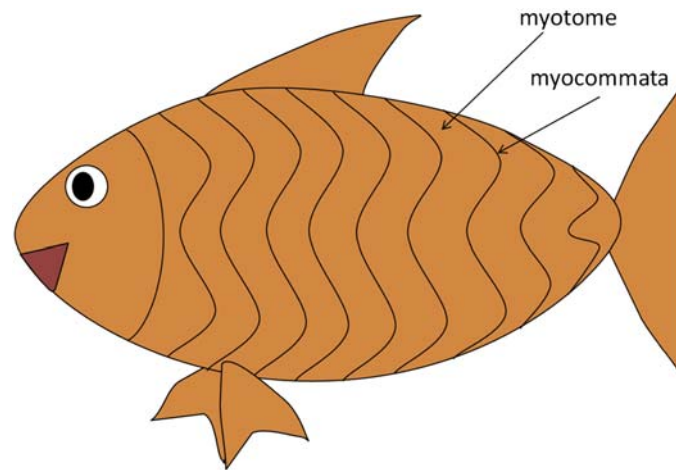
**Figure 1** Structure of skeletal muscle. Adapted from Tortora, G.J., Derrickson, B.H., 2013. Principles of Anatomy and Physiology, fourteenth ed. Wiley Global Education, New York.

## Myofibrillar Proteins

The myofibrillar proteins consist of contractile, structural and regulatory proteins (Hopkins, 2014). The contractile proteins are myosin and actin, which form the thin and thick filaments that control the skeletal muscle contraction and relaxation. The regulatory proteins include troponin and tropomyosin. The structural proteins comprise mainly titin, nebulin,  $\alpha$ -actinin,  $\beta$ -actinin, tropomodulin, desmin, filamin, C-protein, H-protein and myomesin.

## Contractile Proteins

Myosin (molecular weight of 520 kDa) is a filamentous protein that forms the thick filaments of muscle cells and is the principal protein of the A-band (Gault, 1992; Strasburg et al., 2008). A myosin molecule has a quaternary structure consisting of six subunits, which include two myosin heavy chains (MHC), two essential myosin light chains (MLC1) and two regulatory myosin light chains (MLC2), with molecular weights (MW) of approximately 220 kDa, 23 kDa and 20 kDa correspondingly (Clark et al., 2002; Swartz et al., 2009). Each myosin molecule can be hydrolyzed by enzymatic digestion into two portions, which are light meromyosin (LMM) and heavy meromyosin (HMM) (Fig. 4) (Choi and Kim, 2009; Pearson and Young, 1989a; Strasburg et al., 2008). LMM



**Figure 2** Schematic drawing of fish muscle displaying the orientation of myotomes and myocommata.

is important for the construction of thick filaments from myosin molecules while HMM is responsible for the regulation of ATPase activity and actin binding ability. HMM can be further broken down into fragment S1 and S2 where the former contains the globular myosin heads with the binding site of MgATP and actin, while the latter comprises the  $\alpha$ -helix region which binds both MLC1 and MLC2. In adult mammalian skeletal muscle, there are primarily four MHC isoforms, namely MHC I, IIa, IIb, and IIx, which exist in pure slow-twitch fiber type I, fast-twitch fiber type IIa, IIb and IIx, correspondingly (Kohn et al., 2007; Pette and Staron, 2000). Hybrid fiber types containing different myosin isoforms have also been reported.

Actin (molecular weight of 42 kDa) is the building block of thin filaments and is present in two forms namely globular actin (G-actin) and filamentous actin (F-actin) (Gault, 1992; Strasburg et al., 2008). F-actin is formed by the polymerization of G-actin into double-stranded, coiled filaments. F-actin is bound to tropomyosin and troponin as shown in Fig. 5.

During muscle contraction, actin binds myosin to form actomyosin cross-bridges, which activate the myosin ATPase, leading to the pulling of thin filaments by myosin toward the M-line, resulting in shortening of the sarcomere (Lawrie, 2006).

### Regulatory Proteins

Tropomyosin and troponin are two main proteins that regulate muscle contraction and relaxation (Choi and Kim, 2009; Zot and Potter, 1987). They prevent the activation of actomyosin ATPase in the absence of calcium ions by interacting with actin filaments to block the myosin binding site. Tropomyosin is a long, coiled protein (MW 65 kDa) that comprises two  $\alpha$ -helix polypeptide subunits, called  $\alpha$ - and  $\beta$ -tropomyosin. Tropomyosin molecules bind head-to-tail along the F-actin filament. Each tropomyosin molecule is attached to a troponin complex (MW 80 kDa) which is made up of troponin C (MW 18 kDa), troponin I (MW 21 kDa) and troponin T (MW 31 kDa) (Fig. 5). Troponin C acts as the calcium binding site; troponin T connects troponin complex to tropomyosin while troponin I inhibits actomyosin ATPase activity when it is bound to actin (Lehman and Craig, 2008). At high calcium ion concentration, calcium ions bind to Troponin C, which initiates a conformation change in the tropomyosin-troponin complex, dislocating troponin I, allowing the action of actomyosin ATPase for muscle contraction.

### Structural Proteins

The structural proteins control the filamentous structure and integrity of myofibrils (Fig. 3) (Obinata et al., 1981). Titin, also known as connectin, with a molecular weight of 4200 kDa, serves as the backbone of thick filaments in the A-band. It also acts as a molecular spring in the I-band, which provides elasticity to the sarcomere during muscle contraction (Fig. 3) (Labeit and Kolmerer, 1995).

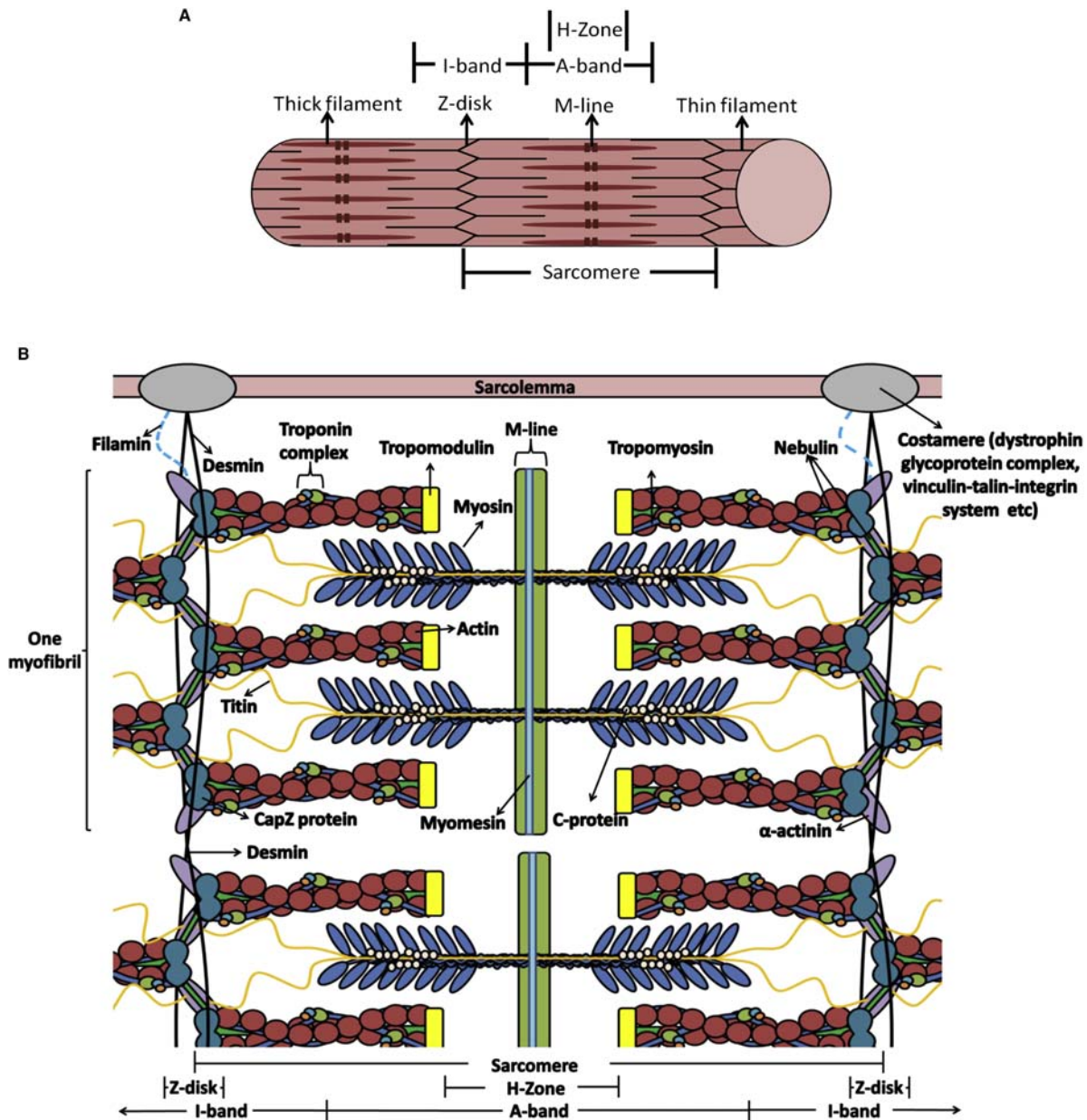
Nebulin (MW 800 kDa) is a structural protein that regulates the length of the thin filaments (McElhinny et al., 2003; Strasburg et al., 2008).

$\alpha$ -Actinin (MW 95 kDa) is the principal constituent of the Z-disk, supporting and attaching actin to the Z-disk (Obinata et al., 1981).

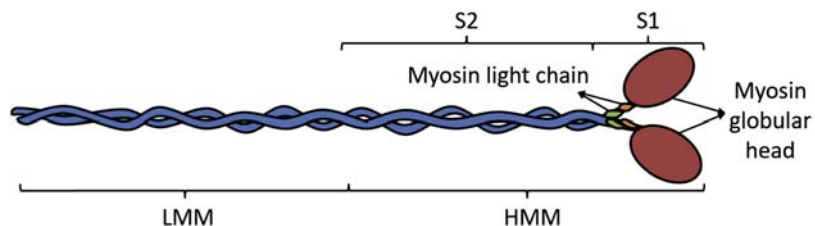
$\beta$ -Actinin, also known as CapZ protein, is a heterodimer consisting of  $\alpha$ - and  $\beta$ -subunits (MW 37 and 34 kDa respectively). It binds  $\alpha$ -actinin in the Z-disk and prevents network formation between the actin filaments (Swartz et al., 2009).

Tropomodulin (MW 40 kDa) binds tropomyosin and actin as well as controlling the length of thin filaments by maintaining the number of G-actin monomers (Clark et al., 2002).

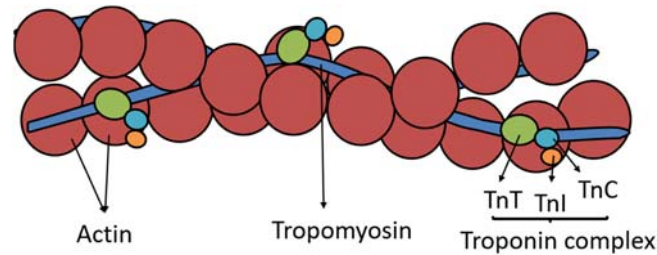
Both desmin (MW 55 kDa) and filamin (MW 300 kDa), play a major role in linking the myofibrils to the sarcolemma as well as stabilizing muscle structure (Capetanaki et al., 1997; Strasburg et al., 2008; Vander Ven et al., 2000).



**Figure 3** (A) Basic functional unit of a myofibril and (B) the arrangement of filaments within a sarcomere. Figure based on Au, Y., 2004. The muscle ultrastructure: a structural perspective of the sarcomere. *Cell. Mol. Life Sci. CMLS* 61, 3016–3033; Tortora, G.J., Derrickson, B.H., 2013. *Principles of Anatomy and Physiology*, fourteenth ed. Wiley Global Education, New York; Henderson, C.A., Gomez, C.G., Novak, S.M., Mi-Mi, L., Gregorio, C.C., 2017. Overview of the muscle cytoskeleton. *Compr. Physiol.* 7, 891–944.



**Figure 4** Schematic diagram representing a myosin molecule. Modified from Adamovic, I., Mijailovich, S.M., Karplus, M., 2008. The elastic properties of the structurally characterized myosin II S2 subdomain: a molecular dynamics and normal mode analysis. *Biophys. J.* 94, 3779–3789.



**Figure 5** . Schematic diagram of a thin filament comprising actin, tropomyosin and troponin complex. TnT (troponin T), TnI (troponin I) and TnC (troponin C). Modified from Strasburg, G., Xiong, Y.L., Chiang, W., 2008. Physiology and chemistry of edible muscle tissues. In: Damodaran, S., Parkin, K.L., Fennema, O.R. (Eds), *Fennema's Food Chemistry*, fourth ed. CRC Press, Boca Raton FL, pp. 923–973.

C-protein (MW 140 kDa) and H-protein (MW 58 kDa) are myosin-binding proteins that are found in the A-band of thick filaments (Koretz et al., 1993; Xiong, 1997). These proteins are believed to contribute to the alignment and stabilization of the thick filaments.

Myomesin (MW 185 kDa) is the major protein in the M-line. It is responsible for the binding of titin and myosin and for maintaining the structure of the thick filaments.

## Stromal Proteins

Stromal proteins constitute the connective tissue, which provides mechanical support and protection to the muscle in the form of tendons, epimysium, perimysium and endomysium (Fig. 1) (Gault, 1992). Connective tissue is composed mainly of collagen (90%) along with other fibrous proteins including elastin, laminin and fibronectin, and proteoglycans (Chagnot et al., 2012; Voermans et al., 2008). Connective tissue contains different types of cells, including fibroblasts, macrophages, lymphoid cells, mast cells and eosinophils. Collagen and elastin are bound to an amorphous ground substance formed by proteoglycans and glycoproteins for the reinforcement of the connective tissue network.

## Collagen

Collagen is the predominant stromal protein in skeletal muscles and is synthesized by fibroblasts (Bailey and Light, 1989; Duance et al., 1977; Gault, 1992). It is classified into four major types based on its aggregation characteristics: striated and fibrous (Types I, II, III, V and XI); non-fibrous and network forming (Type IV); microfibrillar or filamentous (Type VI), and fibril-associated collagen (Type VII) (Bailey, 1991).

Collagen consists of three polypeptide alpha chains with -Gly-X-Y- repeating units, where X is commonly proline and Y can be any amino acid (except tryptophan), but is often hydroxyproline, that coil to create a triple helix structure, forming tropocollagen (Astruc, 2014a; Bailey and Light, 1989; Gault, 1992; Strasburg et al., 2008). Tropocollagen molecules are polymerized into collagen fibers via covalent intermolecular cross-links that offer substantial tensile strength to collagen fibers. As the collagen fibers age, the divalent reducible cross-linkages interact to form mature trivalent, non-reducible, more heat-stable cross-links, which further enhances their stability and mechanical strength.

Collagen has been linked to the toughness of muscle-based food, and its content and extent of cross-linking differ among different animal species and breeds, age, muscle function, history of exercise and treatment with growth promoter (Strasburg et al., 2008). Small aquatic animals like fish generally have a lesser amount and less cross-linking of collagen compared to larger terrestrial animals that require higher weight-bearing strength. Some authors have reported an increase in meat toughness as the animal aged due to an increase in collagen cross-linking and a decrease in collagen solubility (McCormick, 1999; Purslow, 2005; Taylor, 2004). It was also found that the tender meat cut, bovine Longissimus dorsi, contains only half to two-thirds of the total collagen content and hydroxylslypyridinoline cross-links of that found in the tougher cut, Biceps femoris. Various studies have shown improved meat quality by partial solubilization of collagen in tough meat cuts such as Semitendinosus (Christensen et al., 2011a,b; Combes et al., 2004; Sullivan and Calkins, 2010).

## Elastin

Elastin is a minor constituent of connective tissue that offers elasticity to the blood vessels and ligaments in the muscles (Debelle and Alix, 1999). Elastin is an insoluble, hydrophobic, heat-stable and cross-linked protein fiber that behaves in a highly elastic manner in the presence of water. Elastin content varies among different muscle types. In Semitendinosus muscle, epimysium and perimysium are rich in coarse elastin fibers which are believed to be one of the contributors in meat toughness (Rowe, 1986). In contrast, Longissimus dorsi has limited coarse elastin fibers present in the epimysium and even less in the perimysium.



## Sarcoplasmic Proteins

The sarcoplasmic proteins occur in the sarcoplasm surrounding the myofibrils (Pearson and Young, 1989b). They are involved in various metabolic functions such as protein metabolism, fatty acid oxidation, electron transportation, glycolysis, glycogenesis and glycogenolysis (Pearson and Gillett, 2012; Smith, 2000; Strasburg et al., 2008). The sarcoplasmic proteins include the heme pigments (myoglobin), the glycolytic enzymes (glyceraldehyde phosphate dehydrogenase), the mitochondrial oxidative enzymes (such as succinate dehydrogenase, cytochrome), the lysosomal enzymes (notably cathepsin), the nucleoproteins and others. Among them are the proteolytic enzymes that are involved in the post-mortem muscle tenderization process, and myoglobin that is responsible for meat color. The amount of different sarcoplasmic proteins is largely dependent on the muscle fiber type, which in turn depends on the animal species, breed, age, genetics, and muscle anatomical position and function (Strasburg et al., 2008). Sarcoplasmic proteases are vital in protein catabolism and post-mortem muscle softening. Both calpains and cathepsins are responsible for post-mortem proteolysis, with calpains and more specially calpain 1 or  $\mu$ -calpain considered to be playing a major role (Ouali et al., 2006; Veiseth and Koohmaraie, 2005).

## Calpains

The calpains are a family of calcium-activated, cysteine proteases that have maximum activity at neutral pH (Sentandreu et al., 2002). Calpains degrade myofibrillar proteins during protein turnover for muscle growth (Goll et al., 2008; Huang and Forsberg, 1998). There are two types of calpains responsible for post-mortem proteolysis, which are ubiquitous:  $\mu$ -calpain and *m*-calpain (Table 1) (Raynaud et al., 2005; Strasburg et al., 2008). The calcium ion concentrations for the activation of  $\mu$ -calpain and *m*-calpain are in the range of micromolar and millimolar respectively (Camou et al., 2007). Calpains are found and act along the Z-disk. Calpastatin is the natural inhibitor of  $\mu$ -calpain and *m*-calpain.

## Cathepsins

Cathepsins are sarcoplasmic proteins that are released from the lysosomes in post-mortem muscle, which are active at an acidic pH (Table 1) (Geesink and Veiseth, 2008). Among the family of cathepsins, cysteine cathepsin B, H and L and aspartic cathepsin D are the most abundant in muscles. Cathepsins break down MHC, troponin T, troponin I, tropomyosin and collagen (Bechet et al., 2005) and collagen (Goll et al., 1983). The proteolytic activity of cysteine cathepsins and cathepsin D can be inhibited by cystatins (Geesink and Veiseth, 2008) and pepstatin (Bohley and Seglen, 1992) respectively.

## Myoglobin

Myoglobin is a heme protein that acts as an oxygen carrier in muscle cells and is responsible for the color of both raw and cooked meat. The amount of myoglobin varies between different muscle types. Oxidative muscle fibers type I & IIA have higher myoglobin content than glycolytic muscle fibers type IIB and IIX (Hoekstra, 1969). Poultry has a paler appearance than beef due to a higher content of fast glycolytic fibers and consequently a lower level of myoglobin.

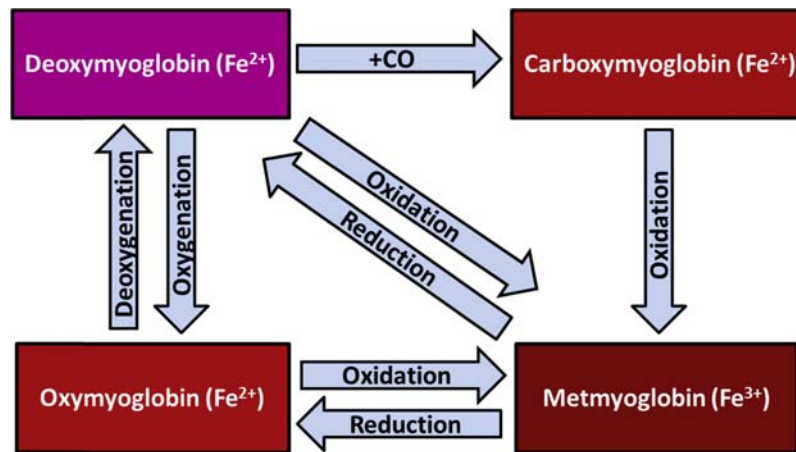
Four forms of myoglobin exist in the muscle, depending on the state of the heme group: deoxymyoglobin (purplish red), oxymyoglobin (cherry red), metmyoglobin (brown) and carboxymyoglobin (cherry red) (Fig. 6) (Suman and Joseph, 2013). The color of the muscle depends on the ratio of these forms of myoglobin (Baldwin, 2012). Cooking of meat results in denaturation of myoglobin and causes the oxidation of heme which induces a change in meat color.

**Table 1** Characteristics of cathepsins and calpains associated with meat tenderness during ageing<sup>a,b</sup>

	Calpains	Cathepsins
Nature	Cysteine proteases; calcium-dependent	Acid proteases
Occurrence	Cytosol	Lysosomes (in sarcoplasm)
Class/Isoform	<i>m</i> -Calpains, $\mu$ -calpains and calpain 3.	Main cathepsins involved in muscle ageing are cathepsins B, L, H and D
pH for optimum activity	Neutral	4–6.5 (varies with enzyme class)
Target proteins	Proteins in the Z-disk and cytoskeleton (mainly titin and desmin). Some breakdown of nebulin, troponin and tropomyosin.	Troponin T, collagen cross-links and mucopolysaccharides of connective tissue. Actin and myosin at pH < 5

<sup>a</sup>Warriss (2010).

<sup>b</sup>Kemp et al. (2010).



**Figure 6** Interconversion of myoglobin redox forms in fresh meats. Modified from Suman, S.P., Joseph, P., 2013. Myoglobin chemistry and meat color. *Annu. Rev. Food Sci. Technol.* 4, 79–99.

### Other Sarcoplasmic Proteins

Most of the enzymes involved in the metabolic processes in muscles are sarcoplasmic proteins (Pearson and Young, 1989b; Strasburg et al., 2008). For instance, the enzymes creatine kinase and adenylate kinase take part in the synthesis and dissociation of high energy phosphate compounds such as ATP, ADP and AMP. Caspases, metalloproteases, thrombin and plasmin have been reported to be involved in post-mortem proteolysis (Sentandreu et al., 2002). Other sarcoplasmic proteins such as hemoglobin play an essential role in the exchange of oxygen and carbon dioxide (Pearson and Young, 1989b; Tahergorabi et al., 2011).

## From Muscle to Meat

### Postmortem Biochemical Changes

Meat comes from the transformation of skeletal muscle after slaughter. After bleeding, cells survive for a short time by regenerating ATP (essential for maintaining ionic and potential membrane gradients) through anaerobic metabolic pathways. The synthesis of ATP is mostly based on the degradation of phosphocreatine and anaerobic glycolysis. When phosphocreatine is depleted, the poor performance of glycolysis does not maintain ATP at a constant value. Its cellular concentration gradually decreases. As the ATP level decreases and glycogen is degraded, protons and lactate molecules accumulate in muscle cells resulting in decreased muscle pH (Bendall, 1973; Greaser, 1986; Monin, 1988). When the ATP level falls by half the resting muscle content, the actin and myosin molecules bind to form an actomyosin complex. The relative sliding of the filaments becomes impossible and the whole of the myofibrillar apparatus is transformed into a rigid system called rigor. Subsequently, the muscle stiffness gradually fades, but not the inextensibility. This 'resolution' of rigor results from the lysis of the proteins constituting the myofibrillar structure during the so-called maturation period.

When the glycogen is depleted and/or when the enzyme co-factor AMP disappears, the pH stabilizes at a value called the ultimate pH (usually close to 5.7). The acidification of the muscle reduces the net charge of myofibrillar proteins and is close to their isoelectric point (around 5). The slightest repulsion of the proteins comprising the myofilaments leads to a tightening of the latter and a lateral contraction of the myofibrils (Pearce et al., 2011), which expels part of the intramyofibrillar water into the sarcoplasm that then migrates into the extracellular space. In addition to the decrease in pH, the osmotic pressure of the muscle evolves after slaughter. Its value doubles during the onset of rigor mortis (from 270–300 mosmoles to 500–600 mosmoles) (Ouali, 1990a, 1990b, 1992). This increase is directly related to the decrease in pH (Ouali, 1992). The resulting increase in ionic strength, close to 0.30, is sufficient to damage myofibrillar proteins and to promote the action of muscle proteases.

### Fast Postmortem Metabolism Effects on Muscle Proteins and Meat Quality

Pronounced stress just prior to slaughter, coupled with the effects of stunning (especially electrical stunning), accelerates the muscle metabolism that continues after the animal dies and leads to a rapid drop in pH while the muscle temperature is still high (Charpentier and Goutefongea, 1963). The low pH/high temperature combination leads to the denaturation of the sarcoplasmic proteins (Bendall and Wismerpedersen, 1962; Charpentier, 1969; Charpentier and Goutefongea, 1963; Fischer et al., 1979; Lawrie et al., 1963; Scopes, 1964; Scopes and Lawrie, 1963) and the myofibrillar proteins, with myosin being the most sensitive (Bendall, 1973; Offer and Knight, 1988; Penny, 1967; Stabursvik et al., 1984). The denaturation of muscle proteins leads to reduction in their water holding capacity. The myofilament lattice retracts, expelling water to the extracellular space (Bendall and Swatland, 1988; Fialik, 1983; Guignot et al., 1993), increasing the ability of meat to reflect light (Macdougall, 1970, 1982). These meats are characterized by strong exudations, a lighter color and a soft texture, hence their name PSE (Pale Soft Exudative) (Adzitey and Nurul,



2011). This phenomenon was first observed in meat from pigs (Bendall and Wismerpedersen, 1962; Briskey, 1964; Hamm, 1960) and then in meat from poultry (Barbut, 2009; Owens et al., 2000; Swatland, 2008) and more recently in meat from sheep (Kim et al., 2014a,b; Warner et al., 2014) and cattle (Aalhus et al., 1998; Guignot et al., 1993; Warner et al., 2014).

Protein denaturation is also observed when the PSE trait is induced by maintaining a muscle at 37 °C for a few hours post slaughter (Penny, 1967, 1977).

## Postmortem Proteolysis

### Proteolytic Systems

Conditions after slaughter and during carcass chilling favor degradation of intracellular muscle proteins by proteolytic systems. Calpains seem to represent the major system involved in tenderizing meat (Goll et al., 2003; Koohmaraie, 1994; Koohmaraie and Geesink, 2006; Raynaud et al., 2005; Taylor et al., 1995a). It has been concluded that post-mortem muscle tenderization is due mainly to the action of  $\mu$ -calpain and to a lesser extent the action of  $m$ -calpain.

Cathepsins and proteasome seem less involved (Sentandreu et al., 2002; Koohmaraie and Geesink, 2006; Koohmaraie, 1994; Taylor et al., 1995b). Other proteolytic systems such as caspases, metalloproteases, thrombin or plasmin could also be involved in the postmortem degradation of muscle tissue (Sentandreu et al., 2002; Ouali et al., 2013).

It has been reported that low pH, elevated temperature and electrical stimulation can all accelerate the release of cathepsins from lysosomes and eventually facilitate muscle tenderization.

### Postmortem Muscle Protein Degradation

The consequences of postmortem proteolysis on the ultrastructure of muscle are well understood (Davey and Dickson, 1970; Davey and Gilbert, 1969; Koohmaraie, 1994; Koohmaraie and Geesink, 2006; Ouali, 1990b; Taylor et al., 1995a). Surprisingly, myosin and actin are little affected by post mortem proteolysis, and it is rather the degradation of cytoskeleton proteins that causes a -modification of myofibril ultrastructure and tenderness of the meat. Overall, post mortem proteolysis leads to a transverse rupture of the myofibrils at the connecting junction of the I-band to the Z-disks. This zone is essentially composed of actin, titin, and neb-ulin. Their postmortem degradation is likely to be responsible for myofibril breakdown (Koohmaraie and Geesink, 2006; Robson et al., 1997; Taylor et al., 1995a).

The cytoskeleton is also disorganized by the degradation of the desmin and the filamin, which bind the Z-disks of myofibrils to each other and also to the sarcolemma from peripheral myofibrils (Huff Lonergan et al., 2010; Taylor et al., 1995b; Koohmaraie and Geesink, 2006). The degradation of the constitutive proteins of the costamere (dystrophin-vinculin-talin-integrin) which constitutes the anchor point of desmin on the sarcolemma (Taylor et al., 1995a; Koohmaraie and Geesink, 2006) is also suspected to change the muscle fiber ultrastructure. The intermediate filaments which bind the myofibrils at the level of the M-lines are also partially hydrolyzed.

Deterioration of these major cytoskeletal proteins causes misalignment of the myofibrils relative to each other, detachment of the sarcolemma and rupture of the myofibrils along the Z-disks. Nevertheless, it appears that the degradation of desmin and titin are the most important of the mechanisms underlying development of tenderness during meat maturation (Koohmaraie and Geesink, 2006). Troponin T, incorporated into the thin filaments, is also degraded during the meat maturation phase, and its disappearance is related to the appearance of a 30 kDa peptide and the tenderness of the meat (Huff Lonergan et al., 2010). This protein is involved in the mechanism of muscle contraction, but it is also suspected to stabilize interactions between myofilaments. Its degradation may favor the disorganization of myofibrils.

Structural changes in intramuscular connective tissue become significant only after 10 days of maturation (Nishimura et al., 1995). In general, maturation increases the solubility of collagen (Purslow, 2005), causing an improvement in the texture of raw meat. However, the effects of postmortem changes in connective tissue on the texture of cooked meat are controversial (Nishimura, 2010; Purslow, 2005).

### Postmortem Protein Oxidation

An increase in myofibrillar protein oxidation is observed during ageing (Martinaud et al., 1997; Rowe et al., 2004a,b), that results in carbonyl derivative formation (Levine et al., 1994; Martinaud et al., 1997) and protein disulfide cross-links (Martinaud et al., 1997; Stadtman, 1990). In general, both of these changes reduce the functionality of proteins (Xiong and Decker, 1995). Calpains are particularly susceptible to inactivation by oxidation (Lametsch et al., 2008). Therefore, oxidizing conditions in postmortem muscle decrease the calpain activity and are likely to affect the tenderization of meat. Myoglobin also undergoes oxidation during the refrigeration of carcasses and meat, with significant effects on the color of the meat (Renerre, 2000).

## Effects of Processing on Meat Proteins

Meat processing affects the physical and chemical properties of the product by the action of physical forces, heat or the addition of salts, additives or processing aids (Lewis, 1992). For instance, meat tenderization through electrical stimulation, ultrasonic waves, blade tenderization and pressure treatment have been reported to modify the muscle structure and protein profile (Hopkins, 2014).

These processes decrease the overlapping of actin and myosin, cause physical damage to the sarcomere and connective tissues or improve proteolysis rates through the activation of calpains by calcium ions released after membrane disruption.

### Electrical Stimulation of Carcasses

Electrical stimulation of carcasses has no detectable influence on the degradation of desmin and troponin T (Ho et al., 1997), but could slightly accelerate the degradation of the cytoskeletal proteins titin and nebulin in postmortem muscle (Ho et al., 1996).

### Pulsed Electric Field

There has been recent interest in meat tenderization applications of PEF. PEF causes the electroporation of muscle cell membranes, which potentially facilitates the meat ageing process by releasing the calcium ions from sarcoplasmic reticulum, which triggers the calcium-activated proteases and/or speeds up pre-rigor glycolysis, and also by releasing the cathepsins from the lysosomes (Warner et al., 2017). Compared to untreated muscles, PEF-treated cold- and hot-boned beef Longissimus lumborum muscles had increased degradation of troponin T and desmin (Suwandy et al., 2015a, b).

### Exogenous Enzyme Technology

Exogenous proteases have been applied in meat tenderization for many years (Payne, 2009; Sullivan and Calkins, 2010). Most plant-based proteases can hydrolyze myofibrillar proteins efficiently (Bekhit et al., 2014; Kim and Taub, 1991). For the stromal proteins, under heating conditions, both papain and ficin were found to be effective in hydrolyzing elastin and collagen whilst bromelain degraded only collagen (more actively than papain and ficin) (El-Gharbawi and Whitaker, 1963; Miyada and Tappel, 1956; Payne, 2009). These enzymes have wide substrate specificity and can break down many of the peptide bonds present in meat proteins (Bekhit et al., 2014; Huff-Lonergan, 2014). Excessive proteolytic action can cause over-tenderization, leading to mushy meat texture (Ashie et al., 2002; McKeith et al., 1994; Weir et al., 1958).

### Shockwave Hydrodynamic Processing

Hydrodynamic processing generates a shockwave up to 1 GPa which travels through water in fractions of a millisecond (Bolumar et al., 2014; Hopkins, 2014). A shockwave process can be set up by subjecting the sealed meat in a water-filled container to electrically-generated shockwaves (Hopkins, 2014). Shockwave treatment caused increased intramyofibrillar and endomysium spaces between muscle fibers (Zuckerman and Solomon, 1998; Bolumar et al., 2014) myofibrillar fragmentation alongside the Z-disk (Zuckerman and Solomon, 1998), destruction of collagen fibrils of the endomysium (Zuckerman et al., 2013) and degradation of C-protein, which is responsible for the integrity of the thick filaments (Spanier and Fahrenholz, 2005a). The physical disruption of muscles may lead to the release of endogenous proteases such as calpains and cathepsins, or their activators such as calcium ions, thus enhancing the tenderization effect. This was seen in a study where troponin T of shockwave-treated samples was degraded more than in an untreated control after ageing (Bowker et al., 2008a,b). Conversely, no enhancement in the activity of endogenous proteolytic enzymes (cathepsins and peptidases) was detected after the shockwave process, suggesting that the tenderization effect is mainly due to physical disruption. The effect of shockwave processing on the protein profile is debatable. It has been reported that there was no distinct difference in the myofibrillar, sarcoplasmic and stromal protein profiles between control and shockwave-treated samples (Bolumar et al., 2014; Schilling et al., 2002). However, it has also been reported that shockwave-treated muscle had higher myofibrillar solubility (Bowker et al., 2008a,b; Spanier and Fahrenholz, 2005a, 2005b) and increased collagen solubility.

### High Pressure Processing

HPP is one of the technologies applied in the meat industry to improve shelf life, safety, and quality characteristics (texture and color) of foods (Bajovic et al., 2012). HPP subjects the meat to high pressure, usually ranging from 100 MPa to 800 MPa, via a surrounding liquid and is sometimes accompanied by heat treatment at 60 °C (Hopkins, 2014; Troy et al., 2016). HPP causes the destruction of microorganism, leads to protein denaturation and inactivation of endogenous enzymes (Sikes and Warner, 2016; Torres and Velazquez, 2005).

HPP alters protein secondary, tertiary and quaternary structures in the muscles by disrupting their conformations and molecular interactions (Campus, 2010; Sikes and Warner, 2016; Strasburg et al., 2008). For example, at pressures above 100 MPa protein tertiary structure unfolds and protein aggregation occurs (Cheftel and Culioli, 1997). However, covalent bonds are mostly unaffected. High pressure has been reported to denature myofibrillar proteins (Anderson and Parrish, 1989), cause depolymerization of actin (Ikkai and Ooi, 1966), and release cathepsins into the cytoplasm by degrading muscle membranes (Homma et al., 1994; Jung et al., 2000). For example, at pressures between 100 MPa and 300 MPa, myosin and actin were denatured (Angsupanich and Ledward, 1998; Sikes et al., 2010). The effect of high pressure on muscle structure depends on the applied pressure, processing temperature and time, and the muscle type (pre- or post-rigor) (Cheftel and Culioli, 1997). For instance, increases in

intermyofibrillar spaces and myofibril shrinkage were observed in Atlantic salmon meat with increasing applied pressure (from 400 to 900 MPa) and time (Gudbjornsdottir et al., 2010).

The tenderness of pre-rigor meat can be improved by HPP (Sikes and Warner, 2016). Accelerated glycolysis of pre-rigor meat may occur when high pressure is applied, resulting in shear force reduction of myofibrillar-based food (Elgasim and Kennick, 1982; Hopkins, 2014; Kennick et al., 1980). The effect of high pressure on the texture of post-rigor meat depends on the processing temperature (Sikes and Warner, 2016). In most studies, high pressure treatment at 30 °C or below did not have a tenderization effect in post-rigor meat. The tenderness of post-rigor sheep muscle (Macfarlane et al., 1981; Macfarlane and Morton, 1978) and chicken breast (Kruk et al., 2011; Zamri et al., 2006) remained unchanged after high pressure, low temperature processing. In contrast, high pressure processing combined with heat treatment was effective in improving meat tenderness (Bouton et al., 1977; Rusman et al., 2007; Sikes et al., 2010).

High pressure processing affects the color of meat (Cheftel and Culioli, 1997). At pressures higher than 400 MPa, the oxidation of heme iron and denaturation of myoglobin in raw meat occur and cause an increase in lightness and decrease in the redness of meat. This increase in lightness was also observed in bluefish *Pomatomus saltatrix* as the applied pressure increased from 100 to 300 MPa (Ashie and Simpson, 1996).

## Effects of Cooking on Meat Proteins

### Sous Vide Cooking

Sous vide is a culinary technique developed in fine dining restaurants, where food is sealed under vacuum and cooked at a controlled temperature, usually in a water bath (Baldwin, 2012; Schellekens, 1996). Cooking in this way results in uniform and efficient heat transfer from the water to the food, overcoming the drawback of uneven heating encountered in conventional cooking processes, while retaining water, soluble components and volatiles in the food and avoiding oxidation. During the cooking process, heat denatures proteins, leading to physical changes in the meat. The effect of cooking on muscle texture largely depends on the cooking time and temperature as well as the heating rate.

The myofibrillar proteins (mostly myosin and actin) shrink during heating, causing the contraction and shrinkage of the muscle fibers (Baldwin, 2012; Christensen et al., 2011a,b). When meat is subjected to heat from 40 °C to 60 °C, muscle fibers shrink transversely which causes a widening of the gap between them (Palka and Daun, 1999; Tornberg, 2005). As the temperature further increases, muscle fibers shrink longitudinally, and the water held between thick and thin filaments is expelled. The sarcoplasmic proteins start to aggregate and gel when heated at 40 °C to 60 °C (Baldwin, 2012). At sous vide cooking temperatures, most of the endogenous proteases remain active and contribute to enhancing the tenderness of the meat (Laakkonen et al., 1970a,b). At 55 °C, cathepsins B and L were found to be active during up to 24 hours of cooking, whereas both  $\mu$ - and *m*-calpains were inactivated within 10 minutes (Ertbjerg et al., 2012). Connective tissues, such as collagen, denature at around 60 °C (Baldwin, 2012). This leads to the formation of water-soluble random-coiled gelatin, decreasing the adhesion between muscle fibers. Collagen in beef perimysium was observed to melt when cooked at 50 to 60 °C, while elastin remained heat stable at 100 °C (Taylor, 2004). DSC analysis showed that prolonged heating at 53 °C (for 19.5 hours) resulted in diminishing of myosin and collagen peaks of meat from bull Semitendinosus muscle in the thermograph, suggesting potentially improved meat tenderness (Christensen et al., 2013).

The temperature of sous vide cooking should be set where it is high enough for collagen solubilization and microbiological inactivation, yet has minimum myofibrillar shrinkage to achieve optimum tenderization action (Ruiz et al., 2013). When a tough meat cut is cooked at a temperature between 55 °C and 60 °C for 24 hours, the tenderness is improved as the collagen is converted to gelatin and the myofibrillar proteins are hydrolyzed by the endogenous proteolytic enzymes (Bouton and Harris, 1981; Tornberg, 2005). A decrease in shear force was observed with an increase in cooking temperature from 50 °C to 65 °C, but the shear force increased when the temperature exceeded 65 °C and up to 80 °C (Baldwin, 2012). It has been suggested that the reduction in shear force is due to the gel formed from the sarcoplasmic protein, which filled the channels between fiber bundles, resulting in a decrease in elastic modulus that requires lesser tensile stress to fracture the meat (Tornberg, 2005).

### Other Cooking

Cooking of meat other than sous vide does not have the same precise control of temperature and generally exposes at least part of the meat to much higher temperatures than encountered in sous vide cooking, although other parts may be hardly heated at all (for example in the center of a "blue" steak). Chefs and consumers often judge the "doneness" of meat by its color, and this is mostly due to the degree of heme oxidation of myoglobin. During the cooking process, deoxymyoglobin, metmyoglobin and oxymyoglobin undergo oxygenation, oxidation and reduction reactions, and the ratio between them determines the color of final product (Liu and Chen, 2001). The color of the cooked meat also depends on the rate in achieving the designated core temperature and the duration held at that temperature. Cooked meat tends to be redder when the rate of heating is faster and paler when it is held at specific temperatures longer (Baldwin, 2012).

Important temperature effects are summarized in Table 2 (McGee, 2004).

**Table 2** Effects of heat on meat proteins and qualities

Temperature (°C)	Meat qualities	Proteolytic enzymes	Fibrillar proteins	Collagen	Protein-bound water	Myoglobin status
40	Soft Smooth Translucent	Active	Beginning to unfold		Begins to escape	
50	Firming Becoming opaque	Very active	Myosin begins to denature			
55	More fibrous, Juicy when cut	Denature and lose activity	Myosin coagulated			
60	Shrinking Losing resilience Losing juice Pink		Other proteins denature	Collagen sheaths shrink, squeeze cells	Flows from cells under collagen pressure	Begins to denature ("medium" doneness)
70	Shrinking Stiff Little juice Gray-brown			Begins to denature and dissolve	Flow ceases	
80			Actin denatures Cell contents compacted			

Adapted from McGee, H., 2004. *On Food and Cooking – The Science and Lore of the Kitchen*, second ed. Scribner, New York.

## Nutritional Value and Digestion

Muscle-based food is an excellent source of protein nutrition. The compositions of lean tissue in muscle-based foods are consolidated in **Table 3** (Foegeding et al., 1996; Strasburg et al., 2008; Williams, 2007). About 17% to 23% of lean muscle is made up of protein that contains all the dietary essential amino acids. Consuming meat together with plant-based foods, such as cereals and legumes, can compensate for the lower levels of lysine (cereals), and sulfur amino acids (legumes) in the diet (Bodwell and Anderson, 1986). Protein from meat is highly digestible and bioavailable compared to plant protein (Černá, 2011), except after severe processing which can modify amino acid side chains (Soladoye et al., 2015). Muscle-based foods, especially red meats, are a rich source of highly bioavailable iron, due to their myoglobin content.

Protein digestibility, which is largely dependent on the source and processing history of food, is considered to be an important factor determining the amount of undigested dietary protein reaching the large intestine (Yao et al., 2016). It has been reported that increasing cooking temperature and time can decrease meat protein digestibility (Astruc, 2014b; Li et al., 2017; Kaur et al., 2014). High pressure-treated meat was found to be more digestible than the untreated control sample, both in vitro (Kaur et al., 2016) and in vivo (Elgasim and Kennick, 1980). Long-time salting and drying rendered the meat protein to be less digestible by pepsin in the stomach (Li et al., 2017).

## Sustainability of Meat as a Source of Protein

### Real Cost of Meat, Water and Carbon Footprints

There has been much comment on the environmental cost of producing meat. A commonly quoted "fact" is that it takes 10 kg of plant protein to produce 1 kg of meat protein. This is a worst-case figure and largely incorrect because:

**Table 3** Composition of lean muscle tissues

Species	Proximate composition (%)			
	Water	Protein	Lipid	Ash
Beef <sup>a,b,c</sup>	70–73	20–23	3–8	1
Pork <sup>b,c</sup>	68–70	19–20	9–11	1.4
Chicken <sup>b,c</sup>	73.7	20–23	4.7	1
Lamb <sup>a,b,c</sup>	73	20–22	5–6	1.6
Cod (lean fish) <sup>b,c</sup>	81.2	17.6	0.3	1.2
Salmon (fatty fish) <sup>b,c</sup>	64	20–22	13–15	1.3

<sup>a</sup>Williams (2007).

<sup>b</sup>Strasburg et al. (2008).

<sup>c</sup>Foegeding et al. (1996).

- It assumes the animal is eating food that could otherwise have been used by humans
- It ignores other contributions made by the animal – often a lifetime of milk or egg production or of providing motive power, as well as the other products from the carcass – offal, hides and gelatin, for example
- Animals are often farmed on land that is too steep or inaccessible to use for cropping, thus any opportunity cost for land use is, in the case of these animals, illusory
- In developing countries (and increasingly in developed countries) animals are fed on waste food that is no longer fit for human consumption.

Production of meat requires a lot of water. Meat animals are often farmed in regions where there is an excess of water available through plentiful rainfall – where no opportunity cost for water exists. Water management will be an increasingly important consideration for all kinds of farming. Farming for animal products, including meat, is appropriate under certain conditions.

The carbon footprint of meat production arises mostly because of production of methane by the animals during their lifetime. Methanogenesis occurs mostly in the rumen from the metabolism of methanogenic bacteria in the rumen population. It is generally undesirable, not only because it produces atmospheric methane, but because it results in lost energy and carbon that could otherwise benefit the animal. Research is underway globally to address this issue and there is hope that in the future ruminants will be able to be largely methanogen-free.

### Importance of the Ruminant

It is a simple fact that almost all food must originate from plant material, because photosynthesis is the ultimate source of almost all organic material. The route by which this material can become food for humans depends on the palatability and digestibility of the plant material. Ruminant animals, by virtue of their completely different digestive systems, can consume plant material such as grasses and the leaves of bushes and trees and convert it into forms that can be valuable nutrition for humans (milk and meat). Without the intervention of the animal, these food sources would be lost to us.

### The Future of Meat

The future of meat and the prospects of meeting future demand for meat and other animal-based sources of protein have been addressed by several groups, and potential solutions include more production through intensification and increasing grasslands (at an environmental cost that may be unacceptable), recycling food waste to feed animals and using alternative sources of animal fodder, such as insects. Laboratory-grown meat has also made an appearance, albeit only as a prototype, but may be expected to have an influence in the future, as will plant-origin meat substitutes, but meat will continue to occupy an important place in the planet's food ecosystem.

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# Natural Antioxidants in Foods

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## Glossary

**Antioxidant** A compounds that can inhibit oxidative processes.

**Free radical** A compound with an unpaired electron that can promote oxidative reaction.

**Free radical scavenger** A compound that can absorb a free radical to decrease the radical energy thus making it less likely to cause oxidation.

**Hydrogen atom transfer** Antioxidant transfer hydrogen to the lipid radicals.

**Metal chelator** Compounds that can bind metals and decrease their reactivity.

**Phenolic compounds** A group of chemical compounds found primarily in plants that act as antioxidant and are beneficial to health.

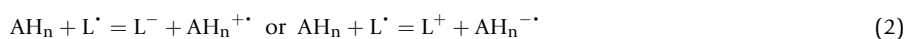
## Overview

Atmospheric oxygen is a low energy biradical (i.e. contains 2 unpaired electrons) in its ground triplet state. However, alterations can occur to produce highly reactive oxygen species (ROS) during metabolism of oxygen. This can cause oxidation of food constituents, including lipids, pigments, vitamins and proteins leading to off-flavor formation, discoloration and loss of important nutrients. Foods, which are derived from a variety of different biological tissues, contain a host of different antioxidant defense systems to prevent the damaging effect of reactive oxygen species. However, during the processing of biological tissues into foods, the formation of oxidizing species can increase and antioxidant systems can be overwhelmed leading to uncontrolled oxidative reactions resulting in loss of quality, decrease in shelf-life, and formation of potentially toxic oxidation products. In order to protect food quality and safety, antioxidants are often added to processed foods. These antioxidants can be synthetically derived compounds such as butylated hydroxytoluene (BHT) and ethylene diaminetetraacetic acid (EDTA). Concern over the use of synthetic food additives and customer's movement toward clean-label have driven the food industry to find effective natural antioxidants additives that are derived from biological sources.

In addition to the association of natural antioxidants with food quality, these compounds have also been associated with health benefits. The association of the protective effect of fruits and vegetable in the diet against metabolic diseases has been established for years. The consumption of an ample supply of fruits and vegetables provides a wide variety of phytochemicals that have been shown to have health benefits and antioxidant activity. In the context, natural antioxidants to protect biological system or food quality induced by free radicals, transition metals and singlet oxidation are reviewed.

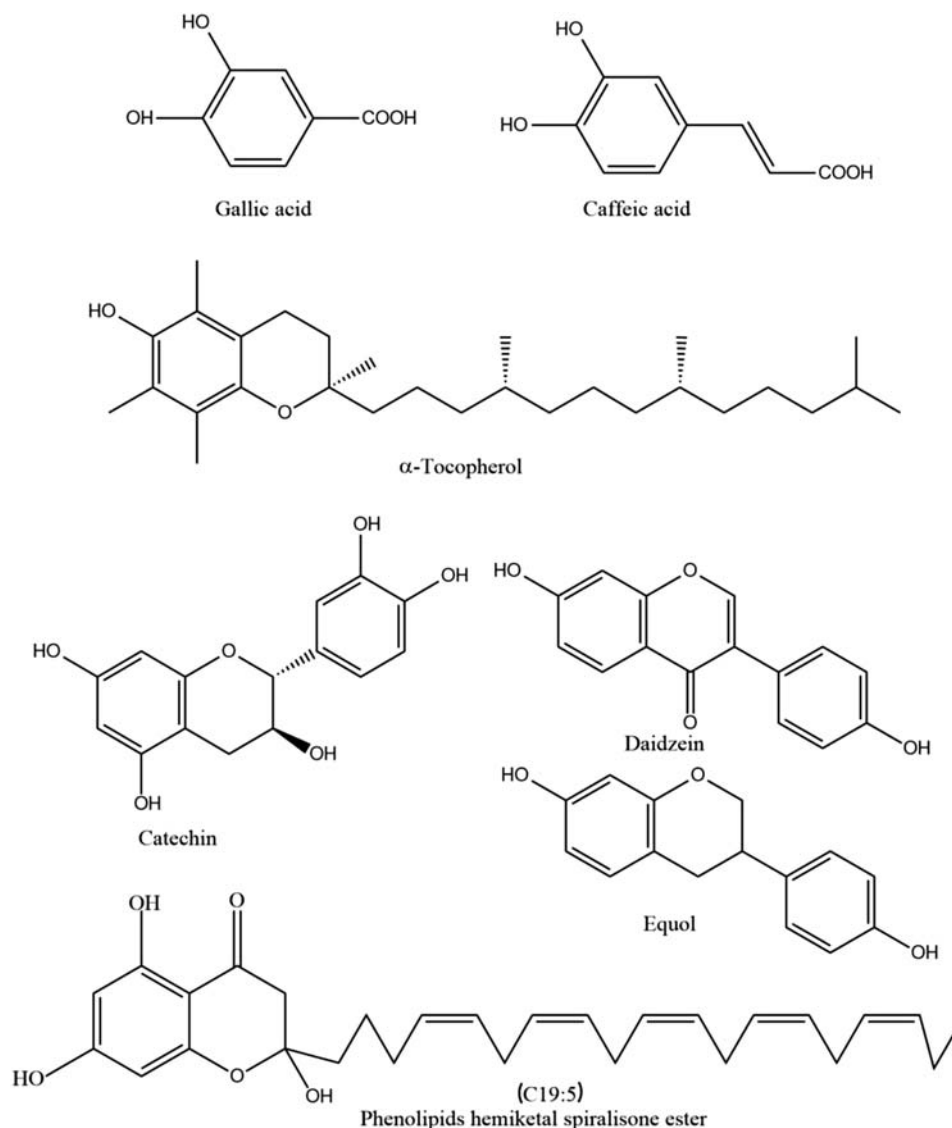
## Free Radical Scavenger

Radical scavenging capacity of natural antioxidant can be classified as two major mechanisms: hydrogen atom transfer (HAT) and electron transfer (ET). Natural antioxidants possessing such capacity is named free radical scavenger (FRS). HAT means antioxidant transfer hydrogen to the lipid radicals for suspending the propagation step of lipid oxidation such as the mechanism of phenolic compounds (Eq. 1). Bond dissociation enthalpies (BDEs) are used to explain the principle of HAT. Lower BDEs of the antioxidant O—H bond are easier to transfer hydrogen, thus have a faster hydrogen transfer reaction. ET-based antioxidant reaction is a redox reaction with the transfer of electron (Eq. 2), such as carotenoids with  $\cdot\text{NO}_2$  and  $\cdot\text{CCl}_3\text{OO}$ , and catechin analogs with peroxy radicals ( $\cdot\text{OH}$ ). One electron reduction potentials ( $E^\circ$ ) is a typical parameter for indicating the capability antioxidants for electron transfer. The lower  $E^\circ$ , the easier the natural antioxidants to transfer electron. Generally, natural antioxidants always involve both HAT and ET mechanisms.



## Phenolic Antioxidants

Phenolics are compounds that have a hydroxyl group associated with an aromatic ring structure. There are numerous variations of natural phenolics (see Fig. 1 for examples). Natural phenolics are found predominately in the plant kingdom. Vitamin E or



**Figure 1** Chemical structures of some examples of phenolic antioxidants.

$\alpha$ -tocopherol is a plant phenolic required in the diet of humans and other animals. Phenolic compounds primarily inhibit lipid oxidation through HAT mechanism to scavenge free radicals and convert the resulting phenolic radicals into a low energy form that does not further promote oxidation. Physical partitioning of phenolics will also influence their reactivity. Initially, antioxidant efficiency is dependent on the ability of the free radical scavenger to donate a hydrogen to a high energy free radical. As the oxygen-hydrogen bond dissociation energy (BED) of the free radical scavenger decreases, the transfer of the hydrogen to the free radical is more energetically favorable and thus more rapid. The ability of a FRS to scavenge a free radical can sometimes be predicted from one electron reduction potentials ( $E^{\circ'}$ ). If a compound has a reduction potential lower than the reduction potential of a free radical found in a food or biological tissue (e.g. fatty acid based peroxy radical), it can donate hydrogen to that free radical unless the reaction is kinetically unfeasible. For example, FRS including  $\alpha$ -tocopherol ( $E^{\circ'} = 500$  mV), urate ( $E^{\circ'} = 590$  mV), catechol ( $E^{\circ'} = 530$  mV) and ascorbate ( $E^{\circ'} = 282$  mV) all have reduction potentials below peroxy radicals ( $E^{\circ'} = 1000$  mV, a common free radical in lipid oxidation reactions) and therefore can convert the peroxy radical to a hydroperoxide through hydrogen donation.

The efficiency of an antioxidant FRS is also dependent on the energy of the resulting antioxidant radical. If a FRS produces a low energy radical then the likelihood of the FRS radical to promote the oxidation of other molecules is lower and the oxidation reaction rate decreases. Phenolics are effective FRS because phenolic free radicals have low energy due to delocalization of the free radical throughout the phenolic ring structure. Standard reduction potentials can again be used to help illustrate this point. Radicals on  $\alpha$ -tocopherol ( $E^{\circ'} = 500$  mV) and catechol ( $E^{\circ'} = 530$  mV) have lower reduction potentials than polyunsaturated fatty acids ( $E^{\circ'} = 600$  mV) meaning that their radicals do not possess high enough energy to effectively promote the oxidation of unsaturated fatty acids. Effective phenolic antioxidants FRS also produce radicals that do not react rapidly with oxygen to form hydroperoxides



that could autoxidize, thus depleting the system of antioxidants. Instead, antioxidant radicals may undergo additional reactions that remove radicals from the system such as reactions with other antioxidant radicals or lipids radicals to form nonradical species through electron transfer (ET) mechanism. This means that each FRS is capable of inactivating at least of two free radicals, the first being inactivated when the FRS interacts with the initial oxidizing radical and the second when the FRS radical interacts with another radical via a termination reaction to form a nonradical product.

Phenolic compounds that act as antioxidants are wide spread in the plant kingdom. Plant phenolics can be classified as simple phenolics, phenolic acids, hydroxycinnamic acid derivatives, and flavonoids. The consumption of natural plant phenolics have been estimated to be up to 1 g per day. Overall, the presence of phenolics in the diet has been positively associated with the prevention of diseases such as cancer and atherosclerosis. Plant foods high in phenolics include cereals, legumes and other seeds (e.g. sesame, oats, soybeans and coffee); red, purple and blue colored fruits (e.g. grapes, strawberries and plums); and the leaves of herbs and bushes (e.g. tea, rosemary and thyme). Many natural phenolics are capable of inhibiting oxidative reactions. However, since phenolics have such a wide array of chemical structures, it is not surprising that antioxidant activities and health benefits vary greatly. This section will focus on the best studied natural phenolics from plant.

Tocopherols and tocotrienols are a group of lipophilic monophenolic FRS ( $\alpha$ ,  $\beta$ ,  $\delta$  and  $\gamma$ ; see Fig. 1 for the structure of  $\alpha$ -tocopherol) originating in plants and eventually ending up in animal foods via the diet. Interactions between tocopherols and fatty acid peroxy radicals lead to the formation of fatty acid hydroperoxides and several resonance structures of tocopheroxyl radicals, which can further interact with other compounds or with each other to form a variety of products. The types and amounts of these products are dependent on oxidation rates, radical species, lipid state (e.g. bulk vs. membrane lipids) and tocopherol concentrations. Tocopherol is found in plant foods especially those high in oil. Soybean, corn, safflower, and cottonseed oil are good sources of  $\alpha$ -tocopherol as are whole grains (in particular wheat germ) and tree nuts. All tocopherol isomers are absorbed by humans, but  $\alpha$ -tocopherol is preferentially transferred from the liver to lipoproteins which in turn transports  $\alpha$ -tocopherol to tissues. For this reason,  $\alpha$ -tocopherol is the isomer most highly correlated with Vitamin E activity.

Tea is an important source of dietary antioxidants for humans since it is one of the most common beverages in the world. Phenolics in tea are mainly catechin derivatives, including catechin (Fig. 1), epicatechin (EC), epicatechin gallate (ECG), gallic catechin (GC), epigallocatechin gallate (EGCG) and epigallocatechin (EGC). Tea originates from leaves harvested from the bush, *Camellia sinensis*. Processing of tea leaves involves either blanching to produce green tea or fermenting to produce oolong or black tea. Green tea leaf extracts contain 38.8% phenolics on a dry weight basis with catechins contributing over 85% of the total phenolics. Condensation of catechins can decrease their solubility; therefore, black tea extracts contain less phenolics (24.4%) of which 17% are catechins and 70% are condensed polyphenols (theaflavins). Ingestion of dietary phenolics from tea has been associated with cancer prevention.

Grapes and wines are also significant sources of dietary phenolic antioxidants. Grapes contain a wide variety of monomeric phenolics including anthocyanins, flavan-3-ols, flavonols (quercetin and rutin), and cinnamates (S-glutathionylcaftaric acid). As with many fruits, the majority of grape phenolics are found in the skin, seeds and stems. During extraction of juice, the pomace is left in contact with the juice for varying times in order to produce products of varying color, with increasing contact time resulting in increased phenolic extraction and thus darker color. Therefore, white grape juices and wines have lower phenolics contents (119 mg gallic acid equivalents/L) than red wines (2057 mg gallic acid equivalents/L). As would be expected, red grape juice and wines have greater antioxidant capacity due to their higher phenolic content. Both grape juice and wines have been suggested to have positive health benefits, however, their phenolic compositions are not the same due to differences in juice preparation and changes in phenolic composition which occurs during both fermentation and storage.

Isoflavones is the primary phenolics in soybeans. Included among the soybean isoflavones are daidzein (Fig. 1), genistein and glycitein and their glycosylation forms. Unlike the phenolics in tea and grapes, soybean isoflavones are associated with proteins and therefore are found in soy flour and not in soybean oil. The concentration of isoflavones in soybeans varies with the environmental conditions under which the beans were grown. In addition, isoflavone concentrations in soy-based foods are altered during food processing operations such as heating and fermentations. Besides whole soybeans, isoflavones are found in soy milk, tempeh, miso and tofu at concentrations ranging from 294–1625  $\mu\text{g/g}$  product. Genistein and daidzein are absorbed into human plasma from products such as tofu and soy milk. Recent research suggested that it is equol (Fig. 1), the metabolized daidzein by human intestinal microflora rather than daidzein itself to contribute to the beneficial effects of soy foods in preventing cardiovascular disorders.

Herbs and spices often contain high amount of phenolic compounds. For example, rosemary contains carnosic acid, carnosol and rosmarinic acid. Crude rosemary extracts are a commercially important source of natural phenolic antioxidant additives in foods meats, bulk oils, lipid emulsions and beverages.

## Phenolipids

Phenolic compounds combined with lipids are called phenolipids or lipophenols acting as oil soluble free radical scavengers. Due to its nature, it has improved solubility in oils compared to phenolics. The health benefits of phenolipids are anti-inflammatory, antiallergic, antimicrobial, antiviral, and anticarcinogenic, which are much the same as common phenolics. That is because the main functional groups are phenolics that still rely on the ability of donating electrons or hydrogen atoms. It means that their antioxidant ability depends on the number and position of the phenolic hydroxyls, the presence of some phenolic ring substituents and the electronic delocalization area.



Although the chemical properties of the phenolipids are much the same as phenolics, the physical properties are different. The lipid part confers special physical properties to the phenolipids, which make it not only react with polar compounds with phenolic aglycone, but also can react with nonpolar compounds through hydrophobic parts.

The natural sources of phenolipids are mainly from marine organisms ranging from 0.1%–15%. They can be related to analogs of n-3 PUFA phenolipids, since phenolics are linked to n-3 unsaturated carbon chain (C11–C21) with double bonds separated by bis-allylic positions. Hemiketal spiralisone (Fig. 1) and chromone isolated from brown algae *Zonaria spiralis*, acylphloroglucinol fatty acid esters from brown algae *Z. tournefortii*, and 5-alkenylresorcinol fatty acid esters from *Cystophora torulosa* have been reported recently. As to the marine animals, the 5-alkenylresorcinols fatty acid esters can also be extracted from the sponge *Haliclona* sp. As regard to the plants on the land, macamide, anacardic, 5-alkenylresorcinols and Z-*p*-coumaryl fatty acid esters can be extracted from Peruvian plant *Lepidium meyenii* (Maca), *Philodendron scadens* subsp. *Oxycardium*, liverwort *Omphalantus filiformis*, and cv. *Annurca* apple fruits, respectively. What's more, the phenolipids have been isolated from microorganism, such as 5-alkenylresorcinols fatty acid esters from heterotrich ciliate *Climacostomum virens*.

### Ascorbate

Ascorbic acid (vitamin C; Fig. 2) acts as a water-soluble free radical scavenger in both plant and animal tissues. Like phenolics, ascorbate ( $E^{\circ'} = 282$  mV) has a reduction potential below peroxy radicals ( $E^{\circ'} = 1000$  mV) and thus can inactivate peroxy radicals. In addition, its reduction potential is lower than the  $\alpha$ -tocopherol radical ( $E^{\circ'} = 500$  mV) meaning that ascorbate may have an additional role in the regeneration of oxidized  $\alpha$ -tocopherol. Interactions between ascorbate and free radicals result in the formation of numerous oxidation products. While ascorbate seems to primarily play an antioxidant role in living tissues, this is not always true in food systems. Ascorbate is a strong reducing agent especially at low pH. When transition metals are reduced they become very active prooxidants that can decompose hydrogen and lipid peroxides into free radicals. Ascorbate also causes the release of protein-bound iron (e.g. ferritin), thus promoting oxidation. Therefore, ascorbate can potentially exhibit prooxidative activity in the presence of free transition metals or iron-binding proteins. This does not typically occur in living tissues due to the tight control of free metals by systems that prevent metal reduction and reactivity.

Ascorbate is found in numerous plant foods including green vegetables, citrus fruits, tomatoes, berries and potatoes. Ascorbate can be lost in foods due to heat processing and prolonged storage. Transition metals and exposure to air will also cause the degradation of ascorbic acid.

### Thiols

#### Glutathione

Glutathione (Fig. 2) is a tripeptide ( $\gamma$ -glutamyl-cysteinyl-glycine) where cysteine can be in either the reduced or oxidized glutathione state. Reduced glutathione inhibits lipid oxidation directly by interacting with free radicals to form a relatively unstable sulfhydryl radical or by providing a source of electrons which allows glutathione peroxidase to enzymically decompose hydrogen and lipid peroxides. Total glutathione concentrations in muscle foods range from 0.7–0.9  $\mu\text{g/kg}$ . The bioavailability of glutathione in rats has also been reported to be low. Lack of, or low absorption of glutathione may be due to the hydrolysis of the tripeptide by gastrointestinal protease.

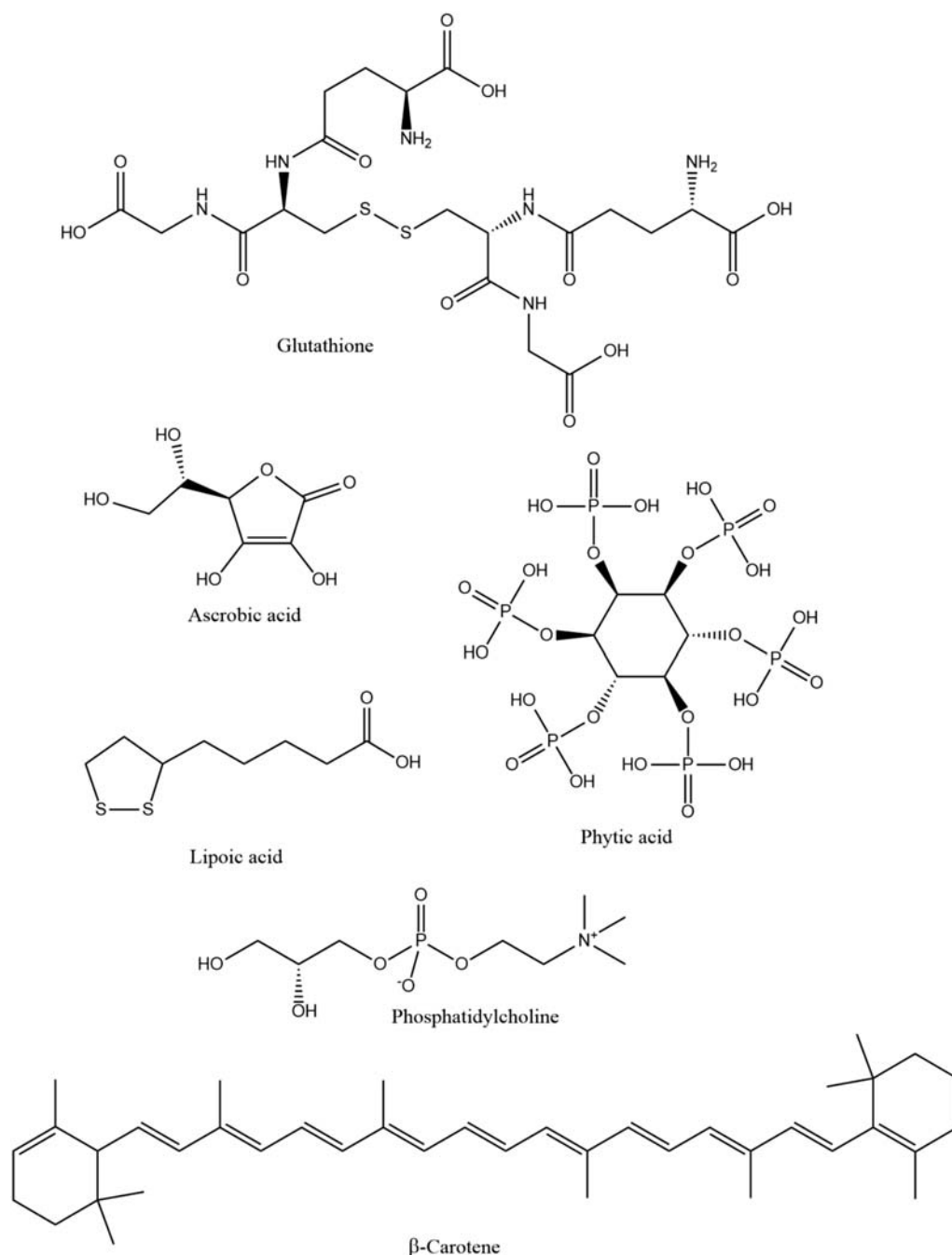
#### Lipoic Acid

Lipoic (thioctic) acid (Fig. 2) is a thiol cofactor for many plant and animal enzymes. In biological systems, the 2 thiol groups of lipoic acid are found in both reduced (dihydrolipoic acid) and oxidized forms (lipoic acid). Both the oxidized and reduced forms of the molecule are capable of acting as antioxidants through their ability to scavenge free radicals. Other than that, they can also quench singlet oxygen, chelate iron and possibly regenerate other antioxidants such as ascorbate and tocopherols. Lipoic and dehydrolipoic acids can protect LDL, erythrocytes and cardiac muscle from oxidative damage.

While lipoic acid has been found in numerous biological tissues, reports on its concentrations in foods are scarce. Lipoic acid is detectable in wheat germ (0.1 ppm) but not in wheat flour and it has been detected in bovine liver kidney and skeletal muscle. Oral administration of lipoic acid (1.65 g/kg fed) to rats for 5 weeks resulted in elevated levels of the thiol in liver, kidney, heart and skin. When lipoic acid was added to diets lacking in vitamin E, symptoms typical of tocopherol deficiency were not observed suggesting that lipoic acid acts as an antioxidant *in vivo*. However, lipoic acid was not capable of recycling vitamin E *in vivo* as determined by the fact that  $\alpha$ -tocopherol concentrations are not elevated by dietary lipoic acid in vitamin E deficient rats.

### Phospholipids

Phospholipids consist of two hydrophobic "tails," which are fatty acid chains, and one hydrophilic "head," which is phosphate group. They connect with glycerol and the "head" is typically found at the sn-3 position. With the amphiphilic structure, phospholipids can form bilayers on the cell membrane, which can be found in all living species. Lecithin, a commercially and widely available product, is a mixture of phospholipids. It mainly contains phosphatidylcholine (PC, Fig. 2), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and phospholipid-phosphoric acid (PA). In addition, lysophospholipids, whose fatty acid chain has been substituted by hydroxy group from either sn-1 or sn-2 position, is also a kind of phospholipids.



**Figure 2** Chemical structures of miscellaneous natural antioxidants.

Phospholipids are reported as natural antioxidants extracted from both animals and plants. The main principle of antioxidant ability can be classified as radical scavenging, synergistically working with other antioxidants, and also metal chelating. Phospholipids such as PE have a primary amine group, a necessary substrate for Maillard reaction. Primary amine group conducts Maillard reaction with carbonyl group from aldehydes or ketones, which are the secondary oxidation products of lipid oxidation. This is the first antioxidant mechanism that phospholipids participate in Maillard reaction. Then, Maillard reaction products such as browning pigments and melanoidin possess high free radical scavenging capability, which can inhibit lipid oxidation from the initiation and propagation steps. In addition, phospholipids can work as synergist with other antioxidants with the mechanism than alteration of location of other antioxidants and regeneration of antioxidant (e.g. tocopherol). In addition, regeneration of other antioxidants also plays an important role for phospholipids functioning as an antioxidant. Metal chelating ability is another antioxidant mechanism of phospholipids. In general, negative charged phosphate head group can react with metal

cations to inhibit lipid oxidation. It was reported that phospholipids acted as metal chelator where 1 ppm ferrous iron was added into bulk oil system.

Although phospholipids are proved to own antioxidant activity with various principles, reaction condition should be controlled to prevent phospholipids become prooxidants. Unlike common antioxidants, polyunsaturated fatty acids are present in phospholipids, which means the phospholipids itself can be oxidized. In addition, phospholipids, as an emulsifier, can increase interaction between hydrophilic and lipophilic compounds by aggregating them on the interface. As a result, initiation and propagation steps of lipid oxidation may be accelerated.

As phospholipids are an essential part of biological membranes, phospholipids can be extracted from all kinds of living plants or animals. The major phospholipids originated from animals are eggs, meats, fishes and milk. Among them, chicken whole eggs contain the highest proportion of phospholipids about 35 g/kg. PC is the main composition of animal phospholipids. Phospholipids from marine animals owning higher omega-3 polyunsaturated fatty acids, have higher demand than warm-blooded animals although they have same concentration of phospholipids. The major phospholipids originated from plants are soybean, corn germ, rapeseed, peanut, and wheat starch. Soybean with the phospholipids content of around 20 g/kg is higher than other plant sources.

## Singlet Oxygen Quencher

### Carotenoids

Carotenoids are a chemically diverse group (>600 different compounds) of yellow to red colored polyenes consisting of 3–13 conjugated double bonds and in some cases 6 carbon hydroxylated ring structures at one or both ends of the molecule.  $\beta$ -Carotene is the most extensively studied carotenoid antioxidant (Fig. 2). The major antioxidant function of carotenoids in foods is its ability to inactivate singlet oxygen. Singlet oxygen is an excited state of oxygen in which two electrons in the outer orbitals have opposite spin directions. Initiation of lipid oxidation by singlet oxygen is due to its electrophilic nature that will allow it to add to the double bonds of unsaturated fatty acids leading to the formation of lipid hydroperoxides. Carotenoids can inactivate singlet oxygen by both chemical and physical quenching. Chemical quenching results in the direct addition of singlet oxygen to the carotenoid leading to the formation of carotenoid breakdown products and loss of antioxidant activity. A more effective antioxidative mechanism of carotenoids is physical quenching. The most common energy states of singlet oxygen are 22.4 and 37.5 kcal above ground state. Carotenoids physically quench singlet oxygen by a transfer of energy from singlet oxygen to the carotenoid resulting in an excited state of the carotenoid and ground state, triplet oxygen. Energy is then harmlessly transfer from the excited state of the carotenoid to the surrounding medium by vibrational and rotational mechanisms. Nine or more conjugated double bonds are necessary for physical quenching with the presence of six carbon oxygenated ring structures at the end the molecule increasing the effectiveness of singlet oxygen quenching.

In foods, light will activate chlorophyll, riboflavin and heme-containing proteins to high energy excited states. These photoactivated molecules can promote oxidation by direct interactions with an oxidizable compounds to produce free radicals, by transferring energy to triplet oxygen to form singlet oxygen or by transfer of an electron to triplet oxygen to form the superoxide anion. Carotenoids inactivate photoactivated sensitizers by physically absorbing their energy to form the excited state of the carotenoid that then returns to the ground state by transfer of energy into the surrounding solvent.

## Metal Chelators

### Organic Acids

Transition metals will promote oxidative reactions by hydrogen abstraction and by hydroperoxide decomposition reactions that lead to the formation of reactive free radicals. Prooxidative metal reactivity is inhibited by chelators. Chelators that exhibit antioxidative properties inhibit metal-catalyzed reactions by one or more of the following mechanisms, i.e. prevention of metal redox cycling; occupation of all metal coordination sites thus inhibiting transfer of electrons; formation of insoluble metal complexes; steric hindrance of interactions between metals and oxidable substrates (e.g. peroxides).

Natural organic acids are employed in the food industry as chelating agents, such as polycarboxylic acids (citric, malic, tartaric, oxalic, and succinic), and phosphoric acids (adenosine triphosphate, and pyrophosphate). The prooxidative/antioxidative properties of a chelator can often be dependent on the amounts of functional groups such as  $-\text{OH}$ ,  $-\text{SH}$ ,  $-\text{COOH}$ ,  $-\text{PO}_3\text{H}_2$ ,  $\text{C}=\text{O}$ ,  $-\text{NR}_2$ ,  $-\text{S}-$  and  $-\text{O}-$ . The metal chelating capability of citric acid, which contains three carboxylic groups, is larger than that of oxalic acid, which only contain two carboxylic groups. In addition, chelator activity is pH dependent with pH below the  $\text{pK}_a$  of the ionizable groups resulting in protonation and loss of metal binding activity. Chelator activity is also decreased in the presence of high concentrations of other chelatable nonprooxidative metals (e.g. calcium) which will compete with the prooxidative metals for binding sites. Organic acids are typically water-soluble but many also exhibit some solubility in lipids (e.g. citric acid) thus allowing it to inactivate metals in the lipid phase. According to the solubility in different solvents, organic acids can synergistically work with radical scavengers. For instance, tocopherol, rosemary extract and citric acids have a synergic effect in sunflower oil.

Organic acid is widely distributed in all kinds of fruits and vegetables, especially citrus fruits, such as pineapples, lemons, oranges and limes. Consequently, vinegars made from these fruits often contain organic acids (e.g. citric, malic, lactic, and tartaric acids).

What's more, fermentation with microorganism is commercially available to produce organic acid, such as fermenting *Aspergillus niger* to produce citric acid.

### Peptides and Proteins

The reactivity of prooxidant metals in biological tissues are mainly controlled by proteins and peptides. Metal binding proteins in foods include transferrin (blood plasma), phosvitin (egg yolk), lactoferrin (milk) and ferritin (animal tissues). Transferrin, phosvitin and lactoferrin are structurally similar proteins consisting of a single polypeptide chain with a molecular weight ranging from 76,000–80,000 D. Transferrin and lactoferrin each bind 2 ferric ions while phosvitin has been reported to bind three. Ferritin is a multisubunit protein (molecular weight of 450,000 D) with the capability of chelating up to 4500 ferric ions. Transferrin, phosvitin, lactoferrin and ferritin inhibit iron-catalyzed lipid oxidation by binding iron in its inactive ferric state and possibly by sterically hindering metal/peroxide interactions.

Reducing agents (ascorbate, cysteine and superoxide anion) and low pH can cause the release of iron from many of the iron-binding proteins resulting in an acceleration of oxidative reactions. Copper reactivity is controlled by binding to serum albumin, ceruloplasmin and the skeletal muscle dipeptide (b-Alanine-His; b-Ala-His), carnosine. Caseinophosphopeptide, which have a main functional domain SerP-SerP-SerP-Glu-Glu, was reported to have transition metal chelating capacity, such as calcium, iron, copper, and zinc. The phosphoserine residues formatted a polar and anionic domain which can be responsible for chelating cationic metal ions.

### Phytic Acid

Phytic acid or myo-inositol hexaphosphate is one of the primary metal-chelators in seeds where it can be found at concentrations ranging from 0.8%–5.3% (Fig. 2). Phytic acid is not readily digested in the human gastrointestinal tract but can be digested by dietary plant phytases and by phytases originating from enteric microorganisms. Phytate is highly phosphorylated thus allowing it to form strong chelates with iron with the resulting iron chelates having lower reactivity. The antioxidant properties of phytic acid are thought to help minimize oxidation in legumes and cereal grains as well as in foods that may be susceptible to oxidation in the digestive tract. Phytic acid has been reported as a preventative agent in iron-mediated colon cancer. While phytate may be beneficial towards colon cancer, it should be noted that it can potentially have deleterious health effects because of its ability to dramatically decrease the bioavailability of minerals including iron, zinc and calcium.

### Polysaccharides

Polysaccharides and derivatives contain at least 20 monosaccharides connected with glycosidic linkages resulting in huge molecular weight. Prevention of oxidative stress with polysaccharide both *in vivo* and *in vitro* have been widely reported and metal chelating capability is the important mechanism accounting for antioxidant activity. In general, compounds contain at least two of the following functional groups:  $-\text{OH}$ ,  $-\text{COOH}$ ,  $-\text{SH}$ ,  $-\text{PO}_3\text{H}_2$ ,  $\text{C}=\text{O}$ ,  $-\text{S}-$ ,  $-\text{NR}_2$  and  $-\text{O}-$  can show metal chelation activity. Consequently, derivatives of polysaccharides such as uronic acid and sulphate substituted polysaccharides have the ability to chelating metals. Polysaccharides fractionated from the leaves of *Ilex latifolia* Thunb containing high contents of sulfuric acid and uronic acid carried stronger ferrous ion chelating ability.

Studies also reported that crude polysaccharides have potential free radical scavenging capability evaluated by DPPH free radical, ABTS free radical, hydroxyl radical scavenging activity, and superoxide anion radical scavenging activity. It is possible that small moieties of flavones, peptide, protein, and polyphenol conjugated on the polysaccharides exert the free radical scavenging capability rather than polysaccharides themselves. In addition, there is an interesting phenomenon that sulphation of polysaccharides can improve their radical scavenging ability although sulphation is not strict related with radical scavenging ability. Moreover, some study indicated that antioxidant activity of polysaccharides might come from the ability to improve the activity of antioxidant enzymes. For example, polysaccharides extracted from *Astragalus membranaceus*, pre-treated mice showed significantly increased antioxidant enzymes including Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px).

Overall, the comprehensive antioxidant properties of polysaccharides are affected by chemical characteristics like molecular weight, degree of substitution, type and ratio of monosaccharides, intermolecular associations of polysaccharides, glycosidic branching, and substitution of functional groups. For instance, lower molecular weight polysaccharides may incorporate into the cells more efficiently and chelating metals more effectively than high molecular weight polysaccharides.

Natural resource with rich polysaccharides are mainly from plant, fungus, and marine organism. Chinese herb medicine is a conventional plant source for bioactive polysaccharides, such as *Dendrobium* plants, *Angelica sinensis*, *A. membranaceus*, *Bupleurum* plants, Jujube fruit, and *Aloe vera*. Pectin, chitin, chitosan, guar and other more complicated polysaccharide extracted from medicine plants have been reported to have high antioxidant activity. Fungal polysaccharides are famous as its antioxidant function, which make it possible for food therapy. The source of novel bioactive compounds from marine organism has been concerned recently. The cell walls of marine algae, red algae, brown algae, and green algae are rich in sulfated polysaccharides such as fucoidans, carrageenans, and ulvans. These natural polysaccharides from algae are reported to be made into medicines with the outstanding antioxidant activity to prevent potential health risks like cardiovascular disease and cancer.

## Antioxidant Enzymes

### Superoxide Dismutase

Superoxide anion is produced by the addition of an electron to molecular oxygen. Superoxide anion can promote oxidative reactions by reduction of transition metals to their more prooxidative state; promotion of metal release from proteins; through the pH dependent formation of its conjugated acid which can directly catalyze lipid oxidation; and through its spontaneous dismutation into hydrogen peroxide (Eq. 3). Due to the ability of superoxide anion to participate in oxidative reactions, the biological tissues from which foods originate will contain superoxide dismutase (SOD).

Two forms of SOD are found in eukaryotic cells, one in the cytosol and the other in the mitochondria. Cytosolic SOD contains copper and zinc in the active site while mitochondrial SOD contains manganese. Both forms of SOD catalyze the conversion of superoxide anion ( $O_2^-$ ) to hydrogen peroxide by the following reaction:



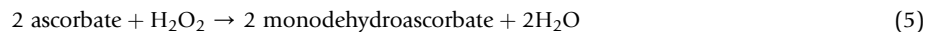
### Catalase

Hydroperoxides are important oxidative substrates since they decompose via transition metals, irradiation and elevated temperatures to form free radicals. Hydrogen peroxide exists in foods due to its direct addition (e.g. aseptic processing operations) and by its formation in biological tissues by mechanisms including the dismutation of superoxide by SOD and the activity of peroxisomes. Lipid hydroperoxides are naturally found in virtually all food lipids. Removal lipid and hydrogen peroxide from biological tissues is critical to prevent oxidative damage. Therefore almost all foods originating from biological tissues contain enzymes that decompose peroxides into compounds less susceptible to oxidation. Catalase is a heme-containing enzyme that decomposes hydrogen peroxide by the following reaction (Eq. 4):



### Ascorbate Peroxidase

Hydrogen peroxide in higher plants and algae may also be decomposed by ascorbate peroxidase. Ascorbate peroxidase inactivates hydrogen peroxide in the cytosol and chloroplasts by the following mechanism (Eq. 5):



Two ascorbate peroxidase isozymes have been described which differ in molecular weight (57,000 D vs. 34,000 D), substrate specificity, pH optimum and stability.

### Glutathione Peroxidase

Many foods also contain glutathione peroxidase. Glutathione peroxidase (GSH-Px) differs from catalase in that it decomposes both lipid and hydrogen peroxides. GSH-Px is a selenium-containing enzyme which catalyzes hydrogen peroxide (Eq. 6) or lipidperoxides (Eq. 7) reduction using reduced glutathione (GSH):



or



where glutathione disulfide (GSSG) is oxidized glutathione and LOH is a fatty acid alcohol. Two types of GSH-Px exist in biological tissues of which one shows high specificity for phospholipid hydroperoxides.

Antioxidant enzyme activity in foods can be altered in raw materials and finished products. Antioxidant enzymes differ in different genetic strains and at different stages of development in plant foods. Heat processing and food additives (e.g. salt and acids) can inhibit or inactivate antioxidant enzyme activity. Dietary supplementation of selenium can be used to increase the glutathione peroxidase activity of animal tissues. These factors suggests that technologies could be developed to increase natural levels of antioxidant enzymes in raw materials and/or minimize their loss of activity during food processing operations.

## Conclusions

The biological tissues from which foods originate contain multicomponent antioxidant systems that include free radical scavengers, metal chelators, singlet oxygen quenchers and antioxidant enzymes. Our understanding of how these endogenous antioxidants protect foods from oxidation is still in its infancy. In addition, how factors that can alter the activity of endogenous food

antioxidants (e.g. heat processing, irradiation and genetic selection of foods high in antioxidants) is still poorly understood. Finally, research is continuing to show that natural food antioxidants in the diet are very important in the modulation of disease. Thus finding mechanisms to increase antioxidative activity of natural food antioxidants may be beneficial to both health and food quality.

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## Natural Sweeteners

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### Introduction

Sweet taste perception is one of the most ancient taste modalities and is associated to the detection of carbohydrate-rich compounds in nutrients. Before the extraction of sucrose (or table sugar) in the 18th century, honey was the main source of sweetness. In the last centuries, sugar beet and sugar cane production grew rapidly to become the major source of sugar. Nowadays the overconsumption of sugar is considered a significant public health problem in industrial countries and has promoted the search for alternatives to high-calorie sweeteners. Many artificial sweeteners have been developed by the food industry but consumer interest in natural high potency sweeteners has grown spectacularly in recent years, fueled by concerns about sugar overconsumption and the use of artificial additives in foods. Though many low-calorie sweeteners are known, only few of them are used by the food industry (O'Brien Nabors, 2012). The search for novel intense sweeteners, possessing the same chemosensory profile as sucrose, remains open and challenging. In addition to physicochemical properties (such as thermal stability or water solubility), production cost and safety, one of the most important sensory properties of a sweetener is its sweetness potency. Sweetness potency can be measured by relative sweetness, which is defined as the ratio of concentrations of substances eliciting equivalent sweetness intensity. Sucrose is taken as the reference and its sweetness is traditionally set to 1. Numerous high-potency sweeteners are known but their noticeable aftertaste (bitter, metallic or licorice taste sensation ...) is a major restraint for consumer acceptability. Another major criterion of a "good" sweetener is its calorie content. For natural carbohydrate type of sweeteners, low calorie content is often associated with low sweetness potency. In the quest for sucrose alternatives, it has been questioned whether ideal sweeteners exist. At the beginning of the 2000s, the identification of the receptor responsible for the sweet taste perception (Nelson et al., 2001) is a major breakthrough in the understanding of the sweet taste modality. It opened new horizons for the rational development of new sweet-taste compounds. In this chapter, we briefly describe the structure and dynamics of the sweet taste receptor, which explain why the chemical space of sweeteners is so extended. Natural sweeteners belonging to saccharides, polyols, terpene glycosides, sweet proteins and other less common chemical families are presented. We also provide an overview of experimental strategies to discover new sweet compounds.

### Only One Sweet Taste Receptor Associated to a Large Chemical Space

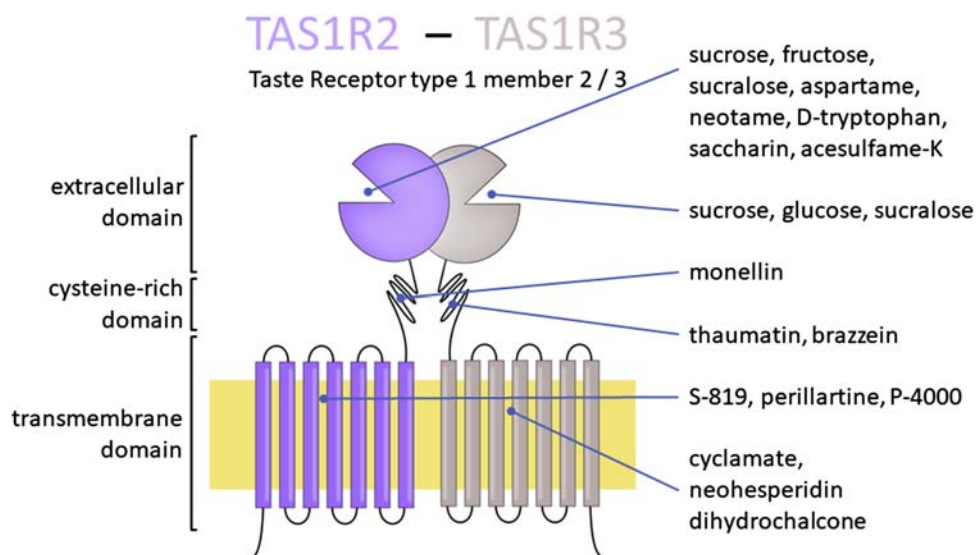
All sweet tasting compounds are detected by a single taste receptor, the heterodimer TAS1R2-TAS1R3 (for Taste Receptor type 1 member 2 or 3), expressed on the surface of our taste buds (Nelson et al., 2001). The sweet taste receptor belongs to the class C G protein-coupled receptors (GPCRs) that share a similar structure: a large extracellular domain containing the orthosteric binding site and a seven-helix transmembrane domain (Pin et al., 2003) (Fig. 1). The two domains are connected by a cysteine rich domain structurally constrained by intramolecular disulfide bonds. Natural sweeteners such as sucrose interact with the orthosteric binding pocket of the receptor (Xu et al., 2004). The consensus mechanism of the sweet receptor activation is that orthosteric ligand binding involves a closure and a rotation of the TAS1R2 and the TAS1R3 extracellular domains. The chemical stimulus is then transmitted via the cysteine rich domain to the transmembrane domains where downstream signaling effectors bind. The transmembrane domain holds an allosteric binding site and the cysteine rich domain may bind sweet taste protein. Considering all the ligand binding sites of both the TAS1R2 and the TAS1R3 subunits, the sweet taste receptor can interact with sweet compounds through six different ways (DuBois, 2016) (Fig. 1). It gives a rational of the large chemical space of sweet-tasting compounds despite the fact that the sweet taste modality is controlled by a single taste receptor.

### Chemical Space of Natural Sweeteners

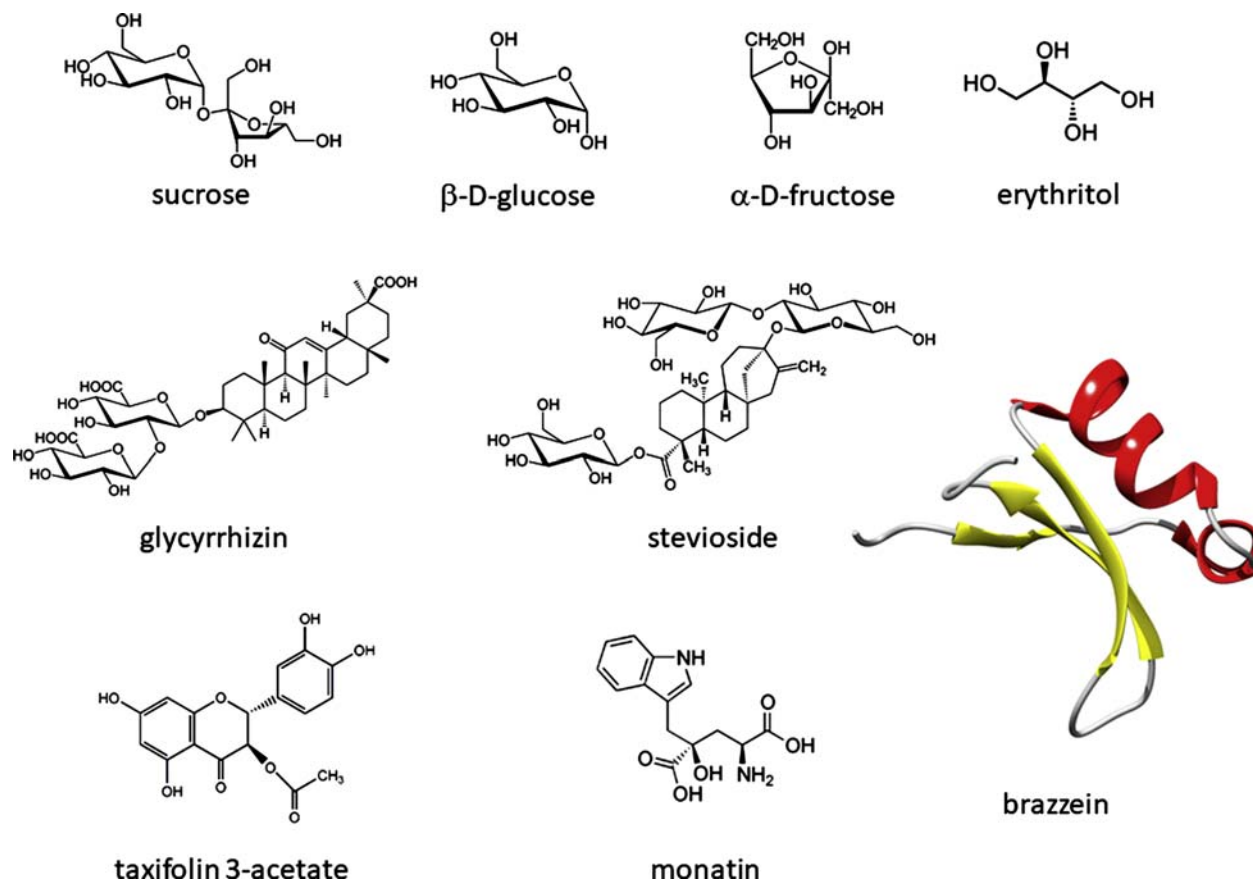
Natural sweeteners include natural sugars, sugar alcohols, terpenoid glycosides as well as some amino acids and polyphenols. In addition, a remarkable class of sweet agents will be reviewed: sweet tasting proteins originating from plants. Sweet taste compounds cover a large chemical space with high structural diversity as illustrated in Fig. 2.

### Natural Carbohydrates

Natural sugars, also called saccharides or carbohydrates, are polyhydroxylated aldehydes or ketones with the brute chemical formula  $C_n(H_2O)_m$  where  $n$  and  $m$  may be different. They are named by the suffix "-ose". There are notably two families: ketoses and aldoses, containing from 3 to 7 carbon atoms for natural oses. The simplest ones are erythrulose ( $C_4H_8O_4$ ) and glyceraldehyde ( $C_3H_6O_6$ ) for

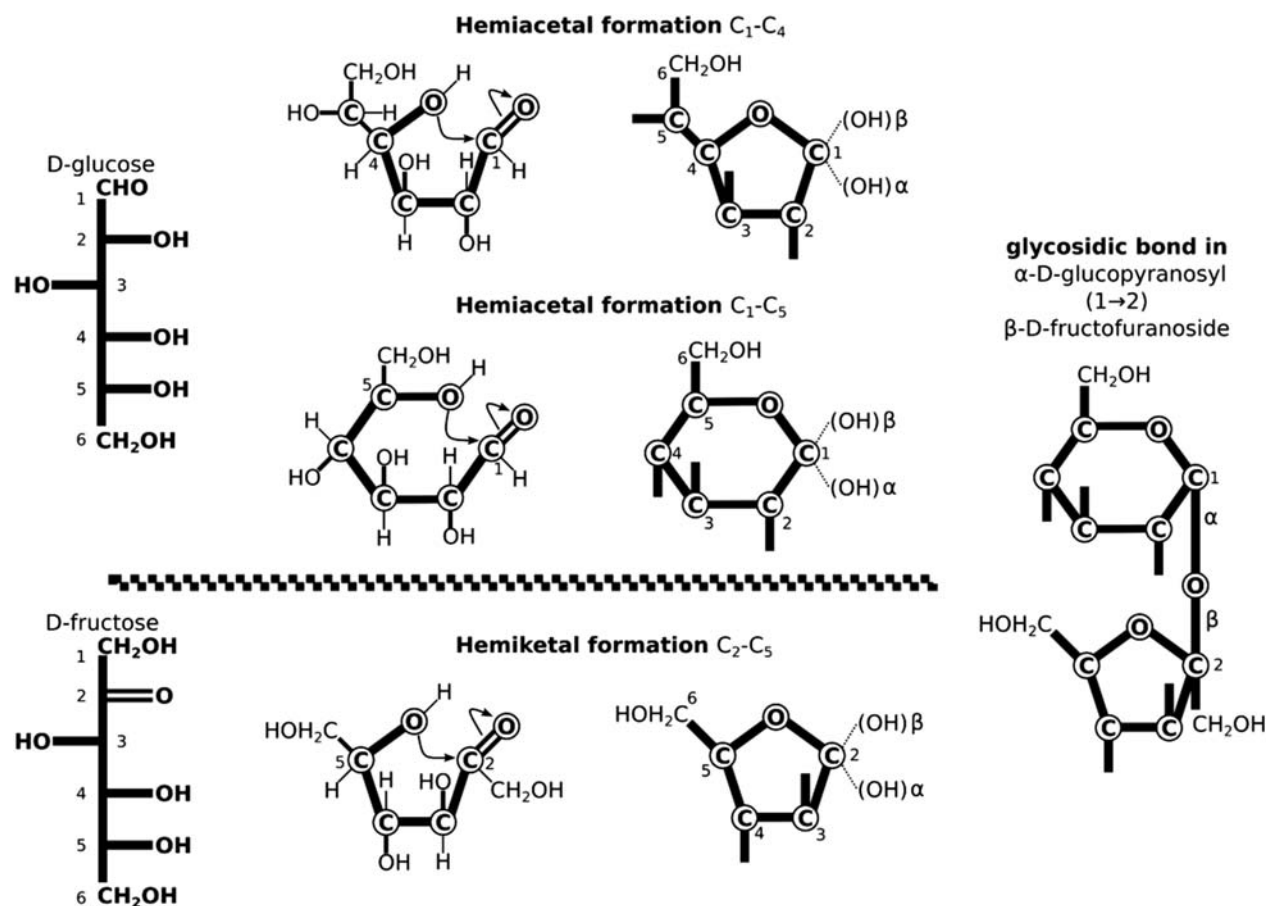


**Figure 1** Structure of the sweet taste receptor and binding sites of various natural and artificial sweet taste compounds (DuBois, 2016).



**Figure 2** Chemical structure of natural sweet taste agents.

each family, respectively. The carbon atom carrying the aldehyde functional group is at position 1 in the carbon skeleton and the one defining the ketone functional group is at position 2. The orientation of the carbon atom at position  $n-1$  defines D or L series. In nature, D-sugars are preponderant. Fig. 3 shows the Fischer representation of linear-chain monosaccharides D-glucose and D-fructose, belonging to the ketose and the aldose chemical family, respectively.



**Figure 3** Fischer projection of monosaccharides D-glucose and D-fructose (left), hemiacetal/hemiketal formation (center) and glycosidic bond in sucrose (right).

The chemical structure becomes more and more complex as the number of carbon atoms increases since each carbon atom (except for the terminal ones) is a chiral center. A linear-chain ose is in equilibrium with its cyclic form that is produced by a hemiacetal or a hemiketal reaction depending on whether the linear form is an aldose or a ketose. The cyclisation of monosaccharides usually produces 5 or 6 atoms rings named furanoses or pyranoses. The orientation of the carbonyl functional group being below or above the plane of the carbon atoms determines the anomers (cyclic isomers) denoted by the  $\alpha$ - or  $\beta$ -prefix as shown in Fig. 3.

Polymers of monosaccharides can be produced via a dehydration reaction called a glycosidic linkage. The anomeric carbon atom is the reactive center to form glycosidic bonds. Two subunits form disaccharides. Oligosaccharides contain three to ten monomers and polysaccharides, more than ten. Sucrose, known as table sugar, is a disaccharide also named  $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -fructofuranoside and is formed by the condensation of the monosaccharides D-glucose and D-fructose (Fig. 3).

Except for fructose, the relative sweetness of saccharides is systematically lower than the sucrose reference (Chéron et al., 2017; O'Brien Nabors, 2012; O'Donnel and Kearsley, 2012) (see Table 1). Fructose, the sweetest natural carbohydrate, occurs naturally in fruits and vegetables. Among monosaccharides, glucose and fructose are the most commonly used sweeteners in industry. High Fructose Corn Syrups (HFCS) are mainly composed of a variable ratio of fructose and glucose and are produced from corn starch through a sophisticated and automated industrial process. Other sources of high fructose syrup exist but HFCS is the most common commercial product. HFCS are used because of its flavor profile (quick perception of sweetness that does not linger) and its texturing capacity (Buck, 2012). Whichever is the fructose:glucose ratio, the relative sweetness is higher than sucrose. Due to health issues that have risen from high consumption of natural carbohydrates, there is a constant increase of low-caloric alternatives.

## Sugar Alcohols

Sugar alcohols are another chemical family of natural sugar substitutes, with the general formula  $\text{CHOH}_n\text{H}_2$ , where  $n = 4-6$ . They are widely used in the food industry for their sweetness and texturing properties (O'Donnel and Kearsley, 2012). They belong to the polyol chemical family which are polyhydric alcohols produced by the hydrogenation of the corresponding reducing sugars. Among the sugar alcohols accepted as food additives, glycerol, erythritol, xylitol, sorbitol and mannitol contain respectively three, four, five

**Table 1** Relative sweetness of saccharides compared to sucrose

Name	Chemical family	Relative sweetness
arabinose	monosaccharide	0.69
D-tagatose	monosaccharide	0.92
fructose	monosaccharide	1.43
fucose	monosaccharide	0.69
galactose	monosaccharide	0.65
glucose	monosaccharide	0.60
mannose	monosaccharide	0.52
psicose	monosaccharide	0.70
rhamnose	monosaccharide	0.32
ribose	monosaccharide	0.70
sorbose	monosaccharide	0.60
tagatose	monosaccharide	0.90
xylose	monosaccharide	0.52
isomaltulose	disaccharide	0.47
lactose	disaccharide	0.25
lactulose	disaccharide	0.50
leucrose	disaccharide	0.50
maltose	disaccharide	0.39
sucrose	disaccharide	1
trehalose	disaccharide	0.43
xylobiose	disaccharide	0.40
raffinose	oligosaccharide	0.25
stachyose	oligosaccharide	0.22

and six (for the two latter) hydroxyl functional groups and are derived from monosaccharides. Maltitol, isomalt and lactitol contain nine hydroxyl functional groups and disaccharide derivatives.

They are used as food additives because of their lower caloric content than sugars despite their lower sweetness (**Table 2**). Their sweetness profile is close to sucrose and they are generally used in combination with intense sweeteners to mask the unpleasant aftertastes of the latter ([Grembecka, 2015](#)). Sugar alcohols produce a cooling sensation in the mouth because their dissolution is an endothermic reaction. In addition to their interesting sensory properties, sugar alcohols are considered tooth-friendly because they are not metabolized by oral bacteria ([Grembecka, 2015](#)). However, like other nutrients that are incompletely digested, they may cause diarrhea, abdominal pain, and flatulence at high dosage level ([Grembecka, 2015](#)).

## Other Natural Sweeteners

Knowledge of the existence of intense sweeteners in plants dates from the mid-nineteenth century ([Kim and Kinghorn, 2002](#)). A non-exhaustive list of natural alternatives to saccharides and sugar alcohols is summarized in **Table 3**. However, most of them elicit licorice, metallic or bitter taste that are incompatible for commercial use. Only few natural high potency sweeteners have regulatory approval as sweetener. For instance, glycyrrhizin is not considered in the United States as a sweetener but as a flavoring agent and flavor enhancer and is still not approved in Europe.

Two types of high-intensity sweeteners have a 'generally recognized as safe' (GRAS) status and are permitted for use in food in the US: certain di- and tri-terpene glycosides obtained from the leaves of the plant *Stevia rebaudiana* and from extracts of *Siraitia grosvenorii* fruit (also known as Luo Han Guo or monk fruit). *Siraitia* fruits are used as food, beverage and traditional medicine in China.

**Table 2** Relative sweetness and glycemic index of sugar alcohols

Name	Chemical family	Relative sweetness	Glycemic index
erythritol	monosaccharide alcohol	0.63	1
xylitol	monosaccharide alcohol	0.97	12
mannitol	monosaccharide alcohol	0.50	2
sorbitol	monosaccharide alcohol	0.58	4
isomalt	disaccharide alcohol	0.54	2
lactitol	disaccharide alcohol	0.35	3
maltitol	disaccharide alcohol	0.87	35

**Table 3** Relative sweetness of various natural high potency sweeteners

<i>Name</i>	<i>Chemical family</i>	<i>Relative sweetness<sup>a</sup></i>
perillaldehyde	monoterpene	12
hernandulcin	sesquiterpene	1345
baiyunoside	diterpene	500
dulcoside A	diterpene	50–120
rebaudioside A	diterpene	250–450
rebaudioside B	diterpene	300–350
rebaudioside C	diterpene	50–120
rebaudioside D	diterpene	250–400
rebaudioside E	diterpene	150–300
rubusoside	diterpene	114
steviolbioside	diterpene	100–125
stevioside	diterpene	300
abrusoside (A-E)	triterpene	30–100
glycyrrhizin	triterpene	90
mogroside IV	triterpene	250–392
mogroside V	triterpene	250–425
osladin	triterpene	500
periandrin (I-IV)	triterpene	85–100
polypodoside A	triterpene	600
pterocaryoside A	triterpene	50
pterocaryoside B	triterpene	100
siamenoside I	triterpene	563
telosmoside A	triterpene	1000
anethole	phenylpropanoid	13
cinnamaldehyde	phenylpropanoid	50
phyllodulcin	coumarin	400–800
selliguaein A	flavonoid	35
taxifolin 3-acetate	flavonoid	80
monatin	amino acid	1200–1400
tryptophan	amino acid	35
brazzein	protein	800
curculin	protein	550
mabinlin	protein	100
monellin	protein	3000
pentadin	protein	500
thaumatin	protein	3000
neoculin	protein	<sup>b</sup>
miraculin	protein	<sup>b</sup>

<sup>a</sup>Some variation in sweetness values can be noticed and may arise from the protocol employed.

<sup>b</sup>Neoculin and miraculin are not sweet by themselves but they are taste modifying proteins.

In Asian and South American countries stevia is cultivated and its extracts are traditionally used to sweeten food. In Europe, only steviol glycosides (E960) have been approved as food additive.

*Stevia rebaudiana* is a plant native to south America has been known to have sweet tasting leaves for centuries by indigenous populations. It is a source of steviol glycosides that contain a common aglycone diterpene scaffold, steviol. Among the terpenoids present in this plant, stevioside and rebaudioside elicit a sweet taste hundreds of times sweeter than sucrose (Table 3), which makes them attractive sweeteners (Soejarto et al., 1982). Rebaudioside A generates a cleaner sweet taste similar to that of sucrose compared to stevioside, which delivers a stronger bitter aftertaste.

Mogrosides are a family of triterpenoid glycosides extracted from a Chinese plant locally known as *Lo Han Guo*. The structure of the two major constituents, mogrosides IV and V, have been characterized in the 1970s (Lee, 1975) and have been described as hundreds of times sweeter than sucrose (Table 3). The sweetness of mogrosides has a slow onset profile, typical of natural high-potency sweeteners (Lindley, 2012). Their liquorice aftertaste coupled with a cooling sensation may limit their use.

There exists another type of sweet agents of strong interest for the food industry: sweet tasting proteins. They are naturally produced by tropical plants and are hundreds to thousands of times sweeter than sucrose (Lindley, 2012). To date, eight intensely sweet-tasting proteins have been identified: thaumatin, monellin, brazzein, pentadin, mabinlin, neoculin and miraculin. Sweet proteins are expected to be digested just as any other dietary protein is. They have been used as sweetener for centuries in their native regions, which eases concerns about their toxicity.

Thaumatin I and II are the major sweet proteins obtained from the fruits of the West African plant *Thaumatococcus daniellii* Benth (also called katemfe). Thaumatin I (generally named thaumatin) is the only sweet protein that has been approved by both the FDA and the European Commission to be used as sweetener in food. Thaumatin has a very different sensory profile to that of sucrose, a very slow onset and a lingering sweet taste leaving a liquorice aftertaste (Lindley, 2012).

Brazzein is a small and heat-stable 54-amino-acid sweet protein isolated from a West African plant, *Pentadiplandra brazzeana* Baillon. Its taste profile is defined as closer to sucrose than any other sweet proteins (Lindley, 2012). Brazzein is intensely sweet with a slight licorice aftertaste and a cooling effect at high concentration levels. Because of its favorable taste profile and thermostability, academic and industrial research efforts have been made to develop various brazzein production protocols, from recombinant systems to fully synthetic solid-phase methods.

## Expanding the Chemical Space of Natural Sweeteners

The research of new natural sweeteners faces two main issues: purification of the molecules from a complex matrix and elucidation of their chemical structure. To achieve these goals, several methodologies have been developed using different analytical techniques.

A common approach to isolate sweeteners from natural extracts relies on targeted separation. Pharmacognosists usually perform bio-guided purifications to isolate active compounds from plants using dereplication and high throughput screening. Similarly, inductive strategies using sensory analysis have been developed to search for natural sweet-tasting compounds. A combination of various separation techniques is often needed. After each step, fractions are tasted and the most active one is kept for further fractionation. Crude plant extracts are partitioned sequentially in various solvents to give fractions containing molecules of different polarity (Kingham and Soejarto, 2002). Further fractionation can be carried on by using physical or chemical techniques such as multiple-step ultrafiltration and chromatography, respectively (Frank et al., 2006; Pickrahn et al., 2014). Recently, centrifugal partition chromatography (CPC) has been hyphenated to taste analysis for the identification of sweet triterpenes from oak wood (Marchal et al., 2011). Unlike solid phase chromatography, CPC presents no risk of irreversible adsorption and its load capacity is high (up to 10 g for a rotor of 1 L). These properties are particularly interesting for the study of natural sweeteners. However, CPC as low-pressure liquid chromatography offers a low chromatographic resolution and a final step using HPLC is often required to isolate pure compounds. Compared to olfactory analysis, tasting is destructive to the samples, so higher quantities of fractions are necessary for the sensory tests. Innovative sensory tools have been developed to replace classical tasting. The taste dilution analysis (TDA) coupled to half-tongue test dramatically decreases the required amount of fraction: serial dilutions of a sample are prepared. These dilutions are successively presented to a taster by applying one aliquot on one side of the tongue and water on the other as control. In this way, the taste dilution factor can be determined; it characterizes the taste-activity of the sample (Scharbert et al., 2004). Recent developments have also given rise to electronic tongues that offer new perspectives for guiding the purification of sweeteners (Zhang et al., 2012).

The identification of new sweeteners requires powerful spectroscopic methods commonly used for chemical elucidation of natural products. While NMR remains the technique of choice, mass spectrometry can also provide reliable structural information. In particular, the composition and the chemical class of an isolated molecule can be determined by high resolution mass spectrometry as well as the number, the type, the position or the sequence of its functional groups (Marchal et al., 2015). Moreover, stereochemistry strongly influences the taste properties of natural compounds and chiroptic techniques such as electronic or vibrational circular dichroism are particularly useful to characterize their absolute configuration (Cretin et al., 2015).

When a high-potency sweetener is identified, the search for structural analogs with similar, or even more interesting, properties is commonly conducted (Bassoli et al., 2011). Chemical synthesis can be a powerful tool in generating these analogs by making slight modifications to the stereochemistry or substituents of the sweetener (Machinami et al., 2002). However, natural biosynthetic pathways may also cause changes of the same type and several isomers and derivatives of a natural compound are often observed in the same plant. This biochemical diversity can be successfully explored to identify new sweet-tasting molecules (Marchal et al., 2015).

## Conclusion

In recent years, there has been growing interest in the replacement of sugars by natural sweeteners and numerous sugar substitutes have been developed and marketed. These include for instance bulk sweeteners (polyols), high fructose corn syrup and more recently steviol glycosides. The billions of years of evolution have allowed nature to create an incredible structural diversity of natural compounds suggesting that many regions of the sweet taste chemical space remain unexplored. The discovery of the sweet taste receptor has provided new opportunities to study the chemical structure – sweet taste relationships. However, the modes of ligand–receptor interactions are mostly unknown or poorly understood and the identification of new natural sweeteners still relies on botanical and ethnobotanical knowledge combined with sensory and analytical experiments.



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# Nitrates

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## Introduction

Nitrates ( $\text{NO}_3^-$ ) are naturally occurring ions formed by the oxidation of nitrogen, and are an integral part of the nitrogen cycle in the environment (Cigulevska, 2002). Apart from the natural occurrence of nitrates in vegetables and fruits, they are also used as food additives for flavoring and preservation of meat and meat products (Song et al., 2015). Though the inclusion of nitrates has beneficial effects in terms of improving the texture, color, flavor and the shelf-life of meat and poultry products, it is, essentially, a double-edged sword as nitrates in food have been implicated in human toxicity and carcinogenicity due to their enzymatic conversion to nitrites ( $\text{NO}_2^-$ ) in the body (Silva and Lidon, 2016). This article discusses the natural and artificial occurrence of nitrates in food, the advantages and disadvantages associated with their presence in food, detection methods, as well as proposed alternatives.

## Occurrence of Nitrates

### Occurrence in Environment

In soil, nitrates are formed by the action of nitrifying bacteria, such as *Nitrosomonas* which oxidise ammonium ions to nitrites, which are further oxidised to nitrates by bacteria such as *Nitrobacter* and *Nitrococcus* spp. (Rao and Puttanna, 2000). These nitrates are absorbed by plants through the roots, and therefore, are natural constituents of plants (Keeton, 2017). A World Health Organization (WHO) estimate states that the average daily human consumption of nitrates is 43–141 mg, and of this, vegetables account for approximately 80% (World Health Organization, 2007; Lairon, 2011). Nitrates can be accumulated in high amounts by plant tissues, and vegetables, such as beetroot, and radishes, and leafy vegetables, such as celery, spinach and lettuce have been shown to accumulate more than 2500 mg of nitrates per 100 g, which is considered to be a very high value (Santamaria, 2006). Nitrate accumulation by plants can be influenced by several biological factors (such as plant cultivar and variety), environmental factors (such as light intensity, soil temperature, rainfall etc.), and processing methods (such as storage temperature) (Hmelak Gorenjak and Cencič, 2013). However, the drastic rise in the use of nitrogen fertilizers for improving crop productivity in face of the increasing global population is the primary reason for higher accumulation of nitrates by plants (Iammarino et al., 2013). Excessive use of nitrogen fertilizers can also cause an increase in the nitrate concentration of drinking water due to leaching into groundwater (Odorog, 2016).

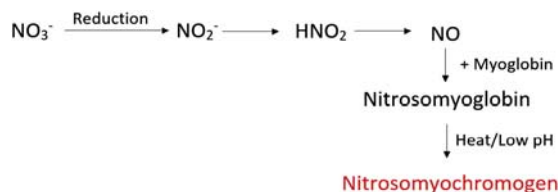
### Nitrates as Food Additives

The earliest use of nitrates for food preservation was recorded in ancient India and China, as the use of “wall saltpeter” or  $\text{Ca}(\text{NO}_3)_2$  for the curing of meat, resulting in a distinctive red color and flavor (Binkerd and Kolari, 1975). It was only in the late 19th century that scientists were able to explain the mechanism of reddening of the meat on treatment with nitrates (Fig. 1) (Hoagland, 1908; Haldane, 1901).

It has been estimated that processed meats account for less than 5% of the total nitrate consumption (Milkowski, 2017). Cured meats have a characteristic reddish to pink color which is attributed to the action of nitrates. First, nitrates are reduced to nitrites by the enzyme nitrate reductase. Nitrites are then converted to compounds such as nitrous acid ( $\text{HNO}_2$ ) and nitric oxide (NO), which is a prerequisite for curing. Subsequently, the NO binds to the  $\text{Fe}^{2+}$  ion present in the porphyrin ring of the globular protein myoglobin, thus forming an unstable compound, nitrosomyoglobin. This intermediate, in the presence of high temperature or acidic pH gives a stable red pigment called nitroso-myochromogen (the nitrosoporphyrin ring), formed by the denaturation of the protein component of nitrosomyoglobin (Honikel, 2008; Govari and Pexara, 2015).

Additionally, nitrates are also added for activity against food spoilage bacteria and pathogens. Nitrates by themselves do not have any direct antimicrobial effect, however, they function as a precursor to nitrites, which are active against pathogens, such as *Clostridium botulinum* (thus preventing botulism), and *Staphylococcus aureus* (Sindelar and Milkowski, 2012; Mahindru, 2008).

Due to the toxic effects of residual nitrite concentrations in processed meat, the use of nitrates was gradually reduced, and currently, sodium nitrate (E 251) and potassium nitrate (E 252) which are classified as Class II preservatives according to the Commission regulation (EC) No. 1129/2011, are most commonly used in curing of meat, and in preservation of fish and poultry (Sebranek and Bacus, 2007; Anand and Sati, 2013). The permissible limits set by the Food and Drug Administration (FDA) for nitrate concentration in foods have been summarized in Table 1.



**Figure 1** Mechanism of formation of red coloration of during curing.

**Table 1** Permissible limits for nitrates in food

Additive	Food	Permitted limit	References
Sodium nitrate (as preservative and color fixative)	Smoked and cured seafood such as sablefish, salmon and shad	<500 ppm	(2017b) <sup>b</sup>
	Smoked and cured poultry and wild game	<500 ppm	(2017b) <sup>b</sup>
Potassium nitrate (as curing agent)	Cod roe	<200 ppm	(2017a) <sup>a</sup>

<sup>a</sup>2017a. Food Additives Permitted for Direct Addition to Food for Human Consumption (Sec. 172.160) [Online]. Available: <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=172.160>.

<sup>b</sup>2017b. Food Additives Permitted for Direct Addition to Food for Human Consumption (Sec. 172.170) [Online]. Available: <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=172.170>.

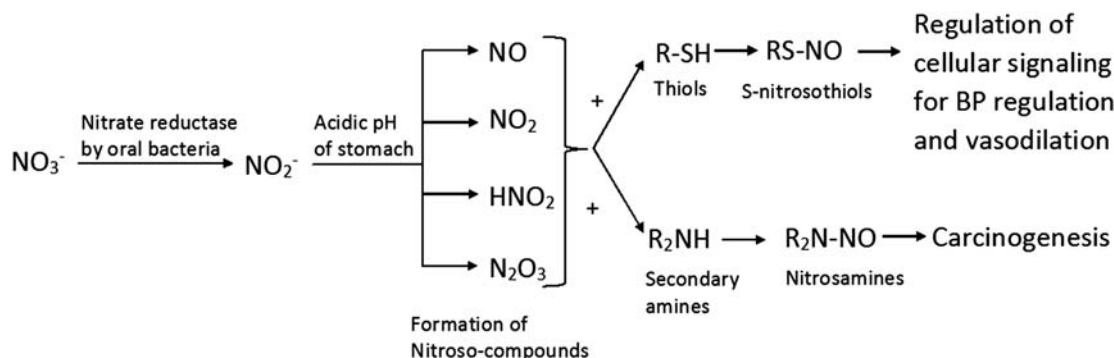
## Effects of Nitrates on Humans

For over a century, several concerns have been raised over the potential of nitrates and nitrites to form carcinogens, leading to strict regulations for their use as food additives. However recent studies have hinted that the dietary supplementation of nitrates may actually have a protective effect with respect to cardiovascular health, however little is understood of the actual mechanism for this effect (Fig. 2).

## Detrimental Effects of Nitrates

While nitrates do not have a direct effect on health, their conversion to nitrites in some physiological conditions in the body has been linked to toxicity and carcinogenesis in humans (Cigulevska, 2002). Chan et al. reported that 5%–7% of the nitrates ingested by humans through food is converted to nitrites by nitrate reducing bacteria present in the saliva in the oral cavity (Chan, 2011). The metabolism of nitrate to nitrite in the digestive system in high amounts (ranging from 0.4 mg kg<sup>-1</sup> to >200 mg kg<sup>-1</sup> body weight) can cause methemoglobinemia, or the blue baby syndrome. In this disorder, the nitrite formed oxidizes the Fe<sup>2+</sup> in hemoglobin to Fe<sup>3+</sup>, thus converting hemoglobin to methemoglobin, and consequently, reduces the oxygen carrying capacity of the blood. Symptoms of methemoglobinemia include tiredness, vomiting, diarrhea, and eventually suffocation and death (Chamandoost et al., 2016).

The indirect carcinogenic effect of nitrates has been attributed to conversion of nitrites to nitrous acid and nitrogen oxides (in acidic conditions such as the stomach), which can further react with secondary amines to form secondary nitrosamines (Butler, 2015; Stojnev et al., 2014). Nitrosamines, in the presence of high temperature or low pH, are converted to di-azonium ions which can react with DNA and cause gene mutations, and ultimately cancer (Chamandoost et al., 2016; Song et al., 2015). Apart from this, a high nitrate concentration in drinking water was implicated in the enlargement of the thyroid gland, as well as the prevalence of



**Figure 2** Mechanism of transformation of nitrates in humans.

Diabetes Mellitus type1 in children aged 1–18 years (Parvizishad et al., 2017). Studies have also linked high levels of dietary nitrates to increased number of deaths from Parkinson's disease and Alzheimer's disease (Anand and Sati, 2013).

Although the exposure to nitrates and nitrites have been linked to the increased incidence of gastrointestinal, nasopharyngeal and brains tumors, a conflicting report by the US EPA (Environmental Protection Agency) raised serious doubts regarding the association of these compounds with the development of cancers in children and adults (Gassara et al., 2016).

### Beneficial Effects of Nitrates

While several studies have proposed the link between dietary nitrates and their subsequent metabolites and adverse effects in humans, there is also emerging research on their health benefits (Lundberg et al., 2009). Several clinical trials have been conducted to evaluate the positive effect of dietary nitrates on cardiovascular health. Nitrate metabolites, such as nitrosothiols and nitroalkenes have been shown to have cryoprotective, hypotensive, antiplatelet effects (Hord, 2011). The DASH (Dietary Approach to Stop Hypertension) plan is based on the hypothesis that increased consumption of "nitrate-rich" vegetables, such as beetroots, spinach etc. is effective for the nonpharmacological management of high blood pressure by promoting vasodilation (d'El-Rei et al., 2016). S-nitrosothiols formed by nitrosation of thiol groups are known to be a part of signalling pathways involved in physiological functions including vasodilation and blood pressure regulation (Habermeyer et al., 2015). Webb et al. (2008) reported a reduction in blood pressure following consumption of beetroot juice, and attributed it to the nitrate load in beetroot. Larsen et al. (2007) reported that a dietary supplementation of nitrate rich vegetables resulted in lower oxygen demand during submaximal work, and a more efficient energy production. Similar results were also obtained by Bailey et al. (2009) on the supplementation of nitrate in the form of beetroot juice. However, so far, the exact mechanism for these hypotheses has not been elucidated.

### Detection of Nitrates

The WHO has set the permissible limits for dietary nitrates to be 300 mg kg<sup>-1</sup> body weight for adults, and 25 mg kg<sup>-1</sup> body weight for babies. Several studies have indicated the harmful effects of nitrates at high doses, therefore it is essential to identify and detect the concentration of these compounds in food. Nitrates can be detected directly, or indirectly by reduction to nitrite. Several techniques have been described for the analysis of nitrates in food, a few of which are summarized below, and in Table 2 (Azmi et al., 2017).

#### Colorimetry

The International Organization for Standardization defined the official method for the detection of nitrates in 1975 (ISO 3091:1975), by which nitrates are estimated indirectly after reduction to nitrites by metallic cadmium (Iammarino et al., 2017). Nitrite quantification is done spectrophotometrically by the Griess-Romijn reaction, which is based on the conversion of aromatic amines such as sulphanilamide to diazonium ions by nitrites. These ions then react with a coupler molecule (e.g., N-(1-naphthyl) ethylenediamine) to form a red compound, which is detected at 540 nm (Ferreira and Silva, 2008). Zatar et al. (1999) developed a method which utilises oxidation of a phosphomolybdenum blue complex by nitrite, causing a decrease in the intensity of the blue color of which was read at 814 nm. The nitrite concentration is proportional to the decrease in absorbance of the phosphomolybdenum blue complex. To account for nitrite present in sample before the reduction of nitrate to nitrite, Merino (2009) used zinc powder to reduce nitrate to nitrite, and nitrite concentration was determined both before and after reduction by the Griess-Romijn

**Table 2** Overview of some nitrate detection techniques

	Colorimetry	Chromatography	Electrochemical detection	Biosensors
Nitrate detection	Indirect	Indirect	Direct	Direct
Mechanism of detection	Spectrophotometer	UV, electrochemical, fluorimetric, mass spectroscopic	Change in current or electric potential	Specific interaction between analyte and biologically derived material
Advantages	<ul style="list-style-type: none"> <li>• Simple assay</li> </ul>	<ul style="list-style-type: none"> <li>• High sensitivity</li> <li>• Rapid analysis</li> </ul>	<ul style="list-style-type: none"> <li>• High sensitivity and selectivity</li> <li>• Rapid detection</li> <li>• Use of non-toxic reagents</li> <li>• Cost-effective</li> </ul>	<ul style="list-style-type: none"> <li>• Cost effective</li> <li>• Highly substrate selective</li> <li>• Portable due to small size</li> </ul>
Disadvantages	<ul style="list-style-type: none"> <li>• Low sensitivity</li> <li>• Use of toxic reagents</li> <li>• Subject to interference from other species</li> </ul>	<ul style="list-style-type: none"> <li>• Non-portable</li> <li>• Expensive</li> </ul>	<ul style="list-style-type: none"> <li>• Increased cost due to sensitive material used</li> <li>• Difficult to store</li> </ul>	<ul style="list-style-type: none"> <li>• Low activity of nitrate reductase</li> <li>• pH and temperature dependent sensitivity</li> <li>• Time-consuming</li> </ul>

reaction, and the nitrate concentration was calculated as the difference between nitrite concentration before and after reduction. Studies have also confirmed to use of Vanadium (III) and nitrate reductase enzyme for reduction of nitrate to nitrite (Miranda et al., 2001).

### Chromatography

Ion chromatography and High-Pressure Liquid Chromatography (HPLC) are the preferred techniques for nitrate detection. The most common pre-treatment technique converts nitrate to  $\text{NO}_2^+$  ions, followed by nitration of 2,4-dimethylphenol. The indicator species is 1,3,5-trimethoxybenzene (Tsikas et al., 1998). Chromatography techniques can be coupled to different detection systems such as UV, electrochemical, fluorimetric, electron capture, and mass spectroscopic for the analysis of the eluent (Moorcroft et al., 2001).

### Electrochemical Detection

Electrochemical detection of nitrates can be voltammetric or potentiometric. Nitrates are reduced to nitrites by a Cu/Cd column or by nitration of aromatic compounds, such as benzoic acid or salicylic acid. The derivative obtained is determined at a glassy carbon electrode by voltammetric analysis. For potentiometric analysis, commercial ion-selective electrodes (ISEs), are used to transfer charged particles from one phase to another, which creates a measurable potential difference (Moorcroft et al., 2001). The sensitivity can be improved by using a nitrate selective membrane. Capillary electrophoresis is a rapid method of detection of anions, and it requires smaller volumes of both the buffer and the sample for analysis. A polyethyleneimine-coated capillary is used for separation of anions, and nitrate detection is done by UV detection at 214 nm (Moorcroft et al., 2001).

### Biosensors

Biosensors consist of a bioreceptor, transducer and detector. The detector signal generated by binding of the analyte to the bioreceptor generates a signal, which is converted by the detector into a measurable signal such as voltage or current (Sohail and Ade-loye, 2016). Biosensors have been fast gaining attention due to their compact size which can reduce overall cost of biosensor use (Azmi et al., 2017). Nitrate reductase is used in synthesis of biosensors as it can convert nitrate to nitrite. Can et al. (2012) studied the efficacy of a carbon nanotube/Polypyrrole/nitrate reductase biofilm electrodes for nitrate detection with promising results.

### Alternatives

Due to the proposed detrimental health effects of nitrates, studies are being carried out to look for alternatives to these nitrates. One approach would be to eliminate nitrates in meat processing by “natural curing,” i.e., by using high-nitrate vegetable sources such as celery powder with nitrate reducing bacteria (Sindelar and Milkowski, 2012). The addition of chemicals such as fumarate esters, butylated hydroxyanisole, sulfur dioxide etc. could be effective, however, the long term effects of such chemicals cannot be predicted (Gassara et al., 2016). Another technique could be the addition of products such as ascorbate and ascorbic acids, which can prevent the formation of nitrosamines from nitrites, thus eliminating the carcinogenic potential of nitrates (Jiménez-Colmenero et al., 2001). The use of coloring agents, such as erythrosine to give the characteristic color of cured meats, as well as a combination of chemical antioxidants to obtain the flavor of cured meats could also be a possible alternative to nitrates (Pegg et al., 1997; Shahidi, 1989). An extensive review by Gassara et al. (2016) describes the potential of different spices as alternatives to nitrates due to their antioxidant and antibacterial properties.

### Future Perspectives

Despite recent years of research, there is still ambiguity regarding both, the positive and negative effects of nitrates. Therefore, extensive clinical studies should be conducted to understand the pharmacokinetics and metabolism of nitrates in humans (Keeton, 2017). While the Acceptable Daily Intake (ADI) value of nitrates is  $3.7 \text{ mg kg}^{-1} \text{ day}^{-1}$ , Rebelo et al. (2015) suggest that due to their potential to cause methemoglobinemia, determining the Acute Reference Dose (ARfD) value for nitrates may be more appropriate to evaluate safety of exposure. The American Academy of Pediatrics suggests that methemoglobinemia in infants can be prevented by excluding nitrate-rich vegetables such as carrots, squash, spinach etc. in their diet till the age of three months (Cunningham, 2013). Since a large portion of dietary nitrates comes from vegetables, the development of new cultivars that have reduced nitrate accumulation and higher stress tolerance could be an interesting option (Cavauiuolo and Ferrante, 2014). Additionally, more eco-friendly agricultural practices such as crop rotation, livestock management etc, can effectively reduce the amount of nitrogen fertilizers, without drastically affecting productivity (Socaci and Stănilă, 2007). Several studies have shown that organic vegetables have a lower nitrate content than conventionally grown ones, and therefore some scientists suggest that the consumption of organic vegetables could reduce dietary exposure of nitrates (Garcia and Teixeira, 2017). Modifications of culinary practices such as cooking at high temperatures could also reduce the toxic nitrosation products of nitrate metabolism (Celada et al., 2016). With respect to the

use of nitrates in food preservation, further studies of alternate technologies such as UV radiation, ionizing radiation, high-pressure processing etc., are being studied to improve their cost efficacy (Carocho et al., 2014).

## Conclusion

The use of nitrates for preservation of meat is an ancient practice. During the late 90s, the use of nitrates of curing reduced drastically, however in recent times there has been some ambiguity regarding the proposed detrimental effects of nitrates in human health. On the one hand, alternatives for addition of nitrates for meat curing are being looked into, and on the other hand, vegetables that are rich in nitrates are being evaluated for their beneficial effect on cardiovascular health. As nitrates are naturally occurring ions, and a part of our regular diet, closer understanding of the mechanism of nitrates in the human body is needed to educate the public regarding the possible risks and benefits associated with nitrates.

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# Oligosaccharides: Structure, Function and Application

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## Glossary

**Galactosylation** The reaction in which a galactosyl donor is attached to a hydroxyl or other functional group of another molecule (a glycosyl acceptor). Likewise, transgalactosylation is the transfer of a galactose residue from one glycoside to another.

**MALDI-TOF-MS** Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry is used to analyze large biopolymers such as proteins, carbohydrates and DNA through ionization with low fragmentation followed by mass spectrometry.

**Microbiota** A microbial community; commonly used to refer to the microbial population itself located within a specific habitat (e.g. the gut microbiota).

**-omics** The high-throughput research methods that are used to analyze the interactions of information objects in the complete sets of molecules within biological systems (-omes), such as metagenomics, transcriptomics, proteomics and metabolomics.

**Prebiotic** Is a food ingredient that is not digestible by humans, and that promotes the growth of beneficial microorganisms in the gastrointestinal tract.

**Probiotics** Are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.

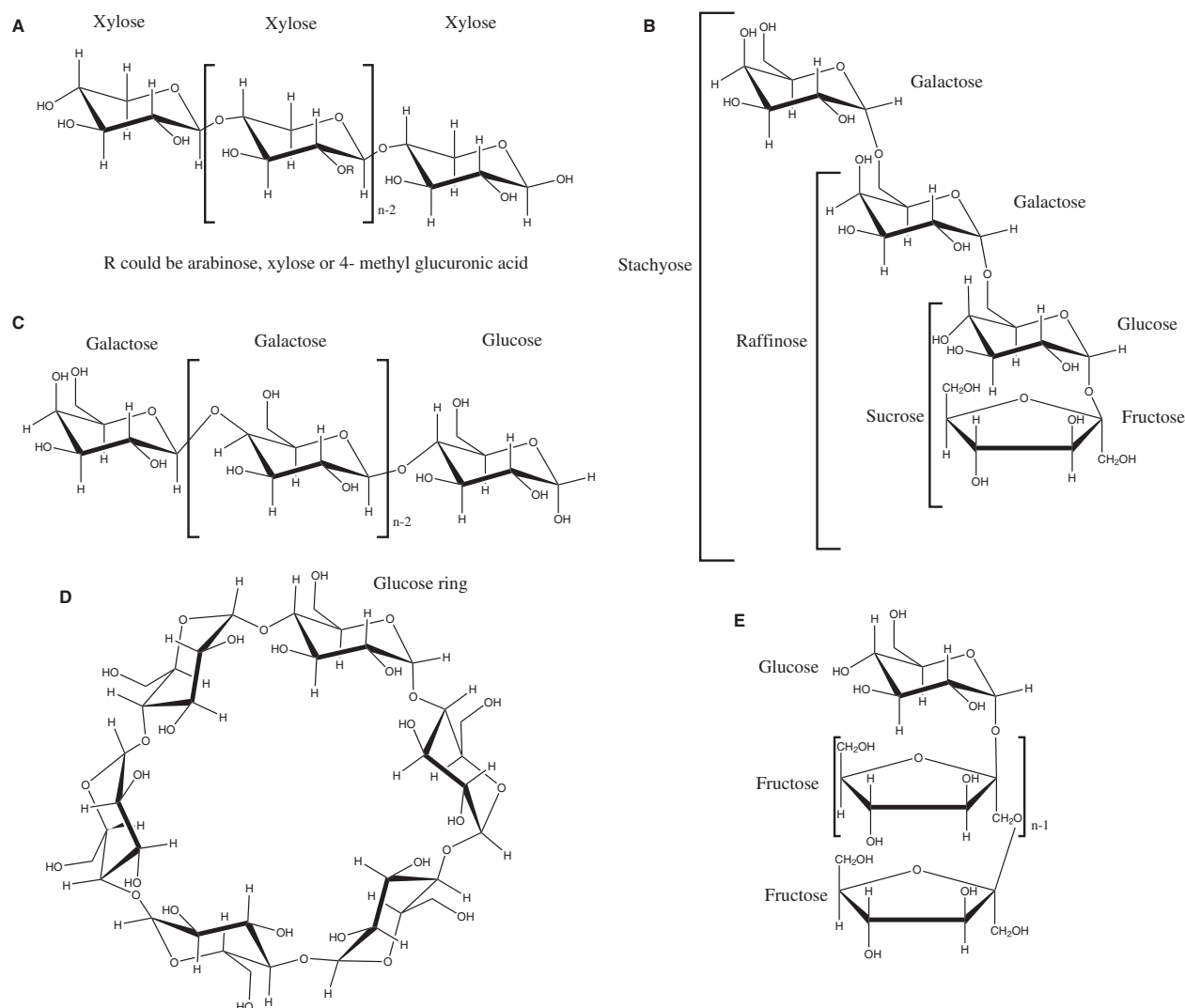
## Introduction

Non-digestible oligosaccharides possess many important physicochemical and physiological properties, e.g. they function as prebiotics to improve gut microecology and immunomodulation and provide protection against pathogen adhesion. These properties have greatly advanced their rapid industrial applications in the last few years.

Compared to polysaccharides, oligosaccharides have much lower molecular weight, therefore high water solubility. By definition (IUBIUPAC nomenclature), oligosaccharides are saccharides with Degree of Polymerization (DP) value ranging from 3 to 10. However, molecules of DP 11 to 19 are generally also recognized as oligosaccharides due to their similar properties. The types of naturally occurring oligosaccharides and their sources have been well summarized in **Table 1** according to a previous review paper (Mussatto and Mancilha, 2007). The typical structural features of oligosaccharides are differentiated by monosaccharides and linkages (**Fig. 1**). For example, fructooligosaccharides are D-fructose residues linked by  $\beta$  (2 $\rightarrow$ 1) bonds with a terminal  $\alpha$ -(1 $\rightarrow$ 2) linked D-glucose; xylooligosaccharides are constructed by  $\beta$ -XylP bonded by 1 $\rightarrow$ 4 linkages, occasionally branched by arabinose, xylose or 4-methyl-glucuronic acid sugar residues according to the source; the raffinose family is comprised of sucrose bonded by one (raffinose) or two (stachyose)  $\alpha$ -galactose residues through 1–6 linkages. However, the low abundance of the naturally occurring polysaccharides cannot meet the high needs for their industrial applications. Most of the oligosaccharide products on the market are commercially produced by either polysaccharide hydrolysis or enzymatic/chemical synthesis from disaccharide substrates, e.g. xylooligosaccharides from hydrolysis of arabinoxylan polysaccharides (**Fig. 2**); fructooligosaccharides from hydrolysis of inulin; galactooligosaccharides and some fructooligosaccharides produced by transgalactosylation (Fischer and Kleinschmidt, 2018) and transfructosylation, respectively (**Fig. 3**). Soybean oligosaccharides and raffinose, however, are still commercially produced by direct extraction.

**Table 1** Sources of naturally occurring oligosaccharides

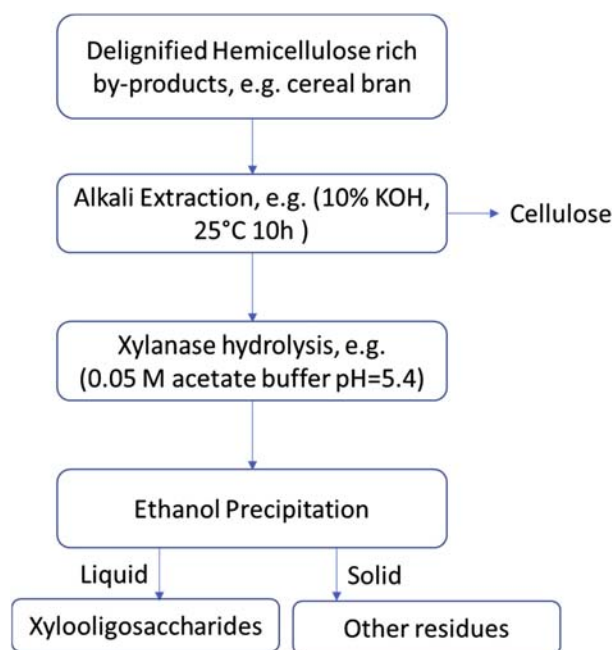
<i>Naturally occurring oligosaccharides</i>	<i>Example sources</i>
Fructooligosaccharides	Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey
Xylooligosaccharides	Bamboo shoots, fruits, vegetable, milk, honey
Galactooligosaccharides	Human milk
Raffinose oligosaccharides	Seeds of legumes, lentils, peas, beans, chickpeas, mallow, composite, and mustard
Cyclodextrins	Water soluble glycans



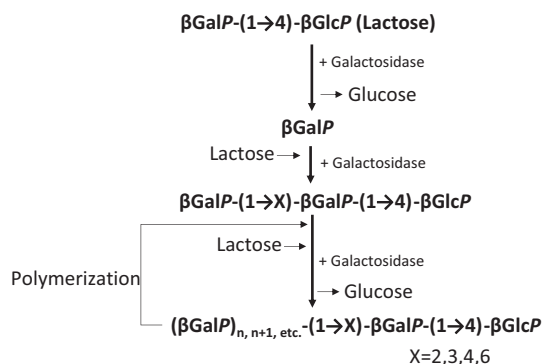
**Figure 1** Demonstration of typical structures of different oligosaccharides. (A): xylooligosaccharides; (B): raffinose family; (C): galactooligosaccharides; (D): cyclodextrin (CD); (E): fructooligosaccharides.

## Structural Characterization

Structural characterization is a must for the labeling of commercial products as well as for establishing their structure and function relationships. However, the commercially produced oligosaccharides differ in degree of polymerization, branching character and concentrations for each oligosaccharide molecule, which increase the difficulties for their structural analyses. Most of the naturally occurring oligosaccharides, and oligosaccharides generated from polysaccharide hydrolysis or enzymatic and chemical synthesis, exist in mixtures. Therefore, isolation, purification and fractionation are always prerequisites prior to structural analysis. For example, galactooligosaccharides produced by  $\beta$ -galactosidase hydrolase could be separated into various DP fractions using bio-gel P-2 column, as previously reported (van Leeuwen et al., 2014; Guo et al., 2017). These fractions can be monitored either by high performance size exclusion chromatography (HPSEC) coupled with an RI detector on a Rezex RSO-01 oligosaccharide Ag+ column (Fig. 4A and B), or by high performance anion exchange chromatography coupled with a PAD detector on a CarboPac PA-1 column (Fig. 4C). The whole mixture can also be monitored by MALDI-TOF-MS (van Leeuwen et al., 2016). The building blocks of the oligosaccharides and corresponding linkage patterns can be revealed by monosaccharide analysis combined with methylation analysis. The detailed sequencing information as well as the configuration ( $\alpha$ - or  $\beta$ -) of each sugar residue can be obtained with the help of 1D & 2D NMR analyses. Using the above mentioned methods, over 40 different molecular structures were successfully uncovered in commercially produced galactooligosaccharide mixtures, providing a structural coverage of over 99% of the products (van Leeuwen et al., 2014).



**Figure 2** Xylooligosaccharides enzymatically produced from hemicellulose rich agricultural by-products. Adapted from [Bian et al. \(2013\)](#).

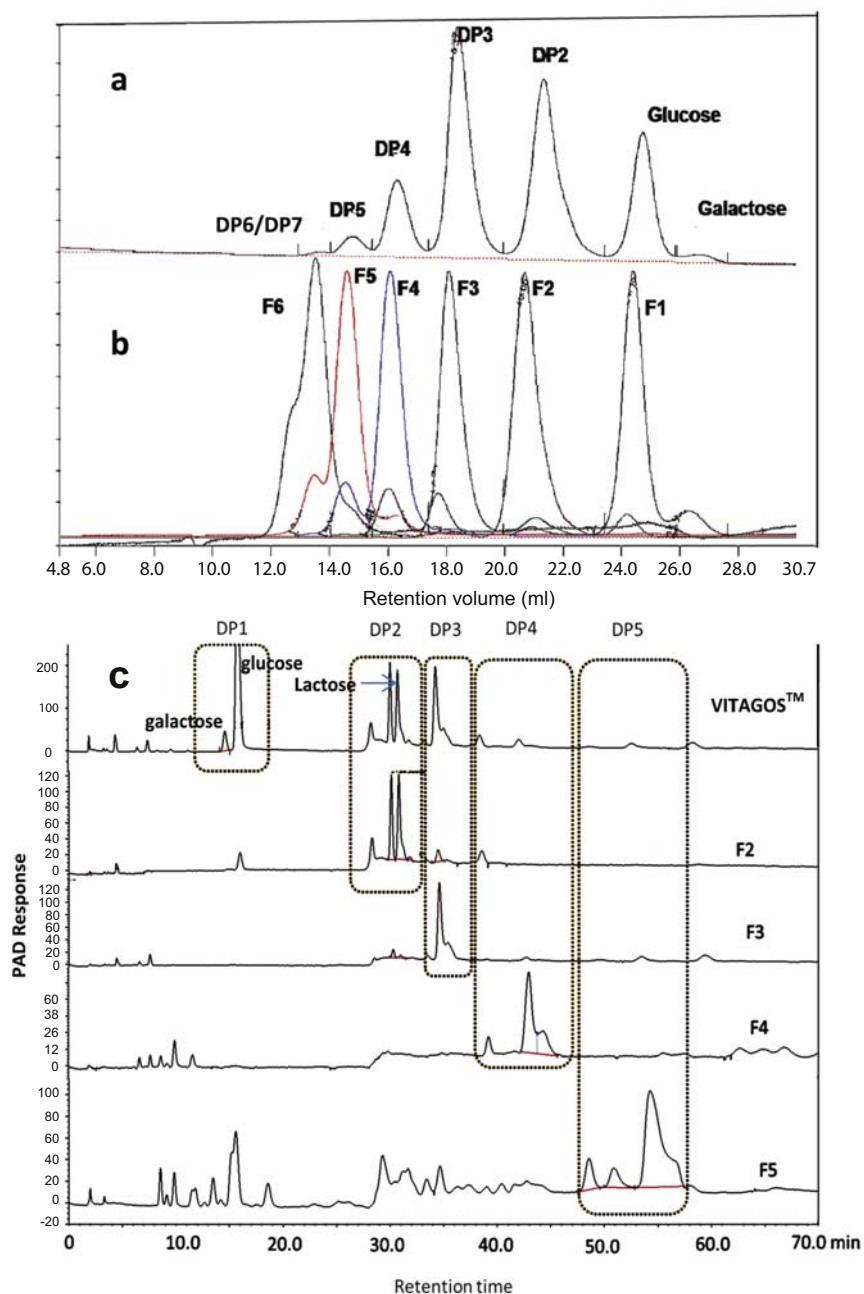


**Figure 3** Demonstration of the main reactions occurring during lactose hydrolysis and GOS synthesis. Numerous other side reactions are also possible to generate free galactose and other by-products.  $X=2,3,4,6$  indicates that the glycosidic bond can occur at several carbon atom positions on the galactose molecule, leading to many different oligosaccharides.

## Commercial Products and Naturally Occurring Oligosaccharides

A number of non-digestible oligosaccharides have been used in the food industry as prebiotic supplement products or food ingredients ([Table 2](#)). As food ingredients, the non-digestible oligosaccharides may be used in beverages, dairy foods, synbiotic products (combining probiotics and prebiotics), bakery, chocolate products, sweeteners and meat products. Aside from their prebiotic or health role, their other main technologically functional role would be as bulking agents or texturing agents to enhance total solids without greatly increasing viscosity, as polysaccharides would.

It should be noted that although many galactooligosaccharides and fructooligosaccharides have been commercially produced using enzymatic synthesis methods to mimic naturally occurring ones in milk, structurally they are quite different. For example, human milk oligosaccharides are comprised of the five monosaccharides glucose, galactose, N-acetylglucosamine, fucose and sialic acid, with N-acetylneuraminic acid. However commercially synthesized galactooligosaccharides and fructooligosaccharides added to infant formula contain galactose and fructose oligomers, which do not naturally occur in human milk. In addition, the fructose monomer itself is not found in human milk. Furthermore, synthesized galactooligosaccharides and fructooligosaccharides are neither fucosylated nor sialylated as they are in human milk. These structural differences could cause some function deficiency in the commercial products, such as antiadhesive antimicrobial effects. However, galactooligosaccharides and fructooligosaccharides could still influence the microbiota composition of the infant's feces and function as prebiotics ([Bode, 2012](#)).



**Figure 4** Oligosaccharides profile of VITAGOS™ (A) and fractions collected from biogel P-2 column (B), from HPSEC (separated by DP value) and HPAEC analysis (C). Adapted from [Guo et al. \(2017\)](#).

**Table 2** Non-digestible oligosaccharides in prebiotic supplement products

Company	Product	Non-digestible oligosaccharide	References
VITALUS	Vitagos	GOS	<a href="#">Guo et al. 2017</a>
Suntory Ltd	Xylo-oligo	XOS	<a href="#">Grizard and Barthomeuf 1999</a>
The Vitamin Shoppe	NutraFlora FOS	FOS	<a href="#">Douglas and Sanders 2008</a>
GlaxoSmithkline, Philadelphia, PA	FiberChoice	Inulin	<a href="#">Douglas and Sanders 2008</a>
Calpis Food Industry Co.	Soya-oligo	SOS	<a href="#">Meyer et al., 2015</a>

## Functional Properties of Oligosaccharides

Non-digestible oligosaccharides elude hydrolysis and absorption in the small intestine due to limited carbohydrate active enzymes in human cells (Sarbin and Rastall, 2011). They are fermented by the anaerobic bacteria in the small and large intestines as energy sources (Mussatto and Mancilha, 2007). In the human gut, most anaerobic bacteria are benign; however, certain species are associated with acute and/or chronic disorders (Mussatto and Mancilha, 2007). Some non-digestible oligosaccharides have prebiotic activity, meaning they are selectively fermented by a limited number of gut bacteria (including probiotics) such as *Bifidobacteria* and *Lactobacillus*, and may promote human health by increasing populations of beneficial microbes and/or their metabolic activity (Sarbin and Rastall, 2011; Mussatto and Mancilha, 2007; Rivi re et al., 2016). For instance, the well-known prebiotic oligosaccharides such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), isomaltoligosaccharides, soybean oligosaccharides and lactosucrose have been shown to increase *Bifidobacteria* and/or *Lactobacilli* populations. This can improve human health due to increasing fecal dry weight and producing more vitamins of the B group and nicotinic acid (Sarbin and Rastall, 2011; Mussatto and Mancilha, 2007; Rivi re et al., 2016).

The gut microbes fermenting prebiotic oligosaccharides produce short chain fatty acids (SCFAs), such as acetate, propionate, and butyrate (Mussatto and Mancilha, 2007; Rivi re et al., 2016). For instance, *in vivo* studies report that arabinoxylan-oligosaccharide (AXOS) consumption increases faecal butyrate concentrations (Rivi re et al., 2016). The pH value in the colon decreases through SCFA production, which can inhibit the growth of pathogenic bacteria, as well as increase mineral absorption by an osmotic effect (Mussatto and Mancilha, 2007). Furthermore, SCFA also exerts anti-inflammatory action and are implicated in the regulation of lipogenesis (Flint et al., 2015), contributing to other beneficial effects on human health. Butyrate is the preferred energy source for epithelial cells, stimulating colonic epithelial cell growth and increase salt and water absorption, thus relieving constipation (Mussatto and Mancilha, 2007; Rivi re et al., 2016). Moreover, butyrate was also found associated with inhibiting proliferation of colorectal cancer cells and changing cancerous cells to a normal cell type (Lim et al., 2005). Depending on the type of prebiotic oligosaccharides and composition of the gut bacteria, the quantity and ratio of SCFA product could be changed (Mussatto and Mancilha, 2007). For instance, *Bifidobacteria* produce acetate, lactate and formate, but no study reported that it could produce butyrate (Sarbin and Rastall, 2011). Butyrate is mainly produced by *Faecalibacterium prausnitzii* and *Eubacterium rectale*, which are two of the most dominant butyrate-producing bacterial species, belong to *Clostridium* clusters IV and XIVa, respectively (Rivi re et al., 2016).

Although non-digestible oligosaccharides have significant beneficial effects on human health, excessive consumption of non-digestible oligosaccharides may cause flatulence through gas production, or osmotic diarrhea in some instances (Mussatto and Mancilha, 2007). Furthermore, recent studies reported that consuming FODMAPs (Fermentable oligosaccharides, disaccharides, monosaccharides and polyols) may exacerbate symptoms of bowel inflammation in certain individuals who either malabsorb or are sensitive to FODMAPs (Rao et al., 2015).

## Relationship Between Non-digestible Oligosaccharides and Human Gut Bacteria

Over the last decade, *in vivo* studies have shown that some non-digestible oligosaccharides change gut microbiota composition at the species level (Scott et al., 2013; Flint et al., 2015). There are many structural factors, such as monomer saccharide composition and glycosidic linkages, that affect the selective utilization of non-digestible oligosaccharides by gut bacteria at the strain level (Sarbin and Rastall, 2011; Hamaker and Tuncil, 2014). This is primarily because the gut bacteria have different oligosaccharide digestive abilities, preferences and metabolite processes (Hamaker and Tuncil, 2014; Sarbin and Rastall, 2011). For instance, the  $\alpha$ -linkage of glucosyl-glucose has higher selectivity than that of galactosyl-galactose, but for  $\beta$ -linkages, the selectivity is opposite (Sarbin and Rastall, 2011). The  $\beta$ -1,3 and  $\beta$ -1,6 linkages of galactose have higher selectivity than  $\beta$ -1,4 linkages of galactose for *Bifidobacteria* (Sarbin and Rastall, 2011). Furthermore, molecular weight or degree of polymerization (DP) of non-digestible oligosaccharides can also influence selective fermentation (Sarbin and Rastall, 2011). Oligosaccharides of low molecular mass or DP are more selectively fermented than high molecular mass or DP by *Bifidobacteria* and *Lactobacilli*. This is because the oligosaccharides with low molecular mass (low DP) have more non-reducing ends that are preferentially attacked, for example by the exo-glycanase produced by *Bifidobacterium* spp (Sarbin and Rastall, 2011). However, low molecular mass or DP of non-digestible oligosaccharides may cause rapid consumption before reaching the distal regions of the colon, and thus leading to loss of its selective function (Sarbin and Rastall, 2011). For instance, a DP of 3 of isomalto-oligosaccharides has a higher selective stimulation than a DP of 2 for human fecal *Bifidobacteria* (Sarbin and Rastall, 2011).

As gut bacterial species differ in oligosaccharide degradation mechanisms and preferences, they may cooperate with each other when they utilize oligosaccharides (Rivi re et al., 2016; Scott et al., 2013). Recently, simple batch modeling, such as mono-culture and co-culture *in vitro* studies, were widely used for tracking the communities of specific gut bacteria and their metabolites when degrading oligosaccharides (Flint et al., 2015). For instance, in a co-culture study, *Bacteroides longum* LMG 11047 was able to consume released fructose and oligo-fructose from inulin, while another strain, *Bacteroides thetaiotaomicron* LMG 11262, had high digestive ability of both oligo-fructose and inulin. When the two strains were co-cultured in a medium with inulin as the sole carbon source, the *B. longum* LMG 11047 was outcompeted by *B. thetaiotaomicron* LMG 11262, which became the predominant strain (Sarbin and Rastall, 2011). Although we have less understanding about how they competed with each other, we know that *Bifidobacteria* have about 5% of their genome coding genes involved in carbohydrate internalization and prefer the transport of short



chain oligosaccharides into the cell (Rivière et al., 2016). The internal or cell-bound degradation is more efficient than extracellular degradation in a highly competitive gut ecosystem (Rivière et al., 2016; Sarbini and Rastall, 2011). However, a strain of *Bifidobacteria* was reported that could cross feed with other gut bacteria by carbohydrate breakdown (Sarbini and Rastall, 2011; Rivière et al., 2016). *Anaerostipes caccae* DSM 14662 and *B. longum* BB536 were co-cultured in the presence of oligo-fructose. The release of free fructose into the extracellular environment by *B. longum* BB536 fed *A. caccae* DSM 14662, a strain without digestive ability of oligo-fructose (Sarbini and Rastall, 2011). These observations indicate that interactions between *Bifidobacteria* and other gut bacteria are strain-dependent (Flint et al., 2015). Many studies also reported that metabolite production could be the substrate for the cross-feeding mechanism as well (Flint et al., 2015). For instance, lactate is associated with certain gut disorders, which is produced by *Bifidobacteria* as one fermentation product (Sarbini and Rastall, 2011). However, lactate can be utilized by *Megasphaera elsdenii* and converted into propionate, or converted into butyrate by *E. hallii* (Sarbini and Rastall, 2011; Flint et al., 2015). Acetate is converted into butyrate via the butyryl CoA: acetate CoA transferase route by butyrate-producing bacteria, such as *F. prausnitzii* and *E. rectale* (Scott et al., 2013). Furthermore, these two species grow better in the presence of acetate (Rivière et al., 2016). However, the result of *in vitro* co-culture studies do not fully reflect *in vivo* complexity of the gut microbiome (Sarbini and Rastall, 2011).

There is presently a need for better understanding of the potential effect of the prebiotic oligosaccharides on human health. Accordingly, molecular microbiological techniques, metagenomics, transcriptomics, proteomics and metabolomics studies are very useful for yielding more detailed information on the interaction between the specific structure of substrate and metabolism of the gut bacteria at the strain level (Sarbini and Rastall, 2011; Scott et al., 2013). Combining *in vivo* and *in vitro* results together will accelerate our understanding about the relationship between probiotics and prebiotics (Scott et al., 2013; Sarbini and Rastall, 2011).

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## Pectin in Foods

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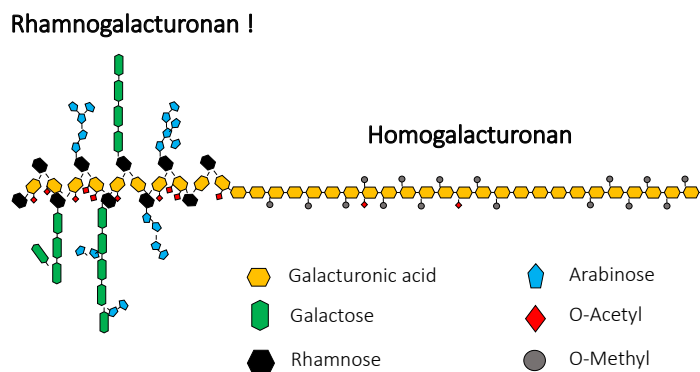
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### Source and Structure

Pectin is a major component of primary cell walls in all flowering plant genera except for the grasses of the Poales (Jarvis et al., 1988; Chan et al., 2017). Consequently, all fruits and vegetables consumed by humans contain pectin (Baker, 1997; Van Buggenhout et al., 2009; Taylor, 2012). Pectin synthesis within a plant cell occurs in the Golgi apparatus and the *Trans*-Golgi Network and is then trafficked to the plasma membrane within secretory vesicles (Driouich et al., 2012; Anderson, 2016; van de Meene et al., 2017). It has been hypothesized that at least 67 different glycosyl, methyl and acetyl transferases are required for pectin biosynthesis (Mohnen, 2008). Pectin's biological functions are numerous and mirror many of its technological applications. Pectin functions in plant cells include cell–cell adhesion, structural support, hydration control, positioning of leaf and floral primordia and as signaling molecules in plant biochemical pathways (Mohnen, 2008; Peaucelle et al., 2008; Atmodjo et al., 2013).

Pectin is composed of up to 20 different sugar moieties (Vincken et al., 2003; Paniagua et al., 2017). Galacturonic acid (GalA) is the dominant component comprising 50%–85% the pectic sugars (Yapo, 2009; Yuliarti et al., 2015; Müller-Maatsch et al., 2016). Arabinose, galactose and rhamnose are the other major pectic sugars. Structurally pectin is a hetero, block-copolymer. Although three dimensional models of pectin structural domains have been described (Pérez et al., 2000) an accurate three dimensional model of its global *in-vivo* structure has been elusive. Pectin has been shown to interact with cellulose, arabinogalactan proteins and xyloglucan to form a single interconnected network (Wang et al., 2016; Phyto et al., 2017). Small angle X-ray scattering (Alba et al., 2017) has also been used to look at the influence of pH on pectin's *in-vivo* structure where it was found that only strongly acidic environments affected structure. Contact between pectin and cellulose has been demonstrated in intact cell walls using solid state NMR (Dick-Perez et al., 2012). The majority of our understanding of pectin structure and functionality has been garnered from pectin that has been extracted from plant tissues. Global structural characterization has been approached with the use of atomic force microscopy (AFM) (Fishman et al., 2015; Kozioł et al., 2017; Paniagua et al., 2017), which has allowed pectin structures to be described as rods, segmented rods, kinked rods, branched chains, spheres and aggregates (Posé et al., 2012; Cybulska et al., 2015; Fishman et al., 2015).

Within these AFM visualized pectin structures are two commonly described dominant regions or block polymers. They are the homogalacturonan (HG) region and the rhamnogalacturonan I (RG I) region (Fig. 1). A structurally minor, but functionally important region is known as rhamnogalacturonan II (RG II) (Mohnen, 2008; Atmodjo et al., 2013; Shi et al., 2017). The HG region is a linear homopolymer of  $\alpha$ -1,4 linked D-GalA. Reports on the degree of polymerization (number of contiguous GalA subunits) for HG regions generally range between 80–120 GalAs (Tanhattan-Nasseri et al., 2011). The GalA subunits within the HG may be methylesterified at the C6 carboxyl group. The pectin being deposited in new cell walls is highly methylesterified but may be subsequently modified by the enzyme pectin methylesterase (PME) (Mohnen, 2008). Consequently the proportion of these subunits that are methylesterified, commonly known as the degree of methylesterification or esterification (DM or DE), varies within a population of pectin molecules. This leads to variability in the distribution of charge within the HG regions RG I is a branched copolymer with a backbone of repeating  $[\rightarrow 2)\text{-}\alpha\text{-L-rhamnosep-(1}\rightarrow 4)\text{-}\alpha\text{-D-galacturonic acidp-(1}\rightarrow 3)]_n$  (Voragen et al., 2009). Both neutral arabinan and (arabino-) galactans may be attached to O-4 of the rhamnose residues (Albersheim et al., 1996; Prade et al., 1999; Ridley et al., 2001). Some of these neutral side chains have been shown to occur in previously unknown configurations (Wefers and Bunzel, 2016). Other minor neutral sugars may also occur (Mohnen, 2008).



**Figure 1** Schematic representation of pectin rhamnogalacturonan I and homogalacturonan regions.

## Functionality

The HG region is widely recognized as the dominant functional region of pectin (Willats et al., 2006; Cameron et al., 2015; Kim et al., 2017) although the RG I and RG II regions also contribute (Sousa et al., 2015; Shi et al., 2017). Pectin functionality is largely dependent on its molecular weight, degree of methylesterification and the spatial distribution of charge within the HG region (Willats et al., 2001; Rolin, 2002). For some functionalities, such as water holding capacity, the distribution of the charge is more important than the DM (Willats et al., 2001). Considerable effort has been undertaken to map the distribution of charged (non-methylesterified GalAs) (Grasdalen et al., 1996; Daas et al., 1999; Neiss et al., 1999; Limberg et al., 2000; Ralet and Thibault, 2002; Kim et al., 2017) and neutral (methylesterified GalAs) domains within the HG region (Ralet et al., 2012; Remoroza et al., 2014; Cameron et al., 2015). The charged domains are required for crosslinking two or more pectin molecules via divalent cations (Powell et al., 1982; Luzio and Cameron, 2008) which is mandatory for gelation in the absence of sugar and low pH. Results typically show that the average number of charged blocks required per molecule must be greater than two and that some fraction of molecules in a given population must be trifunctional or more (contain three blocks or more) for a gel to form (Flory, 1941; Luzio and Cameron, 2008). These types of pectin (calcium sensitive pectin), commonly have a low DM (less than 50%). But in some instances, such as when pectin is used to stabilize acid dairy drinks, high DM pectins (greater than 50%) can also be made calcium sensitive (Rolin, 1994; Luzio et al., 2002; Tromp et al., 2004). Gelation of high DM, non-calcium sensitive pectins is a result of hydrogen bonding, dipolar and van der Waals interactions, and hydrophobic associations (Oakenfull and Scott, 1984; Evageliou et al., 2000). For this type of gelation it is the neutral, methylesterified domains of pectin HGs that are crucial.

Calcium sensitivity can be introduced into high DM, non-calcium sensitive pectins through the use of PME (Duvetter et al., 2006b; Cameron et al., 2011; Ngouémazong et al., 2012) and by cold temperature alkali demethylesterification. Plant and some bacterial PMEs that possess an alkaline isoelectric point are generally most active at neutral to alkaline pHs, whereas fungal PMEs generally have acidic isoelectric points and are most active at lower pHs. At least one plant PME is commercially available in a crude extract from papaya (Vasu et al., 2012). While fungal PMEs have a pseudo-random mode of action (Duvetter et al., 2006a) all plant PMEs have thus far shown the ability to hydrolyze GalA methyl esters with a processive, or blockwise, mode of action and the average sizes of the contiguous demethylesterified GalA blocks produced vary dependent on the enzyme and reaction conditions, such as pH, used (Cameron et al., 2008, 2011; Kim et al., 2013, 2014, 2017). Characterizing the inherent charge distribution, or that resulting from treatments to demethylesterify pectin, has been approached with several techniques including NMR (Grasdalen et al., 1996; Catoire et al., 1998; Kim et al., 2005), capillary electrophoresis (Williams et al., 2003) and enzyme-mediated structural characterizations (Daas et al., 1999; Cameron et al., 2008; Ralet et al., 2012; Kim et al., 2013).

The enzymatic methods developed by Cameron et al. (2008, 2011) estimate the average size of the charged blocks and their average number per molecule using a limited digestion with endo polygalacturonase (EPG). EPG excises the charged blocks from the parent molecules. Subsequently high performance anion exchange chromatography (HPAEC) coupled to an evaporative light scattering detector (ELSD) is used to visualize and quantify the charged oligomers excised by EPG (Cameron and Grohmann, 2005). An alternative enzymatic method developed by Daas et al. (Daas et al., 1999) relies on exhaustive EPG digestion. Products from this reaction are GalA monomers, dimers, trimers and undigested methylester protected domains of the HG. Since GalA monomer, dimer and trimer are commercially available their quantities can be estimated from standard curves produced by either a pulsed amperometric detector or an ELSD coupled to HPAEC (Daas et al., 1999; Cameron and Grohmann, 2005; Kim et al., 2013). The method calculates a parameter defined as Degree of Blockiness (DB) which estimates the amount of demethylesterified GalAs that are located in charged blocks large enough to be susceptible to EPG activity. Guillotin et al. (2005) introduced the parameter of Absolute DB ( $DB_{abs}$ ) which extends the method of Daas et al. (1999) to estimate the percentage of total GalAs that are in charged blocks large enough to be susceptible to EPG activity. Both of these methods, exhaustive and limited EPG digestion focus on charged blocks within the HG region. Other enzymatic methods have been developed to characterize the more neutral, methyl protected domains within the HG region. Ralet et al. (Ralet et al., 2012) coupled a pectin lyase digestion with an exhaustive EPG digestion to describe two new parameters related to HG charge distribution. They were DBMe and  $DB_{absMe}$ . Coupling both of these methods Ralet et al. (2012) were able to clearly differentiate between HGs that had been demethylesterified by chemical (random mode of action), fungal PME (pseudo-random mode of action) or a plant PME. Cameron et al. (Cameron et al., 2015) presented a method to characterize the methyl-protected domains of the HG region that are not susceptible to EPG attack due to the methyl ester of GalA. Cameron et al. (2015) defined methyl-protected HG domains as those that did not have four contiguous demethylesterified GalAs since EPG is presumed to require a minimum of four contiguous non-methylesterified GalA for cleavage (Chen and Mort, 1996). This method incorporated a cold (4 °C) alkaline demethylesterification following an exhaustive EPG digestion. The initial EPG digestion produced GalA monomers, dimers and trimers while the charged oligomers resulting from alkaline treatment of the methyl-protected domains were larger. These larger, charged oligomers were then separated and quantified using HPAEC coupled to an ELSD. Cameron et al. (2015) used the parameters of  $B_{ME}$  (average number of methyl-protected domains per molecule) and  $BS_{ME}$  (the average size of a methyl-protected domain) to describe these domains. Significant correlations between parameters used to describe methyl-protected domains and DM,  $DB_{abs}$ , and rheological properties were observed. Combining both exhaustive and limited EPG digests these analyses show that, based on Pearson's correlation coefficients, the average size of non-methylesterified blocks and the average number of methyl-protected domains per molecule may be the most useful for predicting pectin functionality.

The goal of being able to engineer, or tailor, the functionality of the HG regions in a population of pectin molecules has been a motivating factor behind much of this research. Kim et al. (2017, 2018) hypothesized that it is possible to control the size of

demethylesterified blocks within pectin HG regions with the use of different isoforms of processive pectin methylsterases or a combination of a pseudo-random fungal pectin methylsterase coupled to a processive plant pectin methylsterase (Kim et al., 2017). Values for all four charge distribution parameters (BS, BN, DB and DB<sub>abs</sub>) could be varied by manipulating the extent of an initial demethylesterification by a pseudo-random fungal PME, the total activity units of the processive plant PME and the reaction pH (Kim et al., 2018). When only processive plant PMEs were used for demethylesterification (Kim et al., 2017), and the charged block distributions produced by three different PME isoforms were compared, differences in the distribution pattern could be only be discerned using the results from a limited EPG digest (BS and BN) not from an exhaustive EPG digest (DB and DB<sub>abs</sub>).

## Pectin and Food Quality

Engineering the functionally important properties of pectin (i.e., molecular weight, total charge and distribution of charge) when used as an ingredient for food formulation or its *in-situ* modification in processed food has a long history. Pectin manufacturers routinely modify production conditions, blend different batches to obtain desired functionality and standardize pectins with additives to accommodate a customer's requirements (May, 1990; Rolin, 2002). When used as an ingredient for food formulation pectin is commonly classified as a high DM pectin (HM, DM > 50%) or low DM pectin (LM, DM < 50%) (Chan et al., 2017). If needed the DM can be reduced by an acid or enzyme treatment (Rolin, 2002). In general high sugar amounts are required for gelling with HM pectin (Yoo et al., 2003) while Ca<sup>2+</sup> is used to gel LM pectins (Yang et al., 2017). Calcium sensitivity is dependent on the HG regions having multiple charged, demethylesterified blocks ranging somewhere between 8–20 GalA residues per block (Luzio and Cameron, 2008). A calcium sensitive pectin is one in which two or more HG regions on different molecules can be coordinated to a middle layer of calcium ions (Grant et al., 1973; Kohn, 1975; Braccini and Perez, 2001). HM pectin is generally not calcium sensitive but calcium sensitivity can be introduced with a processive plant PME with a minimal (5%) reduction in DM (Hotchkiss et al., 2002). This calcium sensitive HM pectin is a critical component in acid dairy drinks where it serves to maintain protein suspension via ionic interaction with the positively charged protein surface via the negatively charged blocks of demethylesterified GalA in the HG regions (Glahn and Rolin, 1996; Tromp et al., 2004). The charged blocks within the HG region are not the only structural feature contributing to the stabilization of acid dairy drinks. Non-calcium sensitive pectin can also stabilize acid dairy drinks though not as well as calcium sensitive pectin (Laurent and Boulenguer, 2003). Calcium sensitive pectin also has use in potential applications where softer gels are preferred because they retain more water than LM gels and require less sugar (Joye and Luzio, 2000; Willats et al., 2001).

The *in-situ* modification of pectin in processed food products enables engineering food quality (Christiaens et al., 2015). Unit operations used by food processors such as thermal, high pressure and homogenization treatments alter pectin structure and the organoleptic qualities of the processed product. Texture is a major quality-associated property affected by changes in pectin structure. High temperature processing is the most common method to preserve food and make it microbiologically safe. An unwanted side effect of thermal processing is  $\beta$ -elimination reactions that lead to pectin depolymerization and solubilization. This action is the primary causes of texture degradation in many processed foods (Sila et al., 2006a; De Roeck et al., 2009). High pressure pasteurization is becoming a more common method for making foods microbiologically safe (Huang et al., 2017) with an additional benefit of having a reduced effect on texture. When high pressure pasteurization is coupled with high temperatures (>90 °C) it is believed that  $\beta$ -elimination is reduced due to increased demethylesterification of pectin (De Roeck et al., 2009) since the  $\beta$ -elimination reaction requires an adjacent methyl ester. The increased demethylesterification is believed to be due to increased activity of endogenous pectin methylsterase (Sila et al., 2006b). Additionally, the demethylesterified HG regions are now available for cross linking with Ca<sup>2+</sup> ions which has a positive effect on texture preservation. Ca<sup>2+</sup> soaking or infusion is often coupled to high temperature and high pressure treatments to improve firmness but Smout et al. (2005) concluded that factors in addition to demethylesterification, such as cell damage, are needed to account for the improved firmness.

Food formulation also uses vegetables and fruits that have been processed to disaggregate them into sauces, purees or suspensions (Blatt et al., 2011; Christiaens et al., 2015). Unit operations used to produce these disaggregated products can have a significant effect on their performance as an additive (Lopez-Sanchez et al., 2011). Thermal treatment may activate both pectin methylsterase and endo polygalacturonase (an enzyme that depolymerizes non-methylesterified HG regions leading to a reduction in molecular weight). This is especially important in tomato processing for juice, sauce, puree and paste (Anthon et al., 2008). A low temperature pre-treatment also affected the consistency and separation of pulp and serum in carrot and broccoli due to a decrease DM and an increase in Ca<sup>2+</sup> cross linking (Christiaens et al., 2011a, b).

The most recent innovation focused on pectin in food formulation is the work by Vancauwenberghe et al. (2017, 2018) in which pectin is incorporated into food inks (pumpable liquid or mashed foods that can be solidified) for 3D food printing. While the initial study (Vancauwenberghe et al., 2017) required a post printing incubation in a Ca<sup>2+</sup> bath to insure cross linking the second study (Vancauwenberghe et al., 2018) used a coaxial extrusion method in which both the pectin food ink and the Ca<sup>2+</sup> solution were simultaneously extruded from the print head so no post extrusion Ca<sup>2+</sup> incubation was required.

Pectin also influences quality of fresh fruit and processed juices. Investigations into the role of pectin in fruit softening have been ongoing for many years. Most recently it was reported that pectate lyase, an enzyme that depolymerizes non-methylesterified HG regions has a major role in tomato softening (Wang et al., 2018). Pectin methylsterase also is responsible for cloud loss in citrus juices and other cloudy juices (Cameron et al., 1998; Baker and Cameron, 1999). Galant et al. (2014) was able to demonstrate that

a very small decrease (74.7% to 68.3%) in DM in orange juice pectin was sufficient to initiate juice cloud destabilization, leading to juice clarification.

The targeted modification of pectin to provide precision functionalities as an ingredient or in fresh and processed fruits and vegetables is an area of growing interest. Continued studies on how to engineer pectin structure to produce defined functionality will provide an expanded toolkit for food chemists and food technologists to help feed a hungry world.

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# Phospholipids

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## Structure and Occurrence

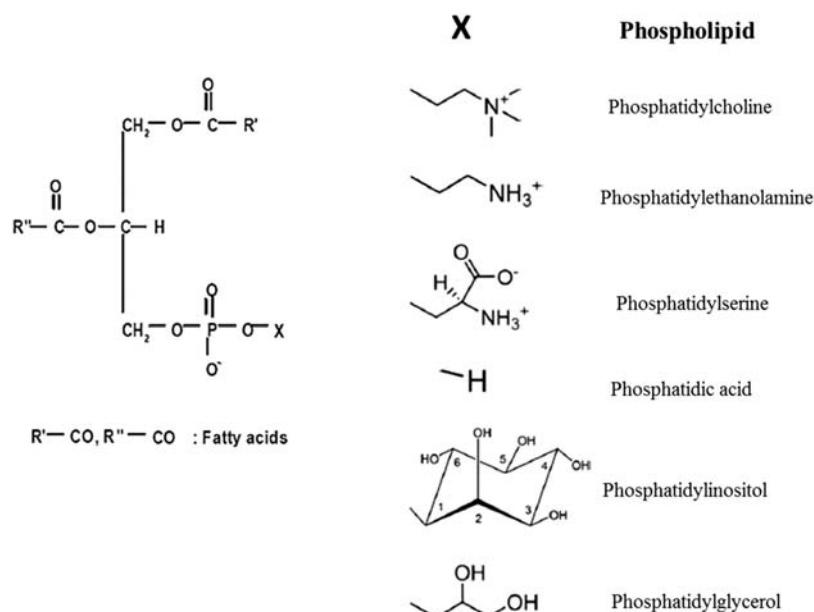
Phospholipids (PL) are a group of polar lipids that consist of two fatty acids, a glycerol unit and a phosphate group which is esterified to an organic molecule (X) such as choline, ethanolamine, inositol, etc. The structure of a PL molecule is shown in Fig. 1. The phosphate group together with the X molecule is called the head group of PL. The common PL classes found in nature include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), and phosphatidylglycerol (PG).

PL are major components of cell membranes, and exist in all living organisms. The contents of PL in various sources are different. In foods, the content of PL range from 11 mg/100 g to 5433 mg/100 g. Good sources of PL include eggs, meats, fish, shellfish, cereal grains and oilseeds (Weihrauch and Son, 1983). The PL composition is different depending on its origin. For example, although PL from both animals and plants contain phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) phosphatidylinositol (PI) and phosphatidic acid (PA), the content of PS in PL from animals is much higher than that in PL from plants (Guo et al., 2005). PG which is rarely found in plants and animals, is a major PL class in microorganism. Fatty acid composition of PL is dependent on the sources as well. PL from oilseeds contain fatty acids including palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3); while PL from egg yolk does not contain C18:3, instead, arachidonic acid (C20:4), eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) are present (Wang, 2007).

## Physiochemical Properties

### pH-Dependency of PL

Although various PL have a similar structure, there are differences in their properties due to the different head groups. For example, PC is positively charged at pH < 4, while at pH > 4, PC exists as a zwitterion, which is a neutral compound carrying electrical charge of opposite signs. Due to steric reasons, the negative phosphate group does not form an intramolecular salt with the positive quaternary amine group. Since the positive trimethylamino group remains isolated at all pH values, PC is hydrophilic at all pH values. As with PC, PE carries positive charge at pH < 4. PE turns into a zwitterion at 4 < pH < 10, and exists as an anion at pH > 10. Unlike PC, PE as a zwitterion can form an intramolecular salt due to the small ethanolamine group. As a result, zwitterion PE is weakly hydrophilic. PI carries no charge at pH < 4, and is negatively charged at pH > 4. PI is highly hydrophilic at all pH values due to the five free hydroxyl groups on the inositol moiety. PA carries no charge at pH < 4; at 4 < pH < 8, PA carries one negative charge; and at pH > 8 it carries two negative charges (Dijkstra, n.d).



**Figure 1** Structure of phospholipid. Adopted from Li et al. (2015) with permission.

## Phase Behavior

When dispersed in water, PL molecules aggregate to form two major structures: lamellar and hexagonal  $H_{II}$  phases (Fig. 2), which are interconvertible. The type of structures adopted depends on temperature, the saturation of the fatty acid chain, the nature of the head group, pH, water content, presence of divalent cations, etc. The increase in temperature usually induces the transition of the lamellar phase to the hexagonal phase, and the lamellar to hexagonal transition temperature varies with different PL classes. In general, PL with fully saturated fatty acid chains form lamellar phase at temperature range of 0–100 °C, regardless of pH and cations concentration. Unlike the other unsaturated PL classes that adopt the lamellar phase under physiological conditions (temperature, pH, salt concentration), the unsaturated PE adopts the hexagonal  $H_{II}$  phase under these conditions. Increase in the unsaturation of PE lowers the lamellar to hexagonal transition temperature. Increasing pH leads to the conversion of the hexagonal phase to the lamellar phase for unsaturated PE, which is uncharged at neutral pH. PS and PA carry negative charge at neutral pH, decreasing pH results in the transition from lamellar to hexagonal phase. In the presence of  $Ca^{2+}$ , PA adopts the hexagonal phase at neutral pH. For PC, lowering the water content induces hexagonal phase formation. As the membrane lipids, the phase preferences of different PL classes are of great importance to membrane functions (Cullis et al., 1990).

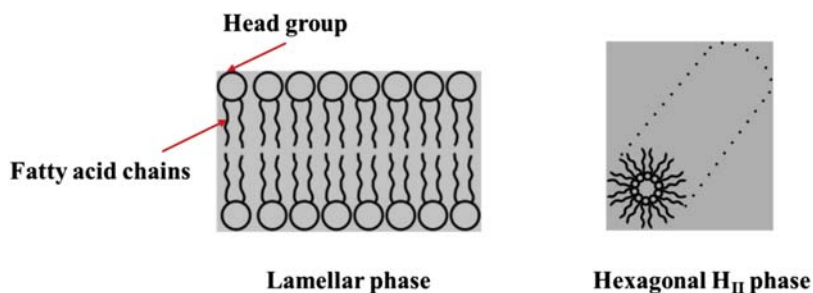
## Emulsifying Properties

PL are amphiphilic molecules containing both hydrophilic (the head group) and hydrophobic (acyl chains) moieties: they are natural emulsifiers with HLB values  $\sim 8$ . Since pH affects the association of the polar head groups of PL, the properties and stabilities of PL-stabilized emulsions are sensitive to pH change. At neutral pH, PC and PE exist as zwitterions. The stability of emulsions stabilized with these two PL are maintained mainly by hydration repulsion; while the stability of emulsions stabilized with PA and PI, which are anionic, are maintained by electrostatic repulsion, and are sensitive to changes in ionic strength and pH. PC has a tendency to favor the formation of oil-in-water (o/w) emulsions, while PE and PI facilitate the formation of water-in-oil (w/o) emulsions (McClements, 2005). PE and PA are sensitive to calcium, which could cause the loss of their function as surfactants. PL from egg yolk (contains mainly PC and PE) can form emulsions with great heat stability, and is usually used in the production of intravenously injectable emulsions. Interaction with proteins can improve the emulsifying properties of PL (Van Nieuwenhuyzen and Tomás, 2008).

## Oxidative and Antioxidative Properties

PL with unsaturated fatty acid chains are susceptible to oxidation. The nitrogen-containing moieties, ethanolamine and choline in PE and PC, respectively, can affect the oxidative stability of the corresponding PL (Corliss and Dugan, 1970). It is reported that PL can act as prooxidant, possibly by causing the decomposition of hydroperoxides which results in the formation of new radicals. However, PL has been demonstrated to exhibit antioxidant activities in various systems including vegetable oils (Doert et al., 2012; Hildebrand et al., 1984), marine oils (Olcott and Van Veen, 1963; Segawa et al., 1994), lard (Koga and Terao, 1994) and aqueous emulsion system (Cardenia et al., 2011.). There are four different mechanisms proposed to explain the antioxidant activity of PL:

1. Synergism between PL and other antioxidants. PL used alone in oil model show little primary antioxidant activities, however, PC, PE and PS work effectively as synergists to phenolic antioxidants such as tocopherol, vitamin C and ethoxyquin. There is no synergism observed between other antioxidants and PG/PI (King et al., 1992). The amino groups in PE and PS is believed to be involved in the regeneration of tocopherol from tocopheroxyl radicals, exerting the synergistic effect (Doert et al., 2012). PC and PE are reported to improve the radical-scavenging activity of tocopherol by making it more accessible to the radicals (Koga and Terao, 1995).
2. Binding of pro-oxidant metals. Acidic PL such as PA, PS and PG have been reported to show effective binding ability for the transition iron ion, which is a prooxidant for lipid oxidation. However, only PS exerts antioxidant activity (Dacaranhe and Terao, 2001).
3. Formation of Maillard-type products between PL and oxidation products



**Figure 2** Phase diagrams of phospholipid. Modified from Li et al. (2015) with permission.

The amino group of nitrogen-containing PL, PE specifically, may react with oxidation products of unsaturated fatty acids (aldehydes) to form Maillard-reaction products, which have been reported to show antioxidant activity (King et al., 1992).

#### 4. As non-radical peroxide decomposer

It is reported that PC can perform as free radical scavenger, and react with peroxy radicals to produce trimethylammonium oxides; while PE can decompose lipid hydroperoxides in a non-radical way to yield imines (Pokorny, 2002).

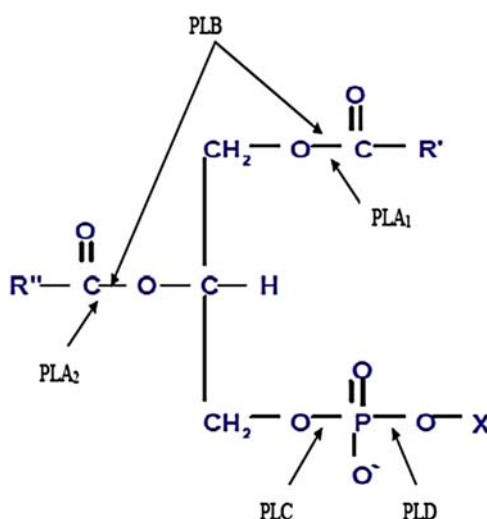
## Enzymatic Hydrolysis of PL

PL can be hydrolyzed by five different phospholipases: phospholipase A<sub>1</sub>, A<sub>2</sub>, B, C and D. The acting sites of these phospholipases are shown in Fig. 3. Phospholipase A<sub>1</sub> and A<sub>2</sub> specifically hydrolyze 1- and 2- acyl ester bonds of PL, respectively. The products of these enzymes are FFA and 1- or 2- LPL. Phospholipase B can hydrolyze both 1- and 2- acyl ester bonds, yielding FFA and water-soluble glycerophosphodiester (Merkel et al., 1999). Phospholipase C cleaves the phosphorus-oxygen bond between glycerol and phosphate, producing diacylglyceride and a phosphorylated head group. Phospholipase D hydrolyzes the phosphorus-oxygen bond between phosphate and the head group, and the products are phosphatidic acid and a free head group (Wang, 2001).

## Production of Phospholipid

Currently, PL is produced commercially in the form of lecithin, which is a mixture of different PL, glycolipids, triglycerides, and other minor components. Lecithin is a by-product of the degumming step in vegetable oil refining. Crude vegetable oils usually contain a certain amount (<3%) of PL, which need to be removed to ensure the quality of the final edible oil products. Degumming is the step designed to remove PL from the oil, where about 2.5% of water is added to the heated oil and mixed vigorously for about 30 min, the hydrated PL is then separated as gum from the oil by centrifugation. Other compounds such as glycolipids, free fatty acids, triglycerides and so on are also incorporated in the gum together with PL. The gum which contains about 50% of water is then dried to a water content of < 1%, resulting in a liquid-form of lecithin product. A liquid lecithin usually contains about 37% of neutral oil (triglycerides, free fatty acid, sterols, etc.), 45% of PL, glycolipids and carbohydrates. Washing the liquid lecithin with acetone would remove the neutral oil, yielding a de-oiled lecithin containing mainly PL (~74%), glycolipids (~17%) and carbohydrates (~5.5%), which are acetone insolubles (AI). Lecithin can be produced from various plant sources. At present, Soy bean is the main source for lecithin production. In food industry, lecithin is commonly used as emulsifier due to the emulsification properties of PL (Van Nieuwenhuyzen and Tomás, 2008).

Several methods are currently used to change the composition or chemical structure of PL in lecithin, in order to improve its emulsifying properties. Fractionation of lecithin with ethanol would yield ethanol soluble fraction enriched in PC and ethanol insoluble fraction enriched in PI. When compared to the parent lecithin, the PC-enriched fraction is a better emulsifier for oil-in-water emulsions, and PI-enriched is better for water-in-oil emulsions. Treating lecithin with peroxide or organic acid can result in the hydroxylation of the unsaturated fatty acids of PL, which can improve the hydrophilicity and emulsifying properties of lecithin. Treating lecithin with acetic anhydride would lead to the acetylation of the amino group in PE, producing acetyl-PE which can enhance the oil-in-water emulsifying properties of lecithin. Enzymatic treatment of lecithin with phospholipase A<sub>1</sub> or A<sub>2</sub> is a method



**Figure 3** Acting sites of phospholipases.

used to hydrolyze PL into lyso-PL (compounds with only one acyl chain compared to PL), which possess better emulsifying properties than PL (Van Nieuwenhuyzen and Tomás, 2008).

## Extraction and Analysis

The Folch extraction method is commonly used to extract PL from plant or animal or microorganism samples. In this method, chloroform/methanol 2:1 (v/v) is used as the solvent to extract all the lipids from samples including triacylglycerol, free fatty acid, PL, glycolipid, etc. PL is then separated from other neutral lipids by column chromatography or solid phase extract (SPE) before further analysis. For the past few decades, qualitative analysis of PL has been carried out through thin layer chromatography (TLC), which is only semi-quantitative. Nowadays, qualitative and quantitative analyses of PL are commonly performed by high performance liquid chromatography (HPLC) with various detectors. Silica columns are the most used stationary phase, but other columns are also used such as diol, cyano, amino, etc. Gradient elution of columns is usually applied with different mobile phases, which are mixtures of various solvents including chloroform, methanol, water, hexane, propanol, and so on. Several detectors can be used for HPLC analysis of PL. UV detector and evaporative light scattering detector (ELSD) are the two most commonly used ones. Although fluorescence detector are highly sensitive for PL, a derivatization of PL into fluorophore compound is required for the analysis, thus it is rarely used. Charge aerosol detector (CAD), which has greater sensitivity and better precision than ELSD, is a relatively new detector for PL analysis (Kielbowicz et al., 2013). <sup>31</sup>P-NMR is considered to be the ideal method for PL analysis. About 15 phospholipid classes (including lyso-phospholipid) can be separated and quantified by this method, while the HPLC method reported now can only separate less than 10 PL classes. However, the extremely expensive instrument required and the low sensitivity make it less popular than the HPLC method (Van Nieuwenhuyzen and Tomás, 2008).

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## Phosphates

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### Introduction

Phosphates are derivatives of phosphoric acid ( $\text{H}_3\text{PO}_4$ ) with positively charged ions of elements, such as sodium, to form salts (inorganic phosphates), or with organic groups, such as phenyl, to form esters (organic phosphates). Phosphates irrespective of whether they are inorganic phosphates or constituents of phosphoproteins and membrane phospholipids, are found in most living organisms. Phosphates are an essential dietary requirement for humans. Organic phosphates from meat, grains, dairy products, and nuts, and inorganic phosphates (Pi) from food additives are readily absorbed in the small intestine, processed in the liver, stored in cells and bone, and reabsorbed in the kidneys (Takeda et al., 2004). Phosphate has many physiological, biochemical and cell signalling roles in the body. Phosphates are also used as additives to a range of food products where they serve as to aid processing, enhance organoleptic properties or improve safety and shelf-life. An appropriate balance between the use of phosphates as additives and possible ill effects associated with excessive consumption needs to be struck.

### Applications of Phosphates in Food

Phosphates are present in a range of foods typically including protein rich products such as meat, poultry, seafood and dairy products. Phosphates are used as additives to food to serve as preservatives on meats, as stabilizers and as melting salts for the processing of cheese products. These food grade phosphates aid in maintaining the structure and hydration of the meat products by enhancing the water holding capacity of muscle and assisting in the oxidative stability of the meat by chelating pro-oxidative metal ions (Lampila, 2013). In dairy products, phosphates are used as a protein dispersant in spray dried milk products. Potassium or sodium salts of phosphates are also used as emulsifiers in food such as processed meat and cheese. Processed food products tend to have a higher phosphate content than that naturally present on food. For instance, processed meat and poultry products were reported to have twice the amount of phosphate as compared to natural products (Sherman and Mehta, 2009). Levels of phosphates permitted for use in foods must be “generally be regarded as safe” by the Food and Drug Administration (FDA) (del Río et al., 2007; Ellerbroek et al., 1996).

### Poultry and Meat Products

Food grade phosphates are commonly used in the meat and poultry industry in the form of polyphosphates or pyrophosphates where they cause the sequestration of metal ions and dissociation of the acto–myosin complex (Fonseca et al., 2011). Application of phosphates on meat may increase the pH of meat, as the pH of most food grade phosphates ranges from 6.3 to 12.5, which will increase the ionic strength and water holding capacity of meat (Lampila, 2013; Vasavada et al., 2006). For this reason they are used as an aid in decreasing cooking loss, controlling pathogen growth, reducing drip loss and purge in chilled storage and improving textural properties (Lindsay, 2008; Lampila, 2013). They also prevent the discoloration of poultry and meat products. In addition, polyphosphates have been found to delay the rancidity of some products due to the sequestration of iron and copper (Coul-tate, 2009). Polyphosphate levels between 0.1% and 0.3% do not appear to pose a risk to health since they are broken down into pyrophosphates which is converted into orthophosphates in most meat products. The presence of phosphates in meat may result in high concentrations of salts, causing protein denaturation under frozen storage.

Sodium tripolyphosphate (STPP) ( $\text{Na}_5\text{P}_3\text{P}_{10}$ ) is commonly used in poultry and meat processing. This compound is used in blends with sodium hexametaphosphate [ $(\text{NaPO}_3)_n$ ,  $n = 10\text{--}15$ ] to improve the tolerance of calcium ions in brines for the curing of meat products. In brines, calcium may be complexed with hexametaphosphate which is soluble and prevents calcium phosphate precipitation. Previous studies have found that the combination of sodium phosphate and sodium chloride decreases the requirement of salt from 3% to 3.5% to 1.4% to 1.8% (Lindsay, 2008). A combination of these compounds solubilizes the myosin with the hydrophobic tail surrounding the fat and the hydrophilic end binds to the water molecule. These two compounds enhance the succulence of the cooked products and maintains the water holding capacity of the product (Lampila, 2013).

The strong antioxidant effect of phosphates against oxidation of lipids in cooked meat products occurs through the binding of metal ions that act as a catalyst for oxidation. Previous studies have reported that encapsulated phosphates enhances the stability and functionality of antioxidant activity and was able to inhibit lipid oxidation in ready-to-eat meat products as well as extend their shelf life (Kilic et al., 2014; Sickler et al., 2013). Sodium pyrophosphate (SPP) and STPP with and without encapsulation were reported to inhibit lipid oxidation in raw and cooked ground chicken and beef (Kilic et al., 2014). Excessive levels of phosphates use will incur higher costs and may affect the organoleptic qualities of the meat due to the amount of hydrogenated oil used in the encapsulation of the phosphate in addition to the high phosphate levels used.



## Dairy Products

Phosphates are added to dairy products, such as milk and cheese, for protein dispersion, acidification, gelation, supplementation of nutrients, chelating calcium and aiding in emulsification (Kapoor and Metzger, 2008). Tetrasodium phosphate is used in chocolate milk to maintain higher viscosity levels for the dispersion of cocoa powder (Lampila, 2013). Acidification by phosphoric acid is used to produce buttermilk. In addition, disodium phosphate is used in milk prior to drying to provide hydration and assist in protein dispersion. The use of trisodium phosphate in evaporated milk ensures that the butterfat is not separated from the aqueous phase (Lindsay, 2008).

There is a provision by the Codex Standards for the addition of low levels of polyphosphates in milk powders (Codex Alimentarius Commission, 2011), although milk powders do not usually contain polyphosphates. The use of polyphosphates such as sodium hexametaphosphate and sodium tripolyphosphate delays gelation via protein denaturation. Ultra-high temperature (UHT) processed milk is well known to gel upon storage. However, sodium hexametaphosphate was reported to enhance the shelf life of UHT milk products by delaying the onset of gelation due to observed proteolysis during the storage by milk proteases such as plasmin and exogenous enzymes from bacterial contamination (Anema, 2015). Another factor for the delay in gelation was the onset of gelation occurred without visible changes to proteins present in the milk (Datta and Deeth, 2001). Alkaline phosphatase is also used to determine if adequate pasteurization has been conducted in the dairy industry (Kilic et al., 2014).

As cheese aging involves proteolysis, it weakens the cheese emulsion. Heat treatment of cheese may separate the emulsion into coagulated protein and butter oil. The role of phosphates will be beneficial as it alters the proteins to form melting salts. During the process of cheese production, dicalcium phosphate and sodium caseinate is formed from disodium phosphate and calcium caseinate. Sodium caseinate generates a layer surrounding the fat droplet to form the desired characteristics of cheese (Lampila, 2013). In addition, sodium aluminium phosphate (SALP) is used as an emulsifier to process cheese and its associated products. The mode of action of SALP is it alters the protein in order to produce a smooth layer surrounding the fat droplets to prevent the separation of fat from the cheese product. SALP will assist in forming a soft texture and aid in easier melting characteristics of the cheese products (Yokel et al., 2008).

## Control of Pathogens Using Inorganic Phosphates

Among the inorganic phosphates, trisodium phosphate (TSP) is a well-recognised antimicrobial used in poultry processing facilities. Trisodium phosphate is formed by neutralizing phosphoric acid with sodium hydroxide as follows:  $\text{Na}_2\text{HPO}_4 + \text{NaOH} \rightarrow \text{Na}_3\text{PO}_4 + \text{H}_2\text{O}$ . Low reactivity of  $\text{Na}^+$  and  $\text{PO}_4^{3-}$  ions which are produced due to the dissociation of TSP when mixed with water causes extremely low by-product formation. This inorganic phosphate is an alkaline sanitizer and is used between pH 10–12. Gram negative bacteria such as *Campylobacter* and *Salmonella* are more susceptible to alkaline sanitizers including TSP at this pH range than Gram positive bacteria due to the presence of the thin peptidoglycan layer (Capita et al., 2002; Dickson et al., 1994). TSP is known for its surfactant properties which aid in preventing *Campylobacter* and *Salmonella* attachment to poultry. In addition TSP has the ability to remove some fat from the meat which indirectly aids in the removal of bacteria attached to the fat on the meat (Sarjit and Dykes, 2015). It is also able to remove the protective lipid layer on the meat enabling it to reach the entrapped pathogens thereby reducing the prevalence of these pathogens on meat (Capita et al., 2002).

TSP is regarded as safe for use on poultry at concentrations between 8% and 12% without hampering the organoleptic qualities of meat. At these concentrations, TSP has the ability to disrupt *Salmonella* spp. cell membranes due to the high pH resulting in the detachment of *Salmonella* cells from poultry and its associated surfaces through sequestration of metal ions (Sam-pathkumar et al., 2003). Effectiveness of TSP on both *Campylobacter* spp. and *Salmonella* spp. on poultry has been studied as indicated in Table 1. The occurrence of carryover of residues in the immersion chillers may alter the pH of TSP in water reducing its effect in reducing the numbers of pathogens in the chillers. To overcome this, air chilling may be used instead of immersion chilling (Buncic and Sofos, 2012).

## Regulations That Control the Use of Phosphates

At an international level, the World Health Organisms (WHO) and the Food and Agriculture Organisation (FAO) play leading roles in regulation of food additives. As both these organisations (WHO and FAO) have an interest in food regulation, they have set up different joint institutions that provide scientific information to United Nation member countries.

One of which is the Joint FAO/WHO Expert Committee on Food Additives (JECFA). This organisation lists over 100 different chemical forms of phosphate that can be used in food for different purposes, some of these applications include as flavouring agents. Phosphates can also be considered a food contaminant as some of its metal can contaminate food. Phosphates also make up the chemical structures of some mycotoxins and can be included in naturally occurring toxicants. They also make up the chemical structures of some veterinary drugs which can find its way into the food chain. The presence of toxicants, naturally occurring or otherwise and veterinary drugs are controlled by the maximum residual level regulations. Maximum residual level

**Table 1** Reduction of pathogens on poultry treated with trisodium phosphate

Sample	Pathogen	Concentration	Exposure time and temperature	Reduction (log units)	References
Chicken drumsticks	<i>C. jejuni</i>	12%	1 min dip at ambient temperature	$3.6 \pm 0.60$	Koolman et al. (2014)
Chicken legs	<i>S. Enteritidis</i>	12%	15 min dip at $20 \pm 1$ °C	$2.91 \pm 1.94$	Alonso-Hernando et al. (2013)
Chicken meat	<i>Campylobacter</i> spp. <i>Salmonella</i> spp.	12%	10 min at 4 °C	~1.81 to less than the limit of detection (<2.02) ~2.46 to less than the limit of detection (<2.02)	Sarjit and Dykes (2015)
Duck meat	<i>Campylobacter</i> spp. <i>Salmonella</i> spp.	8, 10, 12%	10 min at 4 °C	Less than the limit of detection (<2.02)	Sarjit and Dykes (2015)
Chicken legs	<i>S. Enteritidis</i>	12%	15 min at 20 °C	~2.0	del Río et al. (2007)

regulations controls the highest concentration of these chemicals that can be found in a food product that is meant for human consumption. If the concentrations are above this level then they cannot be legally sold as food.

The second WHO/FAO joint organisation is the Codex Alimentarius Commission, commonly known as the Codex. This organisation promotes the establishment of food regulatory frameworks within member countries that are compatible with Codex so as to promote international trade. Codex standard 192 (CODEX STAN 192-1995) is the General Standard for Food Additives which lists all of the food additives and their different chemical forms that can be used in human food. The standard has been revised over the years and the latest revision was in 2017. The Standard states that food additives can only be used when it has an advantage, such as preserving the product or maintaining its quality, and there is no known health risk to the consumer. Food additives cannot be used to mislead the consumer, in other words additives can be used only if it serves a technological function. The amount of a food additive added is controlled to the lowest quantity needed to achieve the required functional effect. The use of these additives are based on the risk assessments and exposure assessments. The JECFA carries out the risk assessment of all these chemicals and this information is provided to the Codex to be included in the evaluation process. The Codex standard (CODEX STAN 107-1981 Rev 2016) states that food additives need to be clearly labelled and must not lead to confusion or mislead the consumer. The Codex standard (CODEX STAN 192-1995) further states that only the food additives listed in it are recognised as suitable for human consumption and that these food additives must have an assigned acceptable daily intake and must be used considering these levels. The Codex lists 30 different chemical compounds of phosphates that are approved for human consumption as shown in **Table 2**. The FAO/WHO food standards not only lists the different chemical forms of phosphate that can be used but it also lists the different food products the chemical forms can be used in and their maximum concentrations. A food manufacturer (if within a Codex signatory country) is obliged to meet these requirements as a minimum level. Some countries or regions have their own maximum usage levels that form part of their regulations within that country.

### The United States of America

The Food and Drug Administration (FDA) of the US Department of Human Services is responsible for developing and administering food law within the United States (US) at a Federal level. The Food Additive Status List provides all of the additives that can be used in food and their different forms. This list needs to be read in conjunction with the different regulations. Some of the phosphate based compounds are listed as Generally Recognised as Safe (GRAS) and can be used considering Good Manufacturing Practises (GMP), which means there may be no regulation that controls its maximum usage levels but it can be used only at levels to achieve the required function. The FDA issues regulations that can be binding obligations and be enforced by law. Title 21 of the Code of Federal Regulations deals with food, Chapter I Subchapter B part 170 of the Federal law deals with Food Additives. Part 172 – Food Additives Permitted for Direct Addition to Food for Human Consumption gives the forms of phosphate that can be used in food for different purposes and the maximum levels that can be used at. For example, monosodium orthophosphate can be used to esterify starch where the residual phosphate in the food does not exceed 0.4% calculated as phosphorus. Part 173 – Secondary Direct Food Additives Permitted in Food for Human Consumption provides the concentrations of different forms of phosphate that can be used in material that may come in to contact with food, for example the concentration of phosphate esters that can be used in lye-peeling solutions, which must not exceed 0.2%.

### Australia

Food law in Australia at a Federal level is governed by the Food Standards Australia New Zealand (FSANZ). Each Australian state and territory has adopted the FSANZ standards as law within their jurisdictions and is enforced by a state government department and the local city councils. FSANZ recognizes that food additives play an important part in ensuring safety and quality required by

**Table 2** Codex list of 30 different phosphate base compounds that are approved for human consumption

<i>Additive name</i>
Ammonium dihydrogen phosphate
Ammonium polyphosphate
Bone phosphate
Calcium dihydrogen diphosphate
Calcium dihydrogen phosphate
Calcium hydrogen phosphate
Calcium polyphosphate
Diammonium hydrogen phosphate
Dicalcium diphosphate
Dipotassium hydrogen phosphate
Disodium diphosphate
Disodium hydrogen phosphate
Magnesium dihydrogen diphosphate
Magnesium dihydrogen phosphate
Magnesium hydrogen phosphate
Pentapotassium triphosphate
Pentasodium triphosphate
Phosphoric acid
Potassium dihydrogen phosphate
Potassium polyphosphate
Sodium calcium polyphosphate
Sodium dihydrogen phosphate
Sodium polyphosphate
Tetrapotassium diphosphate
Tetrasodium diphosphate
Tricalcium phosphate
Trimagnesium phosphate
Tripotassium phosphate
Trisodium diphosphate
Trisodium phosphate

consumers. The Australian food standards, Standard 1.3.1 Food Additives and the Schedules published together with a list of all the different food additives can be found on the Federal Legislation Website. These schedules includes the different chemical forms of phosphate that can be used in food, the purposes that they can be used of and the maximum permitted levels.

The Australian food law, Standard 1.3.1 states that a food additive is a substance added to food for one or more technological or functional purposes as listed in Schedule 14. Schedule 14 lists the different technological functions that a food additive can perform, including their definitions. Schedule 15 and 16 list the substances that can be used as food additives, the food they can be used in and the maximum allowable levels. In these documents different phosphate compounds that can be added to different products at different maximum concentrations are listed. Schedule 7 lists the different classes of food additives which are to be included in the ingredient lists on food product labels. In Australia food additives must be listed on product labels by their class name followed by the name of the food additive or by its number.

## European Union

The European Commission's regulation EC 1333/2008 sets out the regulations on food additives that govern the rules within the different countries in the European Union. The aim of the regulation is to standardise the use of food additives within the different countries of the Union. It provides the different food additives that can be used in food and the maximum concentrations they can be used at, the functions that these can be used for and the labelling requirements. The safety of food additives are evaluated and approved for use within the Union by the Scientific Committee on Food (SCF) and/or the European Food Safety Authority (EFSA). Only additives that are approved by these two organisations can be used in food within the EU and that too only for the purpose(s) it was considered safe. Under Regulation No 1333/2008 Annex II the European Commission has a database of all food additives that are approved for use within the EU. It is designed as a tool to inform stakeholders of the approved food additives, their purposes of use and the maximum levels of usage. In this database there are 17 different phosphate containing phosphate based compounds listed. It also includes the functions for which they can be applied for and the maximum levels they can be included in food.

## Health Impacts Due to Phosphates in the Diet

As a biomolecule, the tetrahedral molecular geometry of phosphate allows it to form bonds with four other molecules and create complex molecular compounds such as nucleic acids, proteins, ATP and phospholipids (Azevedo and Saiardi, 2017). The importance of phosphate in the human body is evident by the fact that 80%–90% of filtered plasma phosphate is actively reabsorbed in the renal tubules in a healthy individual (Prasad and Bhadauria, 2013). However, the amount of phosphate additives in the average North American's diet has more than doubled since 1990, with an estimated half the population of the USA consuming in excess of the recommend ADI of phosphates (Ritz et al., 2012; Uribarri and Calvo, 2013). Elevated blood serum levels of phosphate has been linked to a wide range of cardiovascular diseases (Ritz et al., 2012). The link between elevated phosphate levels in the body and these diseases in at risk patients is a relatively new concept, and there are still many gaps in our understanding as to how they are associated (Selamet et al., 2015). However, it does appear from the evidence currently available that certain foods have a significant impact on homeostatic levels of phosphates which in-turn could be associated with an increased risk of mortality in patients unable to regulate phosphate levels (Moore et al., 2015).

## The Roles of Phosphates in the Body and Hypophosphatemia

The availability of phosphate on Earth when the first living organisms emerged was scarce. It is theorised that early metabolic pathways could have relied on biomolecules composed of ions such as iron and sulphur or carbon-based thioesters (Goldford et al., 2017). If these pathways existed in primitive cells, they have likely been replaced by molecules composed of phosphates suggesting that these phospho-molecules provided a selective metabolic advantage to the cells. Nucleotide molecules are composed of a 5-carbon ring, a nitrogenous base and phosphate groups. In DNA and RNA the phosphate groups bind the hydroxyl group on the 3' carbon and allow for the formation of genetic sequences. In energy-donating nucleotide molecules such as ATP and GTP, the electrostatic repulsive forces between phosphate groups can be released following phosphate hydrolysis and used to power energy dependent processes in the cell (Cooper, 2000).

The main structural component of the cellular membrane are phospholipids; carbon chains with a phosphate group on the extremity of one end of the molecule (Alberts et al., 2002). Phosphate is a polar anion and is therefore attracted to water, whereas the fatty acid tails of the phospholipids are hydrophobic. This configuration causes the phospholipid molecule to orientate itself with the phosphate heads facing the extra- or intracellular environment, whereas the fatty-acid tails aggregate with the fatty acid tails of other phospholipids, forming a membrane bilayer. This configuration allows the membrane to be selectively permeable, meaning it can allow for the passive diffusion of certain molecules into and out of the cell (Alberts et al., 2002).

Bone is comprised of an organic matrix of collagen fibres and an inorganic layer of the calcium phosphate salt, hydroxyapatite (Shaker and Deftos, 2000). Collagen fibres reduce the brittleness of bone so that it has a small degree of flexibility under tensile stress, whereas the hydroxyapatite gives bone structural rigidity. Bone growth is dependent on the maturation, death and subsequent mineralisation of chondrocytes. In addition to the structural roles of calcium and phosphate ions in bone, calcium also regulates the maturation and organisation of chondrocytes whereas phosphate induces apoptosis, resulting in mineralisation. Low calcium and phosphate levels in bone tissue results in the build-up of un-mineralized osteoid tissue characteristic of rickets (Penido and Alon, 2012). In addition to the metabolic and structural roles phosphates play in the body, phosphoric acid and dihydrogen phosphate are also used by the body to maintain the pH of blood within homeostatic ranges (Anderson, 2013).

Phosphate is primarily stored in the bones and teeth of the human body with the rest present as buffering ions or bound to proteins in the blood, or stored as biomolecules in cells. The three major hormones responsible for regulation of phosphate levels in the blood are calcitriol, parathyroid hormone (PTH), and fibroblast growth factor 23 (FGF-23). Broadly speaking, PTH and FGF-23 decrease phosphate levels in blood plasma by reducing the expression of the sodium/phosphate co-transport proteins (NaPiII) in the proximal convoluted tube of the nephron. FGF-23 also indirectly decreases absorption of phosphate in the gut by decreasing the expression of other sodium/phosphate co-transporter proteins. Conversely calcitriol, increases absorption of phosphate in the intestines and inhibits the release of PTH, indirectly increasing phosphate reabsorption in the renal tubules (Penido and Alon, 2012).

Hypophosphatemia is a drop in plasma phosphate levels below 2.5 mg/dL. This can be caused by a variety of different factors such as malnutrition, alcoholism, vitamin D deficiency or resistance to vitamin D, physical trauma, renal disorders, increased serum insulin concentrations, or hereditary factors (Pappoe and Singh, 2010). Hypophosphatemia is associated with general organ dysfunction, impaired bone growth, and structural abnormalities in blood cells (Alberts et al., 2002; Mozingo and Mason, 2007). Patients suffering acute hypophosphatemia have weakened skeletal, smooth and cardiac muscle contractions, which can lead to cardiomyopathy and respiratory problems (Pappoe and Singh, 2010). This is likely due to a drop in intracellular reserves of energy donating phospho-molecules such as ATP which are essential for muscle contractions. In rare cases, low serum levels of phosphate and ATP also increases the rate of haemolysis (Melvin and Watts, 2002).

## Chronic Renal Disease can Induce Hyperphosphatemia

Hyperphosphatemia is abnormally elevated serum concentrations of phosphate (>1.46 mmol/L) generally associated with negative health outcomes if experienced for a prolonged period of time (Shaman and Kowalski, 2016). It is most commonly associated with impaired kidney function but can also be the result of increased dietary phosphate intake, tumor lysis syndrome, and

hyperparathyroidism (Nguyen and Wang, 2012). There is mounting evidence that increased phosphate levels increases the risk of cardiovascular diseases such as atherosclerosis, although there are conflicting arguments over whether this is directly related to hyperphosphatemia (Lau et al., 2010; Nguyen and Wang, 2012).

In healthy patients, roughly 950 mg of phosphorus is absorbed from the gut into the blood stream every day. Being a water soluble ion, phosphorus readily passes through the filtration membranes of the kidney and the majority is actively reabsorbed in the proximal convoluted tubule. In patients with chronic kidney disease the glomerular filtration rate steadily decreases leading to a build-up of serum phosphate levels. PTH and FGF-23 levels are elevated to reduce the phosphate re-absorption NaPi-II molecules in the renal tubules. However, the water soluble phosphate molecules can still passively diffuse from the filtrate into the renal vascular network. Additionally, as the glomerular filtration rate decreases these compensatory mechanisms are insufficient to normalise serum phosphate levels (Shaman and Kowalski, 2016). Interestingly, although PTH decreases the amount of phosphate transporters in the kidneys, it also increases osteoclast activity which leads to bone resorption and elevated calcium and phosphate levels in the blood (Hruska et al., 2008; Shaman and Kowalski, 2016).

### Hyperphosphatemia Association With Cardiovascular Disease

Vascular smooth muscle cells (VSMC) are responsible for the vasomotor properties of the endothelium of blood vessels. However, the contractile phenotype of these cells can be switched to a bone cell-like calcifying state via altered expression of calcification promoter and inhibitor molecules. This switch causes the VSMC to secrete bone matrix proteins, and can induce apoptosis which creates nucleation sites for hydroxyapatite crystals to form (Askar, 2015). The resulting calcification of blood vessels stiffens the walls of the vessels, creates occlusions, and increases blood pressure.

Increased Pi serum levels are associated with increased incidence of vascular calcification (Adeney et al., 2009; Lau et al., 2010). Although the exact mechanisms are still unclear, studies have shown that exposure to concentrations of phosphate similar to serum concentrations of patients with hyperphosphatemia increased the expression and phosphorylation of certain proteins and signaling molecules in VSMC (Lau et al., 2010). For example, exposure to increased phosphate levels increased the expression of Runx2 and Cbfa1 transcription factors which induces the secretion in bone matrix constituents in VSMC (Speer et al., 2010). Increased phosphate levels increases phosphorylation rates of the Bcl2 and ERK1/2 proteins. Although their mechanisms are distinct from each other, phosphorylation of these proteins increases apoptosis of the cells (Lau et al., 2010; Speer et al., 2010). The remains of apoptotic cells provides an “anchoring” site for hydroxyapatite crystals to form, increasing calcification of the surrounding tissue (Lau et al., 2010).

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## Relevant Websites

- Codex Alimentarius Commission (Codex) <http://www.fao.org/fao-who-codexalimentarius/en/>.
- European Food Safety Authority (EFSA) <http://www.efsa.europa.eu/>.
- European Commission database of all food additives [https://webgate.ec.europa.eu/foods\\_system/main/?sector=FAD&auth=SANCAS](https://webgate.ec.europa.eu/foods_system/main/?sector=FAD&auth=SANCAS).
- Food and Agriculture Organization <http://www.fao.org/about/en/>.
- FAO/WHO Food Standards Codex <http://www.fao.org/gsfaonline/groups/details.html?id=18>.
- Food Additive Status List by Food and Drug Administration (FDA) <https://www.fda.gov/food/ingredientspackaginglabeling/foodadditivesingredients/ucm091048.htm>.
- Food Standards Australia New Zealand (FSANZ) <http://www.foodstandards.gov.au>.
- Joint FAO/WHO Expert Committee on Food Additives (JECFA FAO) <http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/en/>.
- Joint FAO/WHO Expert Committee on Food Additives (JECFA WHO) [http://www.who.int/foodsafety/areas\\_work/chemical-risks/jecfa/en/](http://www.who.int/foodsafety/areas_work/chemical-risks/jecfa/en/).
- The Australian food standards, Standard 1.3.1 Food Additives and the Schedules <http://www.legislation.gov.au>.
- Title 21 of the Code of Federal Regulations deals with food, Chapter I Subchapter B part 170 of the Federal law deals with Food Additives <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=170>.
- World Health Organization (WHO) <http://www.who.int/>.



## Phytosterols

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### Glossary

**Blood cholesterol** Cholesterol concentration in the blood, which is used as a marker for the risk of coronary heart disease.

**Oleogel** Gel of a lipid phase, typically involving liquid triglyceride oils and gelling agents different from saturated triglyceride oils. Typically, oleogels can be considered a subclass of organogels, involving edible gelling agents and lipid phases.

**Triglyceride** Triacylglycerol, the main component of edible oils and fats.

**X-ray diffraction** Analytical technique to reveal long range translation order, typically in crystalline materials. The method uses x-ray radiation to probe the structure of the material.

### Sources of Plant Sterols

Steroids are triterpenes, organic compounds with a tetracyclic cyclopenta[*a*]phenanthrene structure (see Fig. 1) having two principal biological functions: signaling molecules that activate steroid hormone receptors, and components of cell membranes that affect the flexibility of the membranes in living cells. The present article concerns itself with sterols, a subclass of steroid alcohols.

Sterols of plant origin are usually referred to as phytosterols, and those of animal origin as zoosterols. These groups are not mutual exclusive, however. The most important zoosterol, cholesterol, can also be found in plant sources, sometimes in relative large quantities (for example in the nightshade and lily families). Further sterols are produced by yeasts and fungi, ergosterol being a common representative. Plant sterols on the one hand and zoosterols and sterols from fungi on the other hand differ in the common intermediate occurring in their biochemical synthesis. Plant sterol synthesis involves cycloartenol as an intermediate, whereas zoosterols and sterols from fungi are synthesized through a route involving lanosterol.

Phytosterols are naturally occurring steroid alcohols having the steroid structure with a hydroxyl group at position 3 and a side chain at position 17, usually containing one or more double bonds in the steroid skeleton. They can be obtained from many sources, but commercial sources include seed oils like corn, soybean and rapeseed oil at typical levels of 0.1%–1%. They are structurally closely related to stanols, which do not have any unsaturated bonds, and can be commercially obtained from hydrogenated sterols from e.g. woodpulp. In fact, stanols are usually considered to be part of the phytosterol group. Typically, sitosterol is the most abundant plant sterol, although stigmasterol and campesterol can often be found in substantial quantities too – depending on the particular source being used. Less common plant sterols include brassicasterol, avenasterol, and spinasterol (Akihisa et al., 1991).

The various phytosterols can be considered as closely related variations of the cholestanol molecule, a molecule with only saturated bonds in the triterpene ring system. Campestanol and sitostanol have an additional methyl or ethyl group respectively attached to the carbon at position 24 compared to cholestanol. Cholesterol, campesterol and sitosterol have an additional double bond between the carbons at position 5 and 6 compared to their stanol counterparts. Brassicasterol and stigmasterol have an additional double bond between carbons 22 and 23 compared to campesterol and sitosterol. Finally, ergosterol has an extra double bond between carbons 7 and 8 relative to brassicasterol (See Fig. 2). Obviously, we have restricted ourselves here to a limited number of well-known sterols, as the full list of sterols is much longer, having ~200 entries (Akihisa et al., 1991).

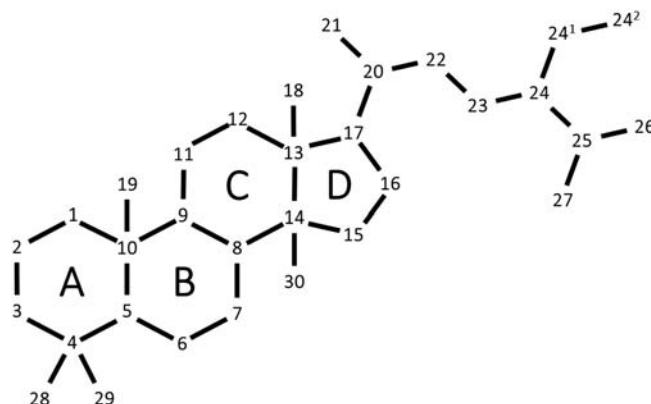
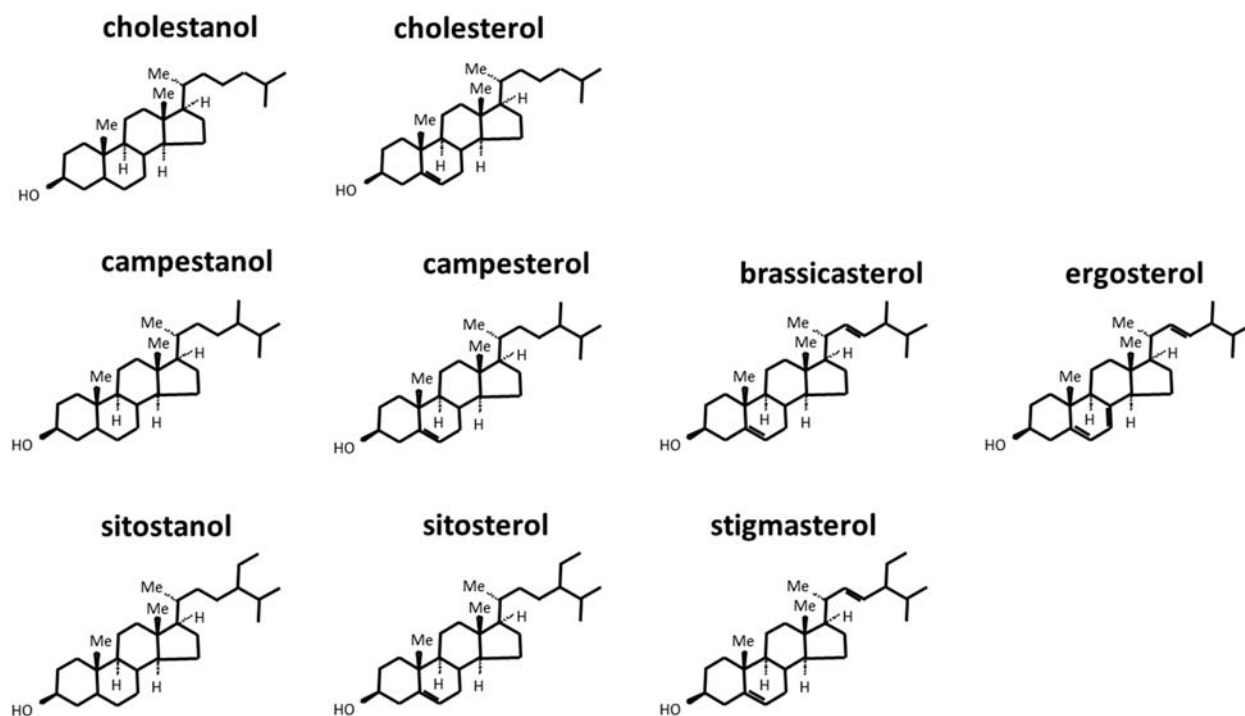


Figure 1 Steroid skeleton nomenclature.



**Figure 2** Structural relationships between selected sterols.

## Chemical Analysis

The official methods for sterol analysis are based on gas chromatography with flame ionisation detection (GC-FID), with an elaborate sequence of sample preparation steps: saponification to remove triglycerides from the sample, extraction of the phytosterol-rich unsaponifiables fraction by liquid extraction followed by fractionation using thin layer chromatography (TLC) or solid-phase extraction (SPE), derivatization to form phytosterol trimethylsilyl-ethers (TMS) or phytosterol acetates, before applying the GC-FID analysis ([International Organization for Standardization, 1999](#); [American Oil Chemists' Society, 2017](#); [Winkler-Moser, 2017](#)). In recent years, more rapid alternative methods have been developed which require a less extensive sample preparation method, often involving mass spectroscopic techniques ([Alberici et al., 2016](#)).

## Crystallization Behaviour

Sterols typically form  $\sim 3.8$  nm thick bilayers in their crystalline state ([Craven, 1986](#); [Argay et al., 1996](#); [Kawachi et al., 2006](#); [den Adel et al., 2010](#)). Depending on the temperature, three hydrated and anhydrous crystal forms are found. For sitosterol, dissolution takes place above  $110^\circ\text{C}$ , an anhydrous form is observed between  $80$ – $110^\circ\text{C}$ , a hemi-hydrate forms between  $45$ – $80^\circ\text{C}$  (1:2 M ratio water/sitosterol), and finally a monohydrate (1:1 M ratio water/sitosterol) occurs for temperatures below  $45^\circ\text{C}$  ([Christiansen et al., 2002](#)).

In x-ray diffraction, all crystal forms share wide-angle x-ray scattering peaks at  $d = 2\pi/q_i = 1.2$  and  $0.59$  nm (here  $d$  is the distance over which the crystal structure repeats itself, and  $q_i$  is the wave vector that characterizes the length scale probed in the scattering experiment). The various crystal forms can be distinguished by the small(er)-angle x-ray diffraction peaks at either  $d = 1.76$  and  $0.880$  and  $0.523$  nm (anhydrous), or  $1.88$  and  $0.481$  nm (hemi-hydrates), or  $1.76$  and  $0.503$  nm (monohydrates). Hydration of the sterols can also be inferred from the existence of an infrared feature at  $\sim 3450\text{ cm}^{-1}$ , which is best explained by the existence of an intermolecular hydrogen bond ([den Adel et al., 2010](#); [Rogers et al., 2010](#)).

The crystal structure of sitosterol in non-aqueous solvents confirm the presence of a bilayer structure and long-range order in three dimensions, with diffraction peaks indicating  $d = 3.76$  and  $1.88$  nm but not at  $0.50$  nm (powder and slurry in oil) and  $d = 3.59$  and  $1.79$  nm and a weaker peak near  $0.50$  nm (slurry in water) ([den Adel et al., 2010](#)). This suggests that the sitosterol exist as a mixture of anhydrous and hemi-hydrate crystals in powder and oil slurry, whereas the monohydrate form seems more abundant in slurries in water.

The infrared spectra show only a very weak band associated with intermolecular hydrogen bonding, around  $\sim 3420$ – $3440\text{ cm}^{-1}$ . The absence of characteristic hemi-hydrate and anhydrate peaks in aqueous slurry and in emulsion suggests the presence of the

mono-hydrate form, despite the surprising similarity of the infrared spectrum for the aqueous slurry and that of the powder (den Adel et al., 2010).

Mixtures of sterols can form mixed crystals. Small structural differences affect the concentration ratio ranges over which mixed crystal formation occurs. Mixed cholesterol + sitostanol crystals form only at a fixed 1:1 wt ratio, whereas cholesterol + sitosterol mixed crystals form over a much wider range around the 1:1 composition (Melnikov et al., 2004). The most likely explanation is that the ring system in the molecules, the sterane core, is more similar in cholesterol and sitosterol than in cholesterol and sitostanol (see Fig. 1).

## Physiological Effects of Plant Sterols

At modest concentrations, phytosterols (and stanols) are part of a normal human diet. Sterols have been added to various food products at enhanced concentrations in order to reduce blood cholesterol by as much as 8.8% at a daily intake of 2.5 g or more (Katan et al., 2003; Demonty et al., 2009). No evidence has been observed of over-consumption of phytosterols as a result of the intake of these fortified products (Lea and Hepburn, 2006), and phytosterols were found to be safe for human consumption - even at higher concentrations (Willems et al., 2013).

An increase in the intake of dietary cholesterol is compensated nearly perfectly in the body by balancing the cholesterol synthesis, leading to relatively constant blood cholesterol levels. For dietary plant sterols, however, the cholesterol synthesis response is weaker. The commonly accepted mechanism explains the effect by a combination of the competition between cholesterol and plant sterols for incorporation in intestinal mixed micelles and the poor intestinal absorption of plant sterols. The reduced uptake of cholesterol does not lead to a matching increase in cholesterol synthesis in the body, and leads to an overall decrease in blood cholesterol levels and an effective increase in fecal excretion of cholesterol (Lecerf and de Lorgeril, 2011).

In addition, there are emerging indications that plant sterol intake may result too in a modest reduction of blood serum triglyceride levels by about 6% for a ~2 g/day plant sterol intake (Demonty et al., 2013).

The large body of nutritional studies resulted in the approval of a number of health claims by major regulatory bodies, like the European Food Safety Authority (EFSA) and the Food and Drug Administration (FDA):

- Europe: Plant sterols have been shown to lower/reduce blood cholesterol. Blood cholesterol lowering may reduce the risk of coronary heart disease (EFSA, 2008).
- USA: Foods containing at least 0.65 g per serving of plant sterol esters, eaten twice a day with meals for a daily total intake of at least 1.3 g, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. A serving of [name of the food] supplies \_\_\_ grams of vegetable oil sterol esters (FDA, 2016).
- Canada: [serving size from Nutrition Facts table in metric and common household measures] of [naming the product] provides X% of the daily amount of plant sterols shown to help reduce/lower cholesterol in adults (Health Canada, 2010)

Serving sizes are dependent on product format. Several related claims exist for stanols, and some additional supporting claims are approved as well.

## Derived Molecules: Sterol Esters and Bile Salts

Sterols can be esterified with other groups to form sterol esters. These occur naturally, like oryzanol, which is a well-known component in rice bran oil and which has a saturated ring systems like the stanols but with additional methyl groups at the 4 and 14 positions and a missing one at the 19 position (cf. Fig. 1). They can also be produced via traditional oil processing technology, like though interesterification of sitosterol with fatty acids obtained from liquid oils like rapeseed or canola (Bot and Flöter, 2011). Plant sterols are often added to functional foods in the form of sterol esters: this form is easier to handle because they are much more soluble in edible oils. However, hydrolysis in the digestive tract ensures that plant sterols become available as free sterols when required.

Bile salts (or bile acids) are steroid acids and act as powerful natural emulsifiers obtained from the cholesterol through enzymic oxidation of cholesterol, and bile salt-like molecules derived from plant sterols will show comparable behaviour.

## Mutual Interactions Between Plant Sterol and Derived Molecules

Mixtures of sterols and their derivatives show rich behaviour, often as a result of co-crystallization. The combination of cholesterol with bile salts, for example, can form helical ribbons in aqueous phases (Konikoff et al., 1992).

Quite a few derivatives of cholesterol have been demonstrated to show fibrillar aggregation in non-triglyceride fluids (Bot and Flöter, 2011). The similarities between cholesterol and plant sterols in terms of aggregation behaviour justify the prediction that analogues based on plant sterols will show the same behaviour.

Sitosterol, and most related plant sterols, self-assembles with oryzanol in triglyceride oil and several other organic solvents (Bot and Agterof, 2006; Sawalha et al., 2012). The molecules cannot stack perfectly parallel, and form curved one-dimensional

aggregates, also known as helical ribbons. These aggregates consisting of equimolar amounts of plant sterol and oryzanol appear as tubules of ~10 nm diameter on longer length scales (Bot et al., 2012; Matheson et al., 2017). The tubules tend to aggregate laterally, but sufficient cross-links are formed between bundles of tubules to establish a network at 3%–4% structurant. Macroscopically, such networks cause the oil to form a transparent or slightly hazy block of gelled oil which are stable for many months.

The presence of water destabilizes the oleogel, however, because the plant sterols have a higher affinity to water than to the oryzanol. The formation of sterol monohydrates can only be avoided if the water activity of the water phase is sufficiently low ( $a_w < 0.9$ ) or delayed if the solubility of water in the organic phase is low enough. The latter cannot be achieved with triglyceride oils (Sawalha et al., 2012).

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## Plant Protein Ingredients

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### Overview

The global protein ingredient market was valued at \$31.8 billion in 2016 and is expected to rise to \$46.4 billion by 2022 with a compound annual growth rate of 6.5% (Anonymous, 2017), with the greatest growth occurring in the plant protein ingredient sector. In 2054, it is estimated that  $\frac{1}{3}$  of all global protein consumed will be of plant origin (Anonymous, 2017). Overarching drivers leading to the market shift towards increased plant protein consumption include: a) population growth; b) increased need for sustainable agronomic practices with reduced water usage; c) changing demographics and lifestyles (Henchion et al., 2017); and d) emerging regulatory influencers, with countries, including China, the Netherlands, France and Denmark, moving to change dietary guidelines to restrict meat consumption in favor of other protein alternatives (Hosafci, 2017). North America represents the largest market for protein ingredient utilization and growth, followed by Europe and Asia Pacific. In these regions, market trends are also shifting towards lower cost, more abundant, plant-based alternatives (although markets are still dominated by animal-based proteins) due to rising costs of dairy-based ingredients, and growing dietary preferences based on religious, moral or ethical beliefs. Other market drivers include the rising demand in the functional food, beverage and breakfast food sectors as consumers move their purchasing power towards healthier ingredients/products. The leading emerging non-soy protein alternative comes from pea, however other legumes (e.g., lentils, faba beans, chickpeas, lupin, peanut and edible beans), oilseeds (e.g., canola/rapeseed, flax seed and hemp) and cereals (e.g., wheat, rice, sorghum, millet, barley, quinoa, amaranth and oats) are of substantial interest as well. Utilization of proteins from agriculture by-products such as from oilseed meals left over after oil extraction or dried distillers grain left over from ethanol fuel processing is also of importance in order to add value to their respective industries, especially based on the sheer volume of feedstock available. In terms of product development, the majority of plant protein research to date has focused on meat alternatives or meat/plant hybrids, sports nutrition bars, high protein non-dairy beverages and breakfast cereals/snacks. However, protein innovations are also occurring across all market segments. Despite experiencing market growth, the wide spread use of plant proteins has been hindered by their reduced solubility (and functionality) relative to animal-based products, in some cases allergenicity (e.g., soy and canola napin), the presence of certain bioactive compounds (e.g., vicine/convicine from faba bean), strong flavor compounds that can negatively affect consumer perception, and tendency to cause flatulence.

### Protein Fractionation

Processing of agriculture crops into flours (20%–30% protein), enriched flours (30%–50% protein), concentrates (50%–80% proteins) and/or isolates (>90% protein) can be accomplished by physical means or dry/wet fractionation processes. Depending on the raw material, dry processing may first involve seed cleaning, dehulling, sorting, and splitting followed by milling into a flour (Wood and Malcolmson, 2011). Milled flours with different particle sizes can be used for various purposes, as they display different functional attributes. Flours may or may not be defatted prior to use. Proteins, due to their size and density differences from starch, can be concentrated by air classification which is a dry separation technique that suspends milled flour in a flow of air allowing for the larger starch particles (known as the coarse fraction) to be collected from the bottom while the smaller protein particles (known as the fine fraction) leave the classifier from the top (Schutyser et al., 2015). The resulting protein concentrates (fine fraction) typically range in protein between 30%–60%. Protein concentrates (65%–80%) and/or isolates (>90% protein) produced by wet fractionation processes can be prepared using flours, enriched flours, defatted oilseed meals or dry fractionated protein concentrates. Alkaline extraction followed by isoelectric precipitation is the most widely used industrial wet extraction method. During this process, flours/concentrates are dispersed in water and pH adjusted to pH 8.0–9.0 to solubilize the proteins. The insoluble materials (e.g., insoluble fibre, carbohydrates and prolamin-type proteins) are removed via centrifugation, leaving a clarified supernatant which is then pH adjusted to the protein's isoelectric point (i.e., zero net charge) to precipitate the protein. The precipitated protein is centrifuged, washed and neutralized then spray dried into a powder (Boye et al., 2010). Alternatively to isoelectric precipitation, proteins can also be recovered from the supernatant using membrane separation technology. Other wet fractionation techniques include salt extraction (Martínez-Maqueda et al., 2013), micellar extraction (Stone et al., 2015), and alcohol washing (Cookman and Glatz, 2009). Depending on the mode of extraction, and the exact conditions used (e.g., flour slurry concentration, pH, temperature, salt concentration, etc.) different protein compositions are achieved leading to different functionality of the final powdered ingredient (Boye et al., 2010).



## Protein Functionality

Protein functionality is dependent on intrinsic factors relating to the protein (e.g., size, conformation, charge, hydrophobicity, amino acid profile and level of folding, etc.) environmental factors (e.g., presence of salt, temperature, solution pH, presence of other additives, etc) and processing factors (e.g., shearing, temperature, enzymatic modification, pressure, etc.). A brief description of the following important functional properties is given below: solubility, water/oil holding, foaming, and emulsification. Protein solubility is correlated with protein surface charge, where solubility tends to be greatest at pHs away from the isoelectric point where protein charge and electrostatic repulsion is highest, and lowest at pHs close to the isoelectric point where repulsion is minimal and aggregation is favored because of non-covalent attractive forces and hydrophobic interactions (Can Karaca et al., 2011). The presence of salts can improve solubility through the 'salting in' effect or decrease solubility through the 'salting out' effect or by charge screening to promote protein aggregation, based on the ionic strength of the solution (Martínez-Maqueda et al., 2013). Water hydration (WHC) and oil holding (OHC) capacities of protein ingredients refers to the amount of water or oil that can be held per g of protein (or protein ingredient) (Boye et al., 2010), and tends to be related to the amount of hydrophilic and hydrophobic amino acids, respectively, on the protein surface (Tiwari and Singh, 2012). These properties are also related to particle size, where smaller particles tend to bind more water (or oil) due to their greater surface area for wetting. The ability of a protein to hold water or oil helps to prevent cook loss from occurring within a product during processing or storage, and improves tenderness and moisture retention (Boye et al., 2010). Foams are described as air-in-water emulsions, where air represents inclusions in the continuous water phase containing the protein. Foams are generated through the addition of mechanical energy in the form of high shear from sparging, homogenization or whipping (Zayas, 1997). During this process, proteins migrate from the water phase to the air–water interface where they then reorient to position the hydrophobic moieties towards the gas phase and hydrophilic moieties towards the water phase to lower the interfacial tension (Boye et al., 2010). Proteins at the interface (also known as the lamellae in foams) form a viscoelastic film around the air bubbles generating a physical barrier between the bubbles (Zayas, 1997). Foam stability is usually the greatest near the isoelectric point of the protein, where the lack of electrostatic repulsion leads to a greater amount of protein–protein interactions and network formation in-between neighboring bubbles (Lam et al., 2017). This network also helps inhibit draining of the continuous phase, which would ultimately lead to foam breakdown. Foams are characterized by their foaming capacity, which refers to the volume of foam generated after mechanical shear for a given amount of protein, whereas foam stability refers to the ability of the foam to retain its structure and resist separation over time (Boye et al., 2010). An emulsion refers to a thermally dynamically unstable mixture of an oil and water phase, in which one phase becomes dispersed within the continuous phase of the other in response to mechanical shear (e.g., homogenization or ultrasonication) and in the presence of an emulsifier (e.g., protein) (Damodaran, 2005). During emulsion formation, proteins migrate in the water phase to the oil–water interface, where like foams, proteins re-orient such that the hydrophobic moieties are positioned towards the oil phase and the hydrophilic moieties are positioned towards the water phase. Protein–protein interactions then occur to form a viscoelastic film that protects against droplet coalescence (Kiosseoglou and Paraskevopoulou, 2011). Emulsions are most stable at pHs away from the protein isoelectric point due to significant electrostatic repulsion between droplets (McClements, 2004). The presence of salts, depending on the increase in solution ionic strength, can destabilize emulsions by screening charges on the protein's surface allowing for closer interactions and droplet flocculation and coalescence (McClements, 2004). Emulsion capacity measures the amount of oil that can be stabilized in an emulsion mixture per g of protein material, whereas emulsion activity index measures the total surface area of the oil droplets being stabilized in the emulsion (Tiwari and Singh, 2012). The stability of an emulsion over time is measured as emulsion stability index (Tiwari and Singh, 2012).

## Nutritional Properties

Protein quality refers to the amount of essential amino acids within the protein itself, as well as their bioavailability or ability for the protein to be uptaken into the metabolic process (Nosworthy et al., 2017a). Essential amino acids include histidine, isoleucine, leucine, methionine, lysine, phenylalanine, threonine, tryptophan and valine. They also include arginine, cysteine and tyrosine for immune compromised individuals and infants. Typically protein digestibility is measured using *in vitro* methods, such as the pH-stat titration method involving one or more digestive enzymes or by *in vivo* methods, such as the Protein Efficiency Ratio, Biological Value, Protein Digestibility Corrected Amino Acid Score (PDCAAS) or the Digestible Indispensable Amino Acid Score (DIAAS) (Nosworthy et al., 2017a). Although more accurate than *in vitro* methods, *in vivo* methods involve the use of animals (e.g., rats or pigs), are more expensive, labor-intensive and time-consuming. As such, *in vitro* methods tend to be favored. Currently, the PDCAAS method is approved for use by the FAO/WHO as the international standard, however, the DIAAS method is being considered for its replacement since it allows high-quality protein sources to be better differentiated (Nosworthy et al., 2017a). Soaked/boiled red kidney beans, green lentils, yellow peas and Kabuli chickpeas have PDCAAS values of 0.55, 0.63, 0.64 and 0.52, respectively (Nosworthy et al., 2017b), whereas raw flours from barley, wheat, oats and maize have PDCAAS values of 0.49, 0.45, 0.71 and 0.41, respectively (Boye et al., 2012).

Despite their nutritional properties, plant materials also contain bioactive compounds that can adversely affect protein and carbohydrate digestion as well as mineral absorption. For instance, phenolic compounds act to cross-link proteins to reduce their digestibility, whereas the presence of enzyme inhibitors (trypsin, chymotrypsin and amylase inhibitors) can reduce the activity of digestive enzymes for proteins and carbohydrates (Dahl et al., 2012). Phytates and oxalates can act as chelators to reduce mineral



**Table 1** Process induced modification of select plant protein ingredients

<i>Material</i>	<i>Processing method</i>	<i>Nutritional properties</i>	<i>Physicochemical properties</i>	<i>References</i>
Pea protein isolate	Extrusion	–	Extrudate expansion, bulk density; solubility; MW, 2° structure	<a href="#">Beck et al., 2017</a>
Rye, winter wheat, quinoa, corn, millet, sorghum (high and low tannin)	Extrusion	IVPD	–	<a href="#">Dahlin and Lorenz, 1993</a>
Canola meal	Extrusion	IVPD	Protein extraction ratio; colour; pH; MW	<a href="#">Zhang et al., 2017</a>
Faba bean flour (air classified fractions)	Fermentation	IVPD; vicine and convicine; TIA; condensed tannins; phytic acid	Free amino acid profile analysis	<a href="#">Coda et al., 2015</a>
Rapeseed and sesame seed meals	Fermentation Pressure cooking Microwave cooking Germination Fermentation		Water/fat absorption capacity; solubility; foam capacity and stability; emulsifying activity and emulsion stability; viscosity	<a href="#">Mahajan et al., 1999</a>
Mung bean seeds	Micronization	Vitamin B <sub>1</sub> and B <sub>2</sub> ; fatty acid profile; phytic acid; TIA; tannins	Water/oil absorption capacity; bulk density; pasting properties; sedimentation value; swelling index	<a href="#">Padmashree et al., 2016</a>
White, yellow and red maize kernels	Soaking/boiling Micronization	Total phenolics; tocopherols; β-carotene; tryptophan; antioxidant activity; peroxidase activity	Protein fractionation and analysis; solubility; viscosity; pasting properties	<a href="#">Zilic et al., 2010</a>
Pea protein isolate	Enzymatic hydrolysis	ACE-inhibitory activity; DPPH scavenging activity;	Amino acid composition; MW; emulsion formation; bitterness evaluation	<a href="#">Humiski and Aluko, 2007</a>
Wheat gluten	Enzymatic hydrolysis (following acid deamidation)		NSI; MW; free amino acid analysis; sensory analysis (taste profile)	<a href="#">Liao et al., 2010</a>
Lentil protein concentrate	High pressure treatment	ACE-inhibitory activity; antioxidant activity; <i>in vitro</i> gastrointestinal digestion of hydrosylates	MW; solubility; proteomics analysis (identification of bioactive peptides)	<a href="#">Garcia-Mora et al., 2015</a>
Peanut protein isolate	High pressure treatment		Surface hydrophobicity; sulfhydryl group & disulfide bond content; MW, differential scanning calorimetric characteristics; water-holding capacity, oil-binding capacity; heat-induced gelling properties	<a href="#">He et al., 2014</a>

(Continued)

**Table 1** Process induced modification of select plant protein ingredients—cont'd

<i>Material</i>	<i>Processing method</i>	<i>Nutritional properties</i>	<i>Physicochemical properties</i>	<i>References</i>
Buckwheat	Microwave cooking	Phytic acid; tannins; saponins; TIA; total flavonoids; IVPD	Amino acid content; free fatty acid analysis; microstructure	<a href="#">Deng et al., 2015</a>
	High pressure treatment			
	Boiling			
Pea, cowpea, white/red kidney bean seeds	Soaking	Tannins; phytic acid; TIA; oligosaccharide content; IVPD; protein efficiency ratio;		<a href="#">Khattab and Arntfield, 2009</a> ; <a href="#">Khattab et al., 2009</a>
	Boiling			
	Roasting			
	Microwave cooking	protein chemical score; essential amino acid index		
	Autoclaving			
	Fermentation			
	Micronization			
Rice (long grain) (processed into rice protein isolate)	Germination		Surface hydrophobicity; MW; emulsifying activity, emulsion stability; foam capacity and stability; amino acid content	<a href="#">Tortayeva et al., 2014</a>
Pea, soy, and rice protein isolates	Ultrasound treatment		MW, protein size; intrinsic viscosity; pH; emulsion properties (droplet size, interfacial tension)	<a href="#">O'Sullivan et al., 2016</a>
Canola seed (canola meal)	Irradiation	IVPD; glucosinolates; phytic acid	Water and fat absorption; foam capacity	<a href="#">Anwar et al., 2015</a>
Soy protein isolate	Pulsed electric fields treatment		Solubility; surface free sulfhydryls; hydrophobicity; protein 2° structure	<a href="#">Li et al., 2007</a>

IVPD: *in vitro* protein digestibility; TIA: trypsin inhibitor activity; NSI: nitrogen solubility index; MW: molecular weight.

absorption (Dahl et al., 2012), whereas lectins can cause diarrhea, vomiting, bloating and red blood cell agglutination (Roy et al., 2010). The presence of oligosaccharides can be fermented by bacteria within the gastrointestinal tract leading to the production of gas, abdominal pain and flatulence (Granito et al., 2005). And in the case of faba beans, the presence of vicine and convicine, which are glycosides, can be associated with favism in certain individuals (McMillan et al., 2001). In canola/rapeseed, glucosinolates can be converted to toxic metabolites (Campbell et al., 2016). Levels of bioactive compounds can be reduced via processing and breeding programs. However further research into specific bioactive compounds, also reveals health promoting effects such as their antioxidant activity, satiety (diet control), anticarcinogenic activity and cholesterol lowering effects (Güçlü-Üstündağ and Mazza, 2007; Chung et al., 1998).

## Tailoring Plant Protein Structure, Functionality and Quality Through Processing

Processing, such as extrusion, roasting, boiling, fermentation, autoclaving (Khattab et al., 2009), microwave, infrared heating and so on, can be used to improve protein digestibility and reduce or eliminate levels of bioactive compounds affecting digestion. Processing causing changes to the protein's quaternary and tertiary structures during partial denaturation to make them more susceptible to digestive enzymes (Park et al., 2010). This also induces changes in the functional properties of the protein/material. Various processes used to modify plant protein-based materials are given in Table 1. For instance, Coda et al. (2015) found that fermentation of a protein-rich faba bean flour, produced by air classification, significantly decreased the concentration of vicine and convicine by >90% and decreased the trypsin inhibitory activity by 86%, however the *in vitro* protein digestibility remained relatively unchanged. Not all conditions lead to a positive impact on protein quality and accordingly are process and material dependent. O'Sullivan et al. (2016) reported that ultrasound treated pea protein isolate produced emulsions with decreased droplet size and lowered the interfacial tension better than untreated pea protein isolate. This was attributed to the ultrasound treatment decreasing the pea protein size and increasing protein surface hydrophobicity, whereas these changes were not seen for a rice protein isolate and no change in the emulsion properties of the rice protein isolate was observed.

## Summary

Plant based protein ingredients have been consistently in the top global food trends as it relates to demand, health, product innovation, sustainability and food security. The nutritional and functional properties of these ingredients can be modified through a wide variety of processing methods with the subsequent induced changes being material and process dependent. Although not discussed in this review, other key areas of interest as it relates to plant protein ingredients are flavour reduction strategies, development of bioactive peptides, utilization of protein-rich waste streams, and ingredient-application specificity.

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## Salts and Salt Replacers

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### Overview

Salt is the name commonly used for sodium chloride (NaCl), which consists of 40% sodium and 60% chloride by weight. In the body, sodium is essential for the maintenance of cellular membrane potential; its presence determines the volume of extracellular fluid and thereby maintains blood volume and blood pressure, [Fig. 1](#). Given its multiple functions in the body, an excessive consumption of sodium can have negative health effects, within the most alarming ones being cardiovascular diseases and elevated blood pressure ([Doyle and Glass, 2010](#)). In industrial countries, about 75%–80% of dietary salt is obtained through processed food consumption, 5%–10% is naturally occurring in the foods that make up the diet and the remaining 10%–15% comes from salt added during cooking or at the table ([Dotsch et al., 2009](#)). In contrast, in developing countries, salt used for seasoning plays a much more important role; In China, for example, this accounts for 76% of total salt intake ([He et al., 2012](#)). An assessment of processed foods in Australia revealed that sauces and spreads contain the most sodium (1280 mg/100 g) followed by processed meats (850 mg/100 g), snacks (800 mg/100 g), fish products (510 mg/100 g) and bread and bakery products (470 mg/100 g); however, a similar study performed in the UK detected lower sodium levels in bread and bakery products, followed by meats, sauces and spreads. These differences indicate that the sodium content of processed food can varies greatly between different markets and between countries ([Kloss et al., 2015](#)).

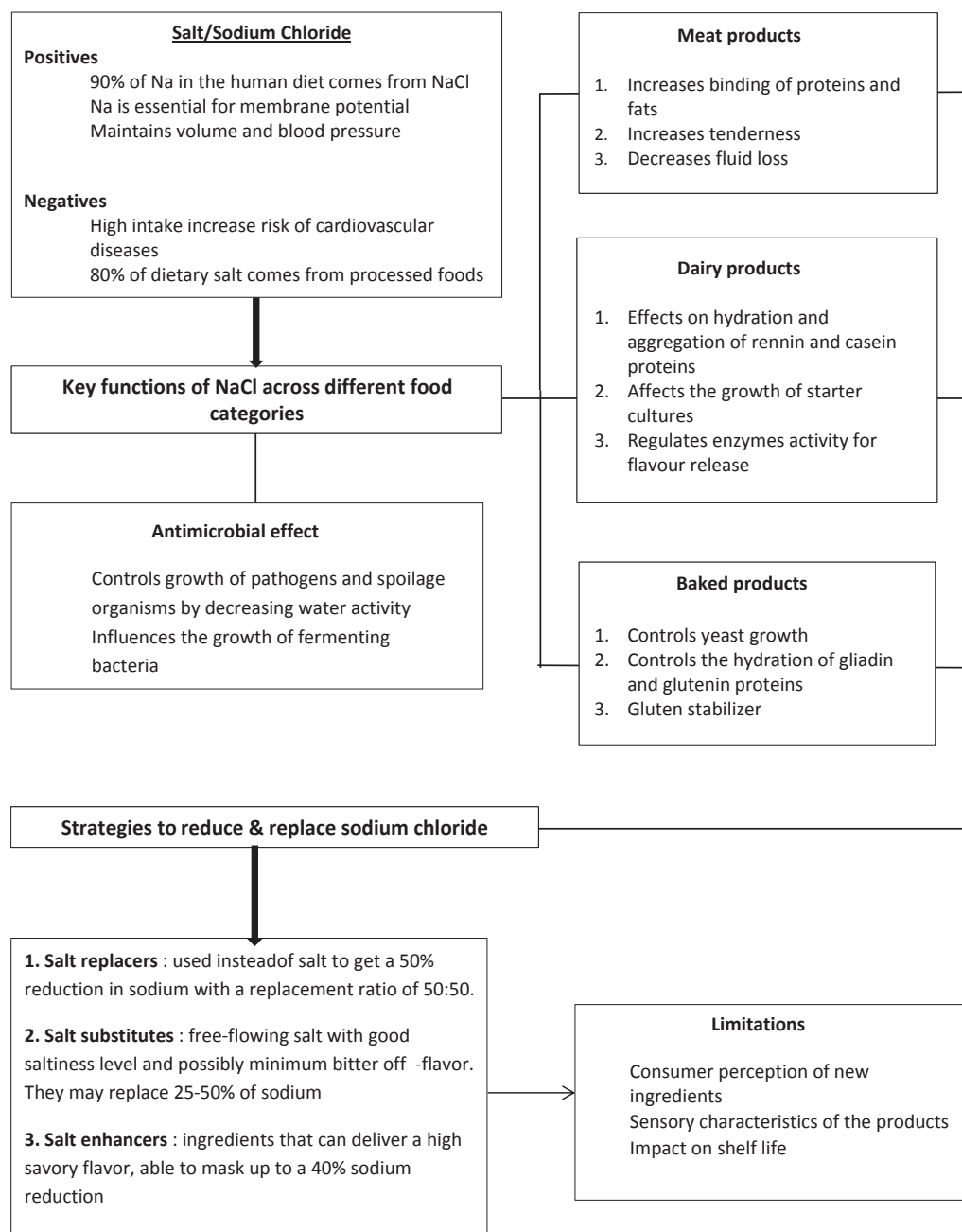
### Roles of Salt in Different Food Categories

#### Texture

Salt play a major role in the development of physical properties of foods that are expected by costumers, one of these being the texture of products ([Desmond, 2006](#)). For example, in **meat**, 1.5% to 2.5% (w/w) of salt can increase hydration and enhance the binding of proteins to each other and to fat; the mechanisms is given by the proteins actin and myosin that by swelling in the presence of salt, can bind more water and thereby increasing tenderness, decreasing fluid loss and allowing the formation of heat-stable emulsions in processed meat products ([Inguglia et al., 2017](#); [Man, 2007](#)). In **dairy products**, the use of salt in concentration of 5% to 6% (w/w), together with pH and calcium level, has a large effect on the degree of hydration and aggregation of the mammalian milk proteins rennin and casein; the mixture of salt and proteins called *para*-casein, precipitates with the calcium ion to form of insoluble curd. Is this that determine the water-binding capacity of the casein matrix, its rheological and textural characteristics and the changes that occur during cooking ([Guinee, 2004](#)). In pasteurized cheeses, emulsifying salts are added to aid in the hydration of the *para*-casein, to help the emulsification of fats, and its stability. Content and composition of emulsifying salts vary in different products, but a levels of about 1.5% are typically used ([Guinee and O'kenedy, 2007](#)). Yeast bread and some other **baked goods** require the addition of salt to control the growth of yeast and to develop an extensible gluten network. Salt in particular helps controlling the hydration of gliadin and glutenin proteins. The development of a robust gluten matrix in the dough is necessary to trap small air bubbles, critical for the production of high-quality bread ([Hutton, 2002](#)). Optimal salt concentrations stabilize gluten and prevent stickiness in dough. Too little salt however allows excessive growth of yeast, resulting in oversized bread with poor texture ([Vetter, 1981](#)). Moreover, it has been shown that salt has a plasticizing effect during heating of cereal products which improves the mobility of reactants and enhances Millard reactions, producing a darker colored product ([Moreau et al., 2009](#)).

#### Flavour

Sodium chloride is the saltiest sodium compound available; it affects the taste of specific foods by providing the flavor of saltiness, by enhancing or masking other flavors, and by controlling the growth of specific microbes that produce flavorful compounds. For example, during cheese ripening, salt significantly affects the growth of starter cultures and activities of lipolysis and proteolysis enzymes that produce important characteristic flavor compounds ([Guinee and O'kenedy, 2007](#)). Growth and metabolic activities of cheese starter cultures and yeast and sourdough starters for bread are also stimulated or depressed depending on sodium chloride levels ([Man, 2007](#)). Moreover, depending on the nature of the food matrix, the use of different particle's sizes may influence the overall perceived saltiness. In general, smaller salt particles showed to have a faster dissolution rate in the saliva resulting in a higher salt perception ([Desmond, 2006](#)). Experiments on fried potato crisps suggested that in a controlled chewing environment with controlled mixing, smaller crystals of NaCl could give a faster delivery of sodium into the saliva, influencing the timing of the maximum perceived saltiness ([Rama et al., 2013](#)).



**Figure 1** Overview of salt functions and salt reduction in different food categories.

### Microbial Safety

A major reason for the use of salt in food products has always been its function to reduce the water activity ( $a_w$ ) in foods and thereby acting as a critical hurdle to control growth of pathogens and spoilage organisms. Available water is a critical factor affecting microbial growth. By definition low water activity foods (dry foods) are those with  $a_w < 0.70$ , while fresh foods, soft cheeses, and low-salt meat products have a high  $a_w$  (0.95–1), as do highly perishable foods such as fresh meat and fish ( $a_w > 0.99$ ) (Blessington et al., 2013; Christian, 2000). Given the abundance of water in these products, the absence of salt would quickly lead to the over growth of bacterial pathogens and spoilage organisms, shortening the shelf life and stability of the products. Most shelf-stable products, processed meats, and hard cheeses rely in fact on salt for safety and preservation. Moreover salt commonly plays a central role in food fermentation. Fermentation is another accepted method use to preserve food due to the action of beneficial types of microbes that by growing inhibit the growth of undesirable spoilage bacteria and fungi (Doyle and Glass, 2010). The presence of lactic acid



bacteria is in fact responsible for distinguish characteristics of fermented foods such as pickles, sauerkraut, some cheeses and fermented sausages.

## Salt Replacers: Functions and Compositions

The link between high sodium intake from the diet and its consequences on the overall cardiovascular health in the population, has led to the development of methods and formulation to help achieving sodium reduction; salt replacers, salt substitutes and salt enhancers have been one of the key strategies used in order to maintain the functional properties of sodium salt with minimal effect on the taste of the product (Doyle and Glass, 2010; Pietrasik and Gaudette, 2014). Salt replacers are used in place of salt to get a 50% reduction in sodium with a replacement ratio of 50:50. Salt substitutes are free-flowing salt with good saltiness level and possibly minimum bitter off-flavor. They may replace 25%–50% of sodium in products like sauces, gravies, dressings, snack seasonings and spices (Desmond, 2006). Salt enhancers are ingredients that can deliver a high savory flavor, able to mask up to a 40% sodium reduction (Brandsma, 2006). However, as already described, sodium chloride influence many steps in the formulation of processed foods, including growth of starter cultures, moisture levels, fat binding properties, flavor of other additives, as well as processing conditions. Reducing sodium chloride levels may require alterations in other parameters to ensure that foods retain acceptable flavors and textures for the specific market.

## Salt Replacers: Taste and Flavor

Generally, sodium and lithium are the only cations with a taste that is primarily salty; lithium however, is toxic in amounts that would be needed as salt substitutes. Potassium and calcium compounds have some component of saltiness but they have other flavors, often described as “metallic” or “bitter” (Grummer et al., 2013). Potassium chloride (KCl), for example, can replace up to about 30% of sodium chloride in many foods but beyond that concentration, foods may become unpalatable (Israr et al., 2016; Inguglia et al., 2017; Grummer et al., 2013). The use of calcium chloride ( $\text{CaCl}_2$ ) and magnesium chloride ( $\text{MgCl}_2$ ) to reduce sodium in Cheddar-style cheese resulted in flavor differences (bitter, metallic, earthy, unclean, soapy, and numbing) compared with full-sodium control. Cheese made with NaCl + KCl and NaCl + modified KCl were similar to cheeses made with NaCl in most respects. Thereby, in products such as cheddar cheese, KCl can be used successfully to achieve large reductions in sodium when replacing a portion of the NaCl (Grummer et al., 2012). Magnesium sulfate or other ammonium compounds, amino acids, and peptides from hydrolyzed proteins have also a salty taste but, their use would need extra additives to mask off-flavors and bitter tastes (Institute of Medicine, 2010). One recommended additive that allows reduction of sodium content of foods, is monosodium glutamate, well known for providing the umami flavor (Kilcast and den Ridder, 2007). However, in the past monosodium glutamate has been claimed to be toxic and potentially leading to adverse effects in humans and animals (Fernstrom, 2007). Sea salts is also a good alternative to high sodium refine salts as it contain different percentages of calcium, potassium, and magnesium compounds that contribute to the different existing varieties and to the different flavors (Kilcast and den Ridder, 2007).

## Salt Replacers: Texture and Quality Characteristics

### Meats and Meat Batters

Formulation of low-salt meat batters is technologically challenging because a reduction in sodium chloride levels requires other ionic compounds to replace the water-holding, protein-binding, and fat-binding functions of the salt that is eliminated (Desmond, 2006). Potassium, calcium, and magnesium chlorides, along with polyphosphate compounds can be used to stabilize meat emulsions in reduced-sodium meats. Since KCl and NaCl, have an equal ionic strengths, this salts can interact identically with the meat proteins; calcium and magnesium chlorides however given their different chemical nature are not as effective (Gordon et al., 1992). Potassium phosphates can bind water and improve stability as well as their sodium counterparts, but high levels of potassium compounds may adversely alter taste. Restructured items such as sausages or deli-style meats are products in which lower-sodium ingredient options have been successfully produced. Here, the structural functions of salt-soluble proteins have been replaced by the addition of soy or milk proteins, gums and starches (Fellendorf et al., 2016). An alternative strategy that does not involve addition of other compounds is the use of different physical forms of salt. Salt companies, such as Morton and Cargill, produce fine flake and dendritic salts whose crystals have a larger surface area and dissolve more rapidly. There have been reports that such salts have the potential to improve water and fat binding in some meat batters and emulsions at lower salt concentrations but further research are needed in the area (Inguglia et al., 2017).

### Baked Products

Sodium chloride in baked products controls the growth of yeast and promotes the development of gluten structure. Therefore, a reduction in salt may cause the yeast to growth to quickly affecting the final texture (Belz et al., 2012). These effects may be mitigated to some extent by decreasing the amount of yeast used and by adjusting mixing and other mechanical processes during manufacture (Cauvain, 2007). Reduced resistance of the dough to extension, extensibility and no changes in the liquid to solid ratio, were observed in wheat bread formulated using decreasing salt concentrations (0.6%, 0.3%, and 0% NaCl). Despite a reduction in the

salt content resulted in no major structural changes compared to the customary level of 1.2% NaCl, complete omission of salt however, produced unpleasant flavors and a significant reduction in structural quality of dough and bread (Lynch et al., 2009).

### Cheeses and Dairy

KCl has been recognized as a potential salt substitute in various food categories given the similarity between the cation of these two salts. In particular this role seems to be the most promising in cheeses. In processed cheese, the replacement of NaCl by KCl has been successfully reported in various research works (Guinee and Sutherland, 2011; Grummer et al., 2013, Guinee, 2004). Most of the functional and microbiological properties of the cheese were not substantially affected as a result of this replacement. Recently, processed cheese was successfully manufactured at full replacement of NaCl by KCl (EL-Bakry et al., 2011). There were no adverse effects on functional properties of the cheese, which included textural, rheological and microstructural properties. However, the microbiological stability of the product was substantially reduced. Other experiments with cheese products where NaCl was substituted with compounds such as  $\text{CaCl}_2$  and  $\text{MgCl}_2$  were however observed to be really soft and crumbly (Guinee and O'Kennedy, 2007).

### Effects on Safety

Whenever changes in the formulation or in the processing method are made, it is necessary to test that the organoleptic and safety of the products are not been altered. The effectiveness of alternative salts relative to sodium chloride seems to vary based on the pathogen of interest (Barbut et al., 1986). KCl seems to be really similar in the way it affect microbes in foods when used at the same molar concentration (Bidlas and Lambert, 2008). Compounds, such as potassium chloride and mixtures of potassium lactate and sodium diacetate, that might be used to replace salt and other sodium-containing preservatives have been also shown to be somewhat effective at retarding growth and toxin production by pathogens (Barbut et al., 1986; Devlieghere et al., 2009; Taormina, 2011). Organic acids are commonly used as chemical preservatives in some foods (Doores, 2005). A list of the chemical compounds that are typically used along with the amount of sodium contributed by sodium salts and the type of products where they are used is presented in Table 1. The majority of the organic acids contain much less sodium than NaCl at the normal concentration used. Potassium, sodium, and calcium lactates are being shown to be equally effective in controlling growth of bacteria in meat packaged in modified atmospheres (Devlieghere et al., 2009). However, many salts of organic acids have strong flavors on their own which is a limitation to their use above certain concentration. Partially replacing salt with other compounds, such as potassium chloride and calcium chloride, may also be possible in fermented products. However, these alternatives may be less effective than salt so, once again, formulations with a higher concentrations may be needed to achieve the same functionality (Bautista-Gallego et al., 2008).

### Conclusion

A comprehensive strategy to reduce salt intake to 6 g/d (from around 8.6 g/d) has been undertaken in the UK in an effort to reduce rates of hypertension and cardiovascular disease. Voluntary salt reduction targets for different categories of manufactured food were first proposed in 2006. Information on current target values can be found at the Food Standards Agency website (<http://www.food.gov.uk/multimedia/pdfs/consultation/consultsalttargets.pdf>). Target levels were set with an eye on effects related to flavor, texture, and safety of foods and are periodically reconsidered in light of experiences to date with these reformulations. Despite the step

**Table 1** Amount of sodium contributed by some common sodium-containing additives and the relative concentrations used in food products

<i>Sodium compound</i>	<i>Amount of sodium contained (%)</i>	<i>Concentration used in foods (%)</i>	<i>Food to which the compound is added</i>
Benzoate	15.95	0.1	Beverages, fermented vegetables, jams, salad dressings
Diacetate	16.18	0.1–0.4	Condiments
Lactate	20.51	1.5–3	Meat products
Propionate	23.93	0.3	Bakery products
Sorbate	17.14	0.3	Cheeses, meats, ketchup, mayonnaise and marmalades
Nitrite	33.32	0.012	Cured meats
Acid pyrophosphate (SAPP)	20.72	0.35	Baked products
Triphosphate (STPP)	31.24	0.35	Seafood, meats, poultry
Pyrophosphate (TSPP)	34.57	0.35	Chicken nuggets, pudding, crab meat, canned tuna and soy-based meat alternatives
Hexametaphosphate (SHMP)	22.55	0.35	Cheese powders and dips, whipped topping, meats and canned fish, frozen desserts, salad dressings, herring, breakfast cereal, and bottled beverages

Modified table from Doyle and Glass (2010) and Institute of Medicine (2010).

forward which has been done in finding ingredients to reduce sodium content, the major limitation in their use used are still the consequences on sensory properties. A number of challenges have to be faced in order to satisfy consumers' opinion about low-salt meat products: taste, colour, flavour, texture, aroma, etc., all parameters which can become unacceptable if too much sodium is removed. Salt replacers and other ionic compounds used needs to be effective at maintaining product safety while being also practical and financially affordable during food formulation.

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# Seaweed Polysaccharides (Agar, Alginate Carrageenan)

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## Introduction

Red seaweeds or macro-algae (*Rhodophyceae*) are phylogenetically the oldest division of marine macrophytes. They contain sulphated galactans, such as carrageenan and agar as the main structural materials of cell walls and intercellular matrices that are commercially exploited across food industry. Carrageenan-containing seaweeds (carrageenophytes) are mainly collected from natural sources around the globe (Table 1). Initially, the production of agar was limited only to several locations where *Gelidium* species grow that produce agars with superior gelling properties. However, alkaline pre-treatment of alternative seaweed species (*Gracilaria*) was introduced later and resulted in expansion of agar industry. Brown seaweeds (*Phaeophyceae*) are primarily used for production of alginates that are also harvested in both northern and southern hemispheres (Table 1). The differences in compositional characteristics and conformational arrangement in solution of carrageenan, agar and alginates produce a set of polysaccharides with a wide spectrum of physical and chemical properties that are utilized in a large number of foods. The focus of the present chapter is on the structure, extraction, physicochemical properties, and food applications of seaweed polysaccharides with major industrial interest.

## Structure – Extraction

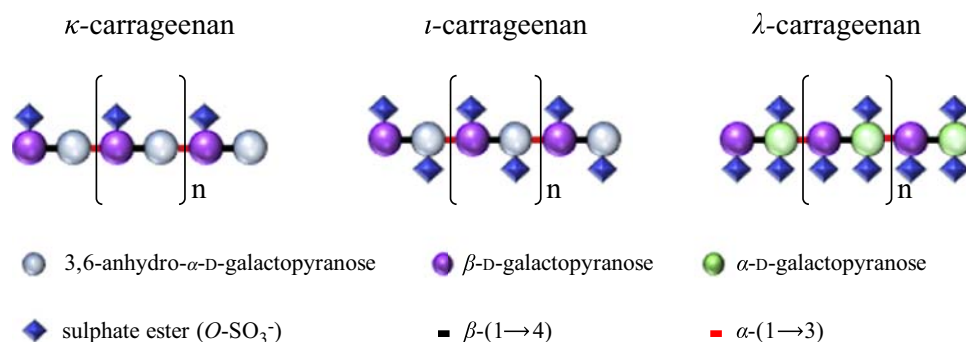
### Carrageenan

Carrageenan is a linear, sulphated galactan that is composed of alternating 3-linked  $\beta$ -D-galactopyranose and 4-linked  $\alpha$ -D-galactopyranose or 4-linked 3,6-anhydro- $\alpha$ -D-galactopyranose, thus forming their disaccharide repeating unit (Fig. 1) (Knutsen et al., 1994). Besides galactose and sulphate groups, other carbohydrate residues (e.g., xylose, glucose, or uronic acids) and substituents (e.g., methyl ethers, and pyruvate groups) may also be present (Van De Velde et al., 2002). These negatively charged polysaccharides are typically classified into six basic forms, namely *iota*- ( $\iota$ ), *kappa*- ( $\kappa$ ), *lambda*- ( $\lambda$ ), *mu*- ( $\mu$ ), *nu*- ( $\nu$ ), and *theta*- ( $\theta$ ) carrageenan based on the number and position of sulphate groups, and the presence of 3,6-anhydro-bridge in galactose residues (Knutsen et al., 1994).  $\kappa$ -,  $\iota$ -, and  $\lambda$ -carrageenan have one, two, or three ester-sulphate groups per repeating disaccharide unit, respectively (Rochas et al., 1986). The most industrially relevant types of carrageenan are *kappa*-, *lambda*-, and *iota*- whereas *mu*- and *nu*-are biological precursors of  $\kappa$ - and  $\iota$ -carrageenan, respectively, and their food applications are limited. The chemical composition of carrageenan varies between red seaweed species and depends on the extraction method. The  $\kappa$ - and  $\iota$ -carrageenan are predominantly extracted from *Kappaphycus alvarezii* and *Eucheuma denticulatum*, whereas  $\lambda$ -carrageenan is primarily sourced from *Gigartina skottsbergi* and *Sarcothalia crispata* (Imeson et al., 2009).

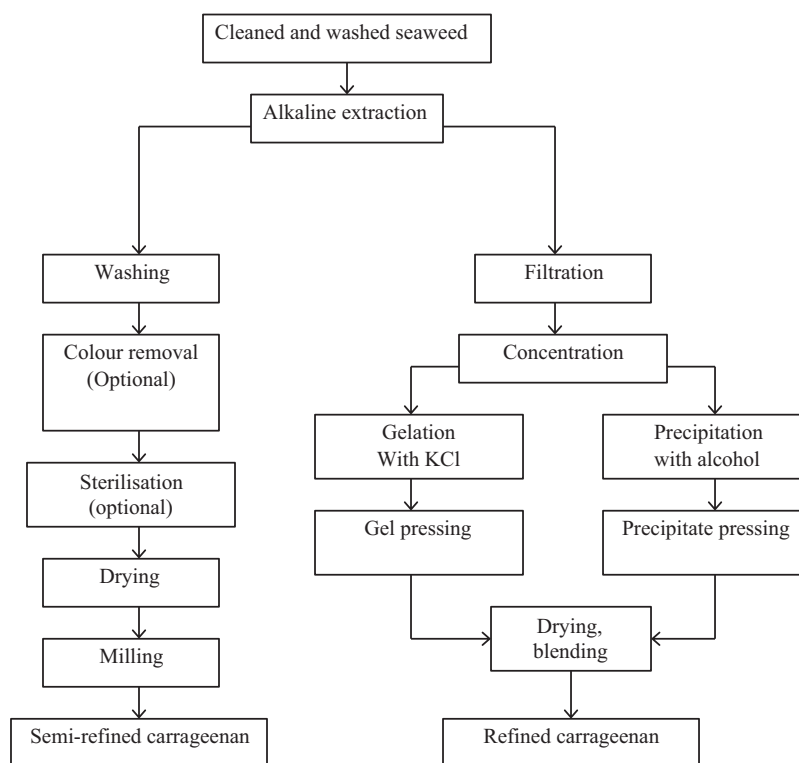
Carrageenan is produced using two extraction methods resulting in isolation of semi-refined or refined powders (Rudolph et al., 2000). During the isolation process of semi-refined carrageenan, hot KOH solution reacts with the sulphate esters of carrageenan precursors ( $\mu$ - and  $\nu$ -) resulting in increase of 3,6-anhydrogalactose (3,6-AG) and production of  $\kappa$ - or  $\iota$ -carrageenan, respectively (Fig. 2). In addition, potassium cations promote gel formation thus preventing carrageenan from dissolving in the hot alkaline

**Table 1** Species and locations of industrially relevant hydrocolloid-producing seaweeds

Species	Location
<b>Carrageenophytes</b>	
<i>Kappaphycus alvarezii</i> , <i>Eucheuma denticulatum</i>	Indonesia, Philippines
<i>Gigartina skottsbergi</i> , <i>Sarcothalia crispata</i>	Central Chile, Argentina
<b>Agarophytes</b>	
<i>Gelidium</i>	Spain, Portugal, Morocco, France, Japan, Republic of Korea, Mexico, Sumatra, Indonesia, lower harvests in Chile, China and South Africa
<i>Gracilaria</i>	Chile, Indonesia, Argentina, Atlantic coast of Canada, China, Vietnam, Namibia
<b>Alginophytes</b>	
<i>Ascophyllum nodosum</i>	Ireland, Scotland, Norway
<i>Laminaria digitata</i>	France, Norway, Iceland
<i>Laminaria hyperborea</i>	Ireland, Scotland, Norway
<i>Macrocystis pyrifera</i>	USA, Mexico, Chile
<i>Laminaria japonica</i>	China, Japan, Russia, France



**Figure 1** Structural features of carrageenan.  $\beta$ -D-Galactopyranose residues in  $\kappa$ - and  $\iota$ -carrageenan are sulphated at C-4, whereas in  $\lambda$ -carrageenan at C-2 position.

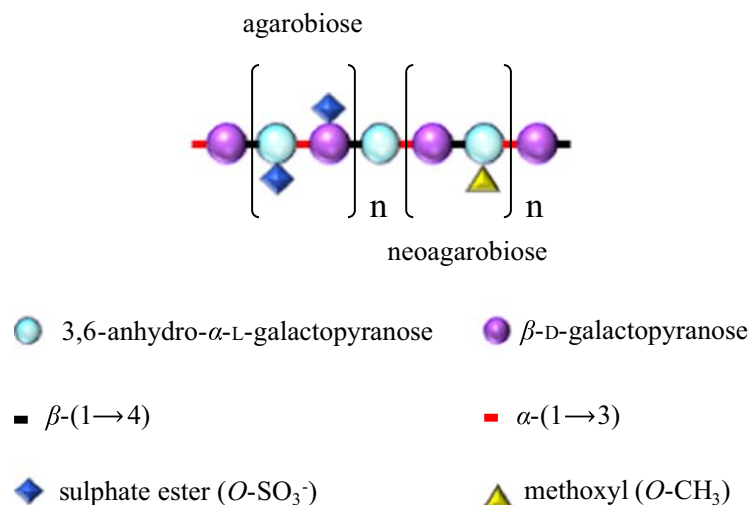


**Figure 2** Generalized extraction strategy of semi-refined and refined carrageenan.

solution. At this stage, seaweed extract is in a gel form that is finally dried and milled into a powder (Fig. 2). This method is only applicable for red seaweed species that contain mainly  $\kappa$ - and  $\iota$ -carrageenan because they form gels with potassium salts, as opposed to  $\lambda$ -carrageenan-containing seaweeds that do not gel in the presence of potassium and would therefore be lost during the alkali treatment. Extraction of refined carrageenan starts with seaweed heating in NaOH solution followed by precipitation with alcohol or by inducement of gelation with potassium salts (e.g., KCl) (Fig. 2). Alcohol-precipitation method can be used for any type of carrageenan, whereas the gel method is only applicable to  $\kappa$ -carrageenan.

## Agar

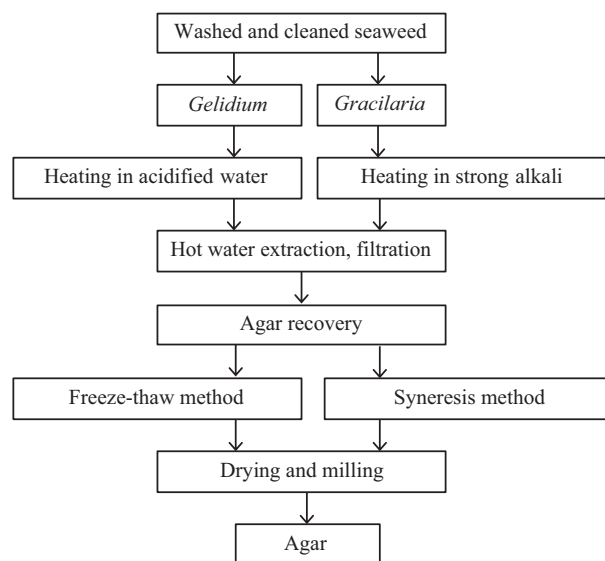
Agar is also a linear galactan with backbone comprised of  $\beta$ -D-galactopyranose and 3,6-anhydro- $\alpha$ -L-galactopyranose linked *via* alternating  $\alpha$ -(1 $\rightarrow$ 3) and  $\beta$ -(1 $\rightarrow$ 4) glycosidic linkages. The two alternating disaccharide units forming the backbone are agarobiose and neoagarobiose (Fig. 3) (Duckworth and Yaphe, 1971). Agar is a heterogeneous complex mixture of related polysaccharides with comparable backbone structure, but differences in the level of substitution of hydroxyl groups of sugar residues (e.g., ester sulphate, methoxyl, etc.) (Duckworth and Yaphe, 1971; Lahaye and Rochas, 1991). Agar is composed of two major polysaccharide fractions,



**Figure 3** Backbone structure of agarose. The repeating disaccharide units are called agarobiose and neoagarobiose. 3,6-anhydro-galactose residues in agar are L-enantiomers, whereas in  $\kappa$ - and  $\iota$ -carrageenan are D-enantiomers.

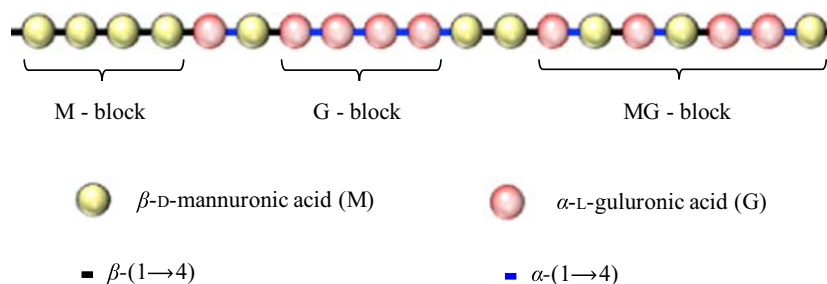
namely agarose that is a neutral, low sulphate/methoxyl substituted fraction that exhibits high gelling capacity, and agarpectin that is charged, heterogeneous, highly-substituted, and has low gelling capacity (Araki and Arai, 1967). The ratio of agarose to agarpectin varies depending on the seaweed species and isolation conditions. In contrast to carrageenan, agar is only lightly sulphated (<0.15%) (Armisen et al., 2009).

The major agar-producing red seaweeds (agarophytes) belong to genera *Gelidium* and *Gracilaria*. The extraction strategies of food-grade agar generally include a washing step followed by pre-treatments and hot water extraction. The pre-treatment stage is performed either with strong alkali (NaOH) for *Gracilaria* seaweed species or with acidified water for *Gelidium* species (Fig. 4). The alkali pre-treatment is a critical step in agar production from *Gracilaria* seaweed species since it results in conversion of the precursor porphyran (sulphated galactose) into the 3,6-anhydro-galactopyranose that is responsible for the gelling capacity and mechanical properties of agar gels (Knutzen et al., 1994). In the hot-water extraction stage, seaweeds of *Gelidium* species are typically extracted under pressure whereas seaweeds of *Gracilaria* species are treated with hot water and the mixture is filtered to remove the residual seaweed (McHugh and Food and Agriculture Organization of the United Nations, 2003). Cooled filtrate forms a gel that contains around 1%–2% of agar and which is later dehydrated using either a freeze-thaw process or by pressing water out (“syneresis” method). The freeze-thaw method results in production of gels that contain 10%–12% of agar, whereas syneresis method yields higher purity gels (~20%) (Armisen et al., 2009). After this treatment, isolated agar is dried in hot-air oven and milled to the desired particle size.



**Figure 4** Generalized extraction strategy for food-grade agar.





**Figure 5** Idealized structure of alginates. Alginates are composed of  $\beta$ -D-mannuronic acid (M) and  $\alpha$ -L-guluronic acid (G). Backbone consists of sequences of blocks of mannuronic acid (M-blocks) or guluronic acid (G-blocks), and regions of alternating sequences (MG-block).

## Alginates

Alginate is the term used for the salts of alginic acid, but it also refers to all derivatives of alginic acid and alginic acid itself. Alginates are structural components of brown micro-algae (*Phaeophyceae*) cell walls that are present in the form of divalent salts of alginic acid and form the intercellular gel matrix. Alginates are linear polysaccharides that are composed of  $\beta$ -D-mannuronic acid (M) and  $\alpha$ -L-guluronic acid (G) residues linked via 1→4 glycosidic linkages (Fig. 5). Accordingly, alginate backbone consists of sequences of blocks of mannuronic acid (M-blocks) or guluronic acid (G-blocks), and regions of alternating sequences (e.g., MG, MMG, GGM) (Grasdalen et al., 1979, 1981). The M-blocks are linked via  $\beta$ -1,4 linkages adopting  ${}^4C_1$  chair conformation that imparts flexibility to the chain, whereas G-blocks are stiff structures of buckled shape due to the  ${}^1C_4$  conformation of guluronate residues linked via  $\alpha$ -1,4 linkages (Atkins et al., 1970; Grasdalen et al., 1977; Penman and Sanderson, 1972). As a result, the stiffness of the blocks on the backbone follows the order (from most to least stiff): GG > MM > MG (Smidsrød et al., 1973).

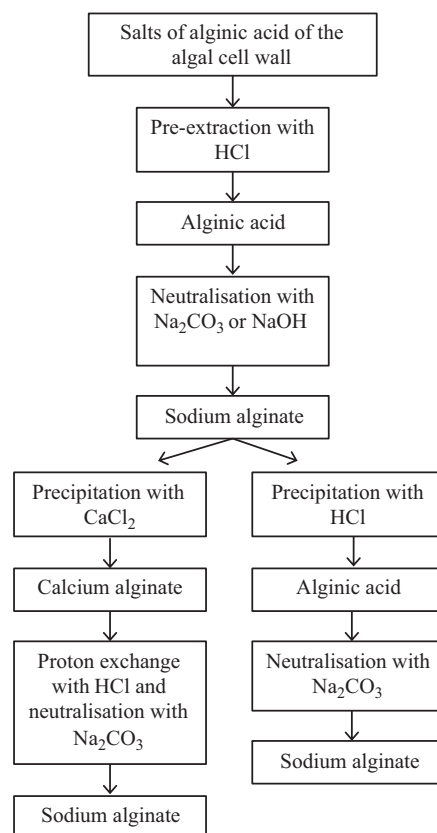
The main brown seaweed species used for commercial alginate production are *Laminaria hyperborea*, *Macrocystis pyrifera*, *Laminaria digitata*, *Ascophyllum nodosum* and to a lesser extent from *Laminaria japonica*. The ratio of mannuronic to guluronic acid, sequence of monomers, length of blocks, and molecular weight of the chains vary with the source and consequently impact the physical properties of alginates (Penman and Sanderson, 1972; Smidsrød and Haug, 1972; Haug et al., 1967). Alginates isolated from *L. hyperborea* typically have the highest guluronic acid content (M/G ratio ~0.62), whereas those extracted from *L. japonica* (M/G ratio: 2.34–3.18) and *A. nodosum* (M/G ratio: 1.29–1.85) are low in guluronic acid and therefore exhibit weak gelling properties (Grasdalen et al., 1979; Penman and Sanderson, 1972; Draget et al., 2006; Haug and Larsen, 1962; Minghou et al., 1984).

Alginate extraction is based on conversion of all insoluble salts of alginic acid that are present within the cell wall of brown seaweed to the soluble ( $\text{Na}^+$ ) salt of alginic that is subsequently recovered as alginic acid or calcium alginate. Isolation of alginates can be divided into three major stages that is pre-extraction, neutralization, and precipitation (Fig. 6). Initially, proton exchange with a strong acid (e.g., HCl) converts salts of alginic acid into free alginic acid. In the next step, insoluble alginic acid is solubilized by neutralization with alkali to form water-soluble sodium alginate, which is consequently recovered from the extraction solution by precipitation with hydrochloric acid, calcium chloride, or alcohol, and finally dried and milled (Fig. 6). Sodium alginate is the major commercial form of alginate, however, there are other forms of soluble alginates such as alginic acid and its calcium, ammonium, and potassium salts, or esters of alginic acid, such as the propylene glycol alginate (PGA).

## Physical Properties

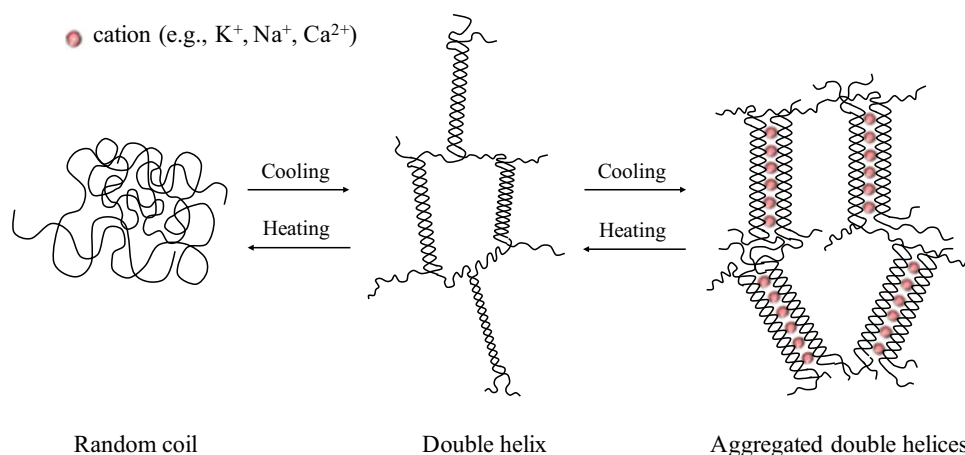
### Carrageenan

All forms of carrageenan are soluble in water and the type of carrageenan, temperature, pH, ionic strength of the medium and the presence of cations are the factors that influence its aqueous solubility. Their hydrophilic character originates from sulphate and hydroxyl groups whereas hydrophobicity from the 3,6-AG units resulting in differences of water solubility between different types of carrageenan. For instance,  $\lambda$ -carrageenan has three sulphate groups and no 3,6-AG content and, therefore, is easily soluble under most conditions. Water solubility of carrageenan follows the order (from most to least soluble):  $\lambda$  >  $\iota$  >  $\kappa$ -carrageenan. The presence of cations in the solution induces aggregation between carrageenan helices resulting in alterations in solubility. For instance, all salts of  $\lambda$ - and sodium salts of  $\kappa$ - and  $\iota$ -carrageenan are soluble in cold water, whereas potassium salts of  $\kappa$ - and  $\iota$ -carrageenan are soluble only in hot water. The viscosity of carrageenan solutions decreases at pH values below 4.3 and in particular at high temperatures (70–120 °C) due to backbone depolymerisation (Imeson et al., 2009). Carrageenan solutions exhibit shear-thinning flow behavior and gel at certain temperatures and cation concentrations. Gelation of  $\kappa$ - and  $\iota$ -carrageenan is comprised of two consecutive steps: a coil-to-helix transition upon cooling and cation-induced aggregation of helices (Fig. 7). First, carrageenan dispersions are heated to about 80 °C. At this stage, chains attain random coil conformation due to the electrostatic repulsions between adjacent polymer chains. On cooling to approximately 40–60 °C, carrageenan solutions demonstrate pronounced increase in viscosity and undergo coil-to-double helix conformational transition. The final sol–gel transition occurs in the presence of cations that leads to the helix–helix aggregation of the adjacent spiral chains that contain sulphate groups and formation of a stable three-dimensional network (Piculell et al., 2006).  $\lambda$ -Carrageenan does not gel, but forms polyelectrolyte



**Figure 6** Generalized extraction strategy of alginates.

solutions and is utilized as a thickening agent in dairy products.  $\kappa$ - and  $\iota$ -carrageenan form thermo-reversible gels at concentrations as low as 0.5% and cation concentrations between 0.2%–0.8%. The strength of carrageenan gels depends on the biopolymer concentration, type of the salt (e.g., KCl, CaCl<sub>2</sub>, NaCl) and concentration of the gelling cation. For instance,  $\kappa$ -carrageenan in the presence of K<sup>+</sup> forms firm, brittle gels, whereas  $\iota$ -carrageenan require Ca<sup>2+</sup> and forms soft, and elastic gels (Kara et al., 2006).  $\kappa$ -Carrageenan has lower gelation temperature (35–65 °C) as opposed to  $\iota$ -carrageenan (40–70 °C) at equivalent gelling conditions (Imeson et al., 2009; Nishinari et al., 1990). Typically, the higher the cation concentration, the greater the gelling temperature and gel strength (Michel et al., 1997). The presence of co-solutes, such as sucrose also increases gelation and melting temperatures of



**Figure 7** Gelation mechanism of carrageenan. Carrageenan dispersions are heated to about 80 °C and chains attain random coil conformation. On cooling to approximately 40–60 °C, they undergo coil-to-double helix conformational transition. The final sol–gel transition occurs in the presence of cations that leads to the helix–helix aggregation of the adjacent chains that contain sulphate groups. Gels are thermoreversible and melt on heating.

carrageenan solutions (Nishinari et al., 1990). Carrageenan gels are thermally reversible (melt at temperatures  $\sim 5\text{--}20^\circ\text{C}$  above the gelling temperature and re-gel on cooling) and also exhibit hysteresis (difference between gelling and melting temperatures).

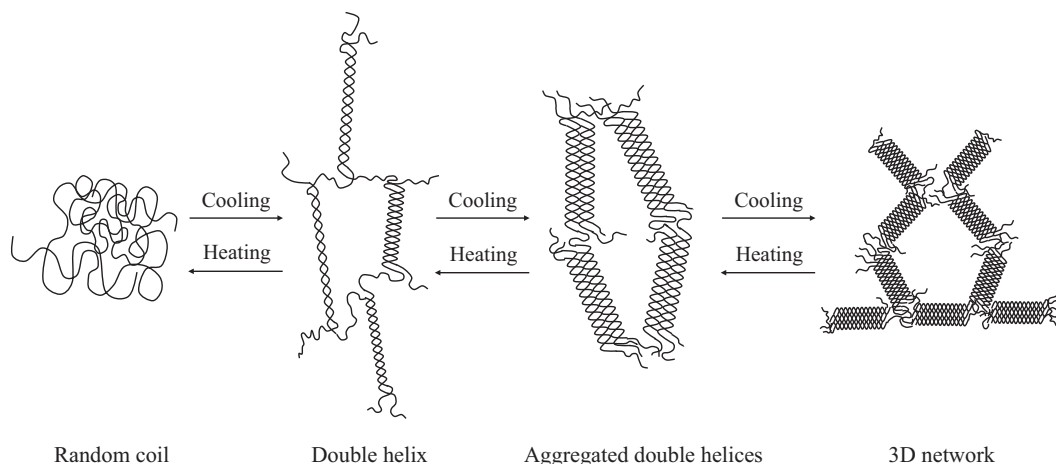
Textural properties and applications of  $\kappa$ -carrageenan gels can be improved by addition of other hydrocolloids (e.g., galactomannans or xanthan) (Williams et al., 1993). Hot solutions of  $\kappa$ -carrageenan and galactomannans form strong and elastic gels with low syneresis in contrast to brittle  $\kappa$ -carrageenan gels. The synergistic effects between  $\kappa$ -carrageenan–galactomannan systems depend on the mannose/galactose ratio of the galactomannan backbone (Dea and Morrison, 1975). The mannose-free regions of the galactomannans, particularly of locust bean gum, are able to associate with carrageenan helices resulting in gel formation (Fernandes et al., 1991). Another notable synergistic interaction of carrageenan is with milk proteins, primarily with casein micelles where carrageenan forms weak gels in the aqueous phase and adsorbs at the surface of casein micelles through interactions with positively charged amino acids (Langendorff et al., 2000).

## Agar

Agar is insoluble in cold water and for dissolution requires heating at temperatures of above  $\sim 85^\circ\text{C}$ . The viscosity of agar solutions at  $45^\circ\text{C}$  is not affected by ionic strength or pH in the pH-range between 4.5–9.0 (Stanley et al., 2006) and it depends on history, as when they are cooled and reheated back to initial temperature, they exhibit higher viscosity (Armisen et al., 2009). Agar is potent gelling agent, as it forms networks at concentrations as low as 0.1%–0.2% w/v in the absence of any cross-linking ions in contrast to carrageenan. Their gelling capacity arises from the agarose fraction and is stabilized exclusively by hydrogen bonding between the 3,6-anhydro- $\alpha$ -L-galactopyranose residues of the chains. The gelation of agar commences with a heating step ( $\sim 85\text{--}95^\circ\text{C}$ ) where chains adopt random coil conformation. Cooling the system to gelling temperature ( $\sim 33\text{--}45^\circ\text{C}$ ) results in the sol–gel transition that proceeds in two steps (Armisen et al., 2009; Normand et al., 2000). Initially, randomly distributed, single agarose coils join to form a double helical association *via* intra-molecular hydrogen bonding. This is followed by aggregation of double helices *via* inter-molecular hydrogen bonding into a three-dimensional gelled network (Fig. 8).

Agar forms thermo-reversible gels, that melt by heating and gel again upon cooling showing considerable hysteresis (melting  $\sim 85\text{--}95^\circ\text{C}$  and gelling  $\sim 33\text{--}45^\circ\text{C}$ ). Agar gels have the most pronounced hysteresis values among gelling polysaccharides that are typically in the range of  $40\text{--}60^\circ$  depending on the source of seaweed. For instance, hysteresis values of  $\kappa$ -carrageenan are in the range of  $15\text{--}27^\circ$ , whereas the lowest values are reported for  $\iota$ -carrageenan ( $2\text{--}5^\circ$ ) (Rees et al., 1969). The gelation temperatures of agar increase with the degree of methoxylation (Guiseley, 1970; Falshaw et al., 1998). Agar gels are susceptible to “syneresis” that is separation of water from the system during ageing. This is attributed to the contraction of the network by slow further aggregation of double helices that reduces the interstitial space available for water. The rate of syneresis is lower for agar gels that contain high ester sulphate content (Lahaye, 2001).

Agar typically forms transparent, stiff gels; however, their rheological properties can be modified by addition of sugars (e.g., sucrose, glucose, trehalose) or incorporation of other polysaccharides (e.g., locust bean gum, xanthan, alginates). The addition of sugars to the agar solutions promotes formation of helices and results in increased gel strength and higher gelation temperatures (Rees et al., 1969; Watase et al., 1990; Vilgis, 2015). This is attributed to the “exchange” of free water by sugar molecules that leads to increased hydrocolloid concentration and decreased distance between polymer chains thus favouring intermolecular polymer–polymer interactions. Agar–locust bean gum mixtures show the most notable synergistic interaction that results in formation of stronger gels and improved mouthfeel making the texture of agar gel similar to gelatin, a property that is widely utilized in food



**Figure 8** Gelation mechanism of agar. The gelation of agar commences with a heating step ( $\sim 85\text{--}95^\circ\text{C}$ ) where chains adopt random coil conformation. Cooling the system to gelling temperature ( $\sim 33\text{--}45^\circ\text{C}$ ) results in the sol–gel transition and formation of double helical associations. This is followed by aggregation of double helices into a three-dimensional network. Double helices of agar are more compact than carrageenan due to the smaller amount of sulphate groups.

formulations (Armisen et al., 2009; Stanley et al., 2006; Sousa and Gonçalves, 2015). Multicomponent gel systems of agar, xanthan and alginate are also formulated and typically aim to expand the range of textures and mouth-feel of agar gels (Russ et al., 2013).

### Alginates

Alginates have a wide range of molecular weight distribution and are charged polysaccharides with electrostatic forces originating from the carboxylic groups within the biopolymer backbone. Therefore, its solubility depends strongly on the pH and ionic strength of the solvent, and the presence of ions in the solution (Mackie et al., 1980). Alginates are soluble in aqueous solutions at pH above its dissociation constant ( $pK_a$ ). The  $pK_a$  of mannuronic and guluronic acids are 3.38 and 3.65, whereas the  $pK_a$  of the global alginate structure varies within this range (Haug et al., 1967). An abrupt decrease of pH results in protonation of carboxyl groups leading to precipitation of alginate, whereas a slow and controlled release of protons results in formation of alginic acid gel. The pH range at which precipitation of alginates occurs is controlled by chemical composition, molecular weight and sequence of M-, G-, and MG-blocks of the backbone. For instance, alginates that contain more MG-blocks will precipitate at lower pH values as opposed to those that contain homogeneous M- and G-blocks. Change in the ionic strength of alginate solution has considerable impact on alginate solubility and viscosity due to the decrease of electrostatic interactions between the biopolymer chains. Monovalent metal ions (e.g.,  $Na^+$ ) form soluble salts with alginate whereas divalent or multivalent cations form gels. Alginates form highly viscous solutions due to the highly extended conformation of the chains and large hydrodynamic volume. The viscosity of alginate solutions depends the length of M and G blocks on the backbone, and on external factors such as pH and ionic strength of the solution.

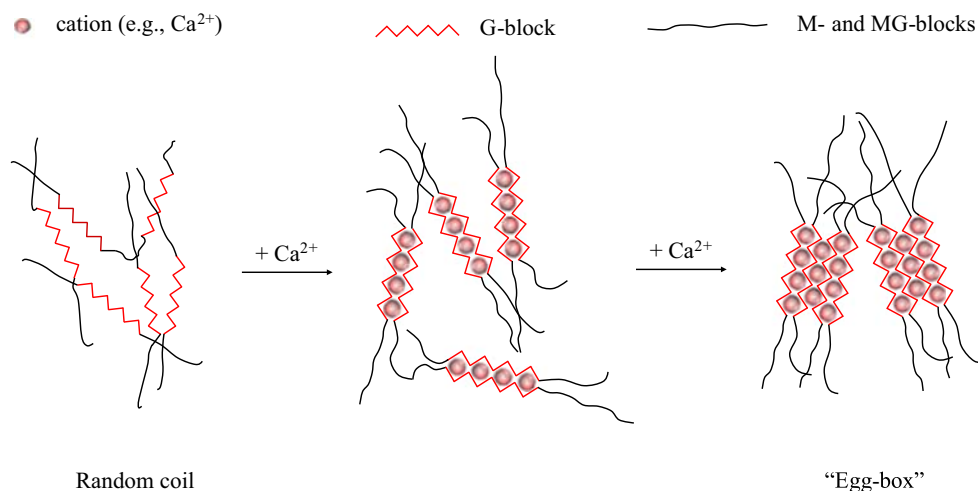
The most prominent physical property of alginates is the selective binding of divalent and multivalent cations that determines their ability to form gels. Cations have different affinity for alginates, although the extent of cross-linking may be also influenced by the chemical composition (M/G ratio). For instance, alginates with high content of G-blocks form tightly held junction zones in the presence of divalent cations (e.g.,  $Ca^{2+}$ ) and form gels of considerably higher strength compared to alginates rich in M- or M-G-blocks that exhibit lower affinity towards divalent cations (Morris et al., 1978; Wang et al., 1993). In contrast, trivalent cations (e.g.,  $La^{3+}$  and  $Pr^{3+}$ ) bind to both M- and G-blocks of alginate backbone (Deramos et al., 1997). The affinity of alginates towards divalent ions decreases in the following order:  $Pb > Cu > Ba > Sr > Ca > Zn > Mn$ , however,  $Ca^{2+}$  is the most commonly utilized to induce gelation. Gelation of alginates is described by the “egg-box” model, according to which alginate chain-chain interactions are induced by the presence of cross-linking divalent cations (Stokke et al., 2000; Donati et al., 2005). Alginate blocks show different mechanisms of interactions with cross-linking cations. For instance, G-blocks integrate cations into pocket-like structures formed between adjacent chains of guluronate residues, whereas M-blocks bind cations externally near the carboxylate groups. In MG blocks, cations are preferentially located in a concave structure formed by MG pairs (Emmerichs et al., 2004). The strongest interactions between junction zones occur in guluronic acid residues, as the buckled chain conformation enables strong gel formation (Donati et al., 2005; Grant et al., 1973). Ionotropic gelation of alginates can be performed by two ion-dependent methods: “diffusion” (sometimes referred as “dialysis”) and “internal setting” method. In the “diffusion” method, alginate solution is dripped into the solution of cross-linking ion (e.g., soluble calcium salts,  $CaCl_2$ ). Calcium ions diffuse into the alginate solution and alginate beads are formed (Fig. 9).

This method is characterized by fast gelation kinetics, heterogeneous distribution of alginate and widely utilized in the production of restructured food products and encapsulation of bioactive compounds. In “internal setting” method, inactive form of cross-linking ion (e.g., insoluble calcium salts,  $CaCO_3$ ,  $CaSO_4$ ) is mixed with alginate solution. Then, a slowly hydrolyzing agent (e.g., D-glucono- $\delta$ -lactone, GDL) is added to the mixture of alginate and inactive cross-linker. The hydrolysis of GDL to gluconic acid results in gradual decrease of the pH of the system and production of protons that convert inactive cross-linker (e.g.,  $CaCO_3$ ) into its active form (e.g.,  $Ca^{2+}$ ). Slow release of cross-linker in the alginate solution leads to the formation of homogeneous gels. Alginates also gel following cation-independent gelation mechanism. In this method, pH of alginate solutions is gradually lowered below the  $pK_a$  of the uronate residues leading to the reduction of electrical repulsion between biopolymer chains and consequently formation of alginic acid gels though intermolecular hydrogen bonding. Alginic acid gels are typically produced either by addition of a hydrolyzing lactone (e.g., GDL) or by conversion of ionically cross-linked (pre-formed) gel to the acid gel by addition of mineral acids. The M/G ratio and length of those blocks in the alginate backbone have a considerable impact on the mechanical properties of the resulting gels. Alginates with low M/G ratios produce strong and brittle gels with good heat stability but with pronounced syneresis after freeze-thaw processing, whereas alginates with high M/G ratios produce more elastic gels with good freeze-thaw stability (Grant et al., 1973; Braccini and Pérez, 2001). The strength of alginate gel increases when the average length of guluronate blocks changes from 5 to 15 leading to a formation of mechanically strong, stable, and porous gel. However, gel strength is relatively unaffected by molecular weight values greater than  $200 \times 10^3 \text{ g mol}^{-1}$ .

## Food Applications

### Carrageenan

Carrageenan is utilized mainly as gelling agent, thickener, and stabilizer at concentrations between 0.005% and 2.0% w/w. Carrageenan is weaker gelling agent than agar, however, their ability to produce gels with a wide variety of textures is highly valued. Food applications of carrageenan depend on whether they are added to dairy or aqueous systems (e.g., water-based dessert gels) (Table 2).



**Figure 9** Gelation mechanism of alginates. G-blocks integrate divalent cations into pocket-like structures formed between adjacent chains of guluronate residues leading to formation of a network. This is known as the “egg box” model.

**Table 2** Food applications of seaweed polysaccharides (Stanley et al., 2006; Hambleton et al., 2009; Fabra et al., 2012)

Applications	Functionality	Seaweed polysaccharide
Desserts (e.g., dessert jellies, low-energy jellies, non-dairy puddings, sorbets)	Gelation, emulsion stabilization, emulsification	$\kappa$ - and $\iota$ -carrageenan, $\kappa$ -carrageenan with galactomannans, alginates, agar
Seasonings (e.g., relishes, sauces, vinaigrette salad dressings)	Gelation	$\kappa$ - and $\iota$ -carrageenan, alginates
Meat (e.g., low fat meats, cooked hams, meat preserves, canned meat, restructured meat and fish products, pet food)	Gelation, thickening, fat stabilization and reduction	$\kappa$ -Carrageenan and galactomannans, $\iota$ -carrageenan and konjac gum, alginates, agar
Beverages (e.g., fruit drinks, beer, wine)	Emulsion stabilization, foam stabilization, flocculating agent	$\kappa$ - and $\iota$ -carrageenan, Na-alginates, agar
Bakery (e.g., gluten-free breads, pastry fillings, pie fillings, icings and glazes)	Improve loaf volume, gel formers/binders, moisture barrier, antistaling agent	$\kappa$ -Carrageenan, alginates, agar
Milk gels (e.g., custard, pudding) and whipped dairy products	Gelation, syneresis control, thickening, emulsion stabilization	All forms of carrageenan and their mixtures
Milks (pasteurized, sterilized)	Suspension, mouthfeel, emulsion stabilization	Combinations of $\kappa$ -, $\iota$ - and $\lambda$ -carrageenan
Frozen desserts (e.g., ice cream, ice milk)	Prevents whey separation, prevents formation of ice crystals	$\kappa$ -Carrageenan, alginates, agar
Processed cheeses (e.g., cheese slices, cream cheese)	Gelation, moisture binding, improve slicing, texture improver	$\kappa$ -Carrageenan with locust bean gum, agar
Food films and coatings	Extended shelf-life	Alginates, all forms of carrageenan
Foods with encapsulation technology	Aroma encapsulation, controlled release of aroma compounds	$\iota$ -Carrageenan, alginates

All forms of carrageenan are utilized at concentrations  $<0.3\%$  in dairy formulations (Anderson et al., 2002; Langendorff et al., 1999). Carrageenan interacts with milk proteins and forms a network that prevents whey separation and aggregation, and stabilizes particles such as cocoa suspensions in chocolate milk. Water-based desserts are the typical non-dairy carrageenan applications, in which  $\kappa$ -carrageenan or  $\kappa$ -/ $\iota$ -carrageenan blends are used at concentrations  $>0.3\%$ . The synergistic interactions of carrageenan with galactomannans are also widely utilized in the production of fruit sorbets, poultry and meat products. The thixotropic nature of  $\iota$ -carrageenan gels is applied in salad dressings formulations to stabilize suspended herbs and vegetable particles. In drink industry, they are used at low concentrations ( $\sim 0.2\%$ ) to stabilize suspensions (e.g., fruit juices) and colloidal systems (e.g., soft drinks) or increase the viscosity and add palatability to liquid food products (Piculell et al., 2006). Recently, carrageenan is also explored in bread making industry with particular interest in improvement of dough proofing characteristics and formulation of gluten-free breads (Rosell et al., 2001; Sciarini et al., 2010).



## Agar

Food applications of agar rest on its high gelling capacity, high hysteresis, and perfect gel reversibility. Agar is largely utilized in the baking industry due to the ability to withstand high temperatures as opposed to carrageenan, and is effective in retarding staling of cakes and bread. In confectionary products (e.g., cakes or doughnuts), agar is primarily utilized (0.2%–1.0% w/v) to prevent the dehydration of the product and maintain the integrity of the icing during storage. Agar is also important in fruit jelly confections, due to its ability to gel without high sugar concentrations. In meat industry, agar is used at levels of 0.5% to 2.0% in gelled canned meat products, fish, poultry and boiled sausages as a structuring and fat reducing agent (Stanley et al., 2006).

## Alginates

Alginates are used as thickeners, gelling agents, and stabilizers of aqueous mixtures, dispersions, and emulsions. Alginates are polyelectrolytes and therefore can interact electrostatically with proteins in mixed systems resulting in increase in viscosity. These types of interactions can be utilized to stabilize and enhance the mechanical properties of gelled networks in some restructured food products. The process of food restructuring is based on binding together sectioned, chunked or milled food components (e.g. meat cuts with high connective tissue content, or homogenized fruits and vegetables) to make them resemble the original or formulate novel food products (Draget et al., 2006). Gelation of alginates is independent of temperature and therefore can be used in the restructuring of foods that may become damaged or oxidized at high temperatures (e.g. meat products, fruits and vegetables). Common restructured foods produced using alginates are reconstituted onion rings, pimento olive fillings, and cocktail berries (Draget et al., 2006; Mancini and McHugh, 2000; Manjunatha and Gupta, 2006). Synergistic interactions between G-rich alginates and pectins with high degree of esterification result in formation of thermo-reversible gels that are commonly utilized in the production of jams (Walkenström et al., 2003; Toft et al., 1986). The advantage of alginate-pectin gels is that they are independent of sugar content and therefore can be utilized in low-calorie food products. PGA is the most common alginate derivative used in food formulations acting simultaneously as surface-active and gelling agent and finds applications in foams (e.g., desserts, beer froth) and emulsions (Nilsen-Nygaard et al., 2016; Jackson et al., 1980). PGA has also high tolerance to  $\text{Ca}^{2+}$  and low pH and is suitable for applications in fermented milk-based products and salad dressings. In food industry, alginate matrices (e.g., alginate, alginate with xanthan gum, chitosan, or pectin) may also be used for encapsulation and delivery of live cells (probiotics) to the large intestine and colon and in immobilization of reactive or volatile molecules (e.g., enzymes, flavours, etc.) (Cook et al., 2012; Desai and Park, 2005). Alginates have been also used as edible coatings in meat products and a number of fresh fruits and vegetables where the coating maintains texture, reduces the rate of browning, inhibits the growth of yeast and molds, decreases weight loss, and provides color and moisture retention (Rojas-Graü et al., 2007; Azarakhsh et al., 2014; Jiang et al., 2013; Sipahi et al., 2013; Robles-Sánchez et al., 2013).

## Conclusions

Seaweed polysaccharides are widely utilized in the food industry and are of particular technological importance due to their broad spectrum of functionality. The physical properties (e.g., gelling, viscosity enhancement etc.) are tunable by controlling molecular properties of the chains and the environmental conditions (e.g., pH, ionic strength, etc.). This results in hydrocolloid systems with remarkably wide spectrum of physical properties that find applications across food industry. The most industrially relevant types of carrageenan are kappa-, lambda-, and iota-that are sulphated anionic galactans. Agar is also a linear galactan with backbone of two alternating disaccharides, agarobiose and neoagarobiose consisting of two major polysaccharide fractions, namely agarose and agarpectin.  $\kappa$ - and  $\iota$ -types of carrageenan are able to form gels ( $\lambda$ -does not form gels) assisted by the presence of cations most commonly potassium. In contrast to carrageenan, agar forms networks in the absence of any cross-linking ions and is more potent gelling agent. Gelling capacity of agar arises from the agarose fraction as agarpectin does not play central role in the process. Alginates are linear polysaccharides that are composed of mannuronic acid (M) and guluronic acid (G) residues. Accordingly, alginate backbone consists of sequences of M- or G-blocks, and regions of alternating sequences (e.g., MG, MMG, GGM). Consequently, the length of sequence of M and G blocks in the alginate backbone controls its physical properties. The most prominent physical property of alginates is the selective binding of divalent cations and in food industry gels are usually formed in the presence of calcium. Use of seaweed polysaccharides in foods is well established and future technologies should focus on their potential applications beyond food industry (e.g., biomedical, pharmaceutical, or drug industries) with the overall aim to create advanced formulations with tailored functionality (e.g., for nutrient and drug delivery, or wound healing).

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# Sequestrants as a Food Ingredient

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## Glossary

**Chelation** a type of bonding of ions or molecules to metal ions; involves the formation or presence of two or more separate coordinate bonds between a polydentate ligand and a single central atom.

**Food additives** substances (synthetically produced or naturally occurring) incorporated in the formulation of a food product with the purpose to contribute in the processing, storage, preparation, or quality of foods.

**Ligand** a molecule, ion, or atom that is bonded to the central metal atom of a coordination compound.

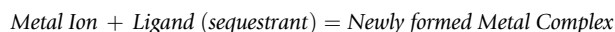
**Sequestrant** a type of a food additive which improves the quality and stability of foods through the formation of chelate complexes.

## Overview

Sequestrants (chelating agents) offer food processors a valuable tool to be used to ensure integrity of whole and processed foods. The reaction caused by sequestrants protects food products from chemical, oxidative, and enzymatic reactions that promote deterioration during processing, storage, or preparation of foods through chelation. Sequestrants form chelate complexes with polyvalent metal ions (i.e. copper, iron and nickel), which can serve a variety of purposes in maintaining food quality (Aamoth and Butt, 1960). The term chelate comes from the Greek word “claw”, and they often resemble a claw with the metal ion clutched between the pincers of the organic molecule (Bessman and Doorenbos, 1957). As a food additive that has been around and used for over 75 years, the mechanistic action and purposes of sequestrants as a food additive are relatively well understood (Bessman and Doorenbos, 1957; Albert and Serjeant, 1984). This book chapter will help readers understand the mechanistic action of sequestrants, provide insight to common examples of sequestrants, and describe the use of sequestrants in foods.

## Mechanistic Action of Sequestrants

The reaction of a sequesterant with metallic and alkaline ions causes new metal complexes to be formed. In the newly formed complex, the metallic or alkaline ion is bound with a charged or uncharged electron donor referred to as the ligand (Damodaran et al., 2007; Furia and Furia, 1972; Yoe, 1958). By definition, the sequesterant is the molecule with the ligand properties (binding sites) that enable the newly formed metal complex to be formed (Furia and Furia, 1972).



Two general conditions must be satisfied for a sequesterant to be effective (Furia and Furia, 1972). First, the sequesterant must have the proper steric and electronic configuration in relation to the metal ion that is being acted upon, and secondly the environmental conditions (pH, ionic strength, solubility, etc.) must be conducive to the formation of the new complex.

## Examples of Sequestrants

While a great number of sequestrants are known to be highly effective as metal complexing agents, a great concern of food processors and government regulatory agencies continues to surface regarding their safety (Furia and Furia, 1972). Thus, most sequestrants used in the food industry are naturally occurring substances such as polycarboxylic acids, hydroxycarboxylic acids, polyphosphoric acids, amino acids, and various other macromolecules (Furia and Furia, 1972). Once safety and efficacy are established, the other determining factors determining the usefulness of a sequesterant is the effect on product quality and sensory attributes (color, flavor, etc.). Depending on the food product being manufactured, other considerations when choosing a sequesterant includes the substrate capability and the particular metal(s) in the food matrix that warrants chelation. Common examples of sequestrants in foods can be broken into three major categories, which will be discussed in the succeeding subsections.

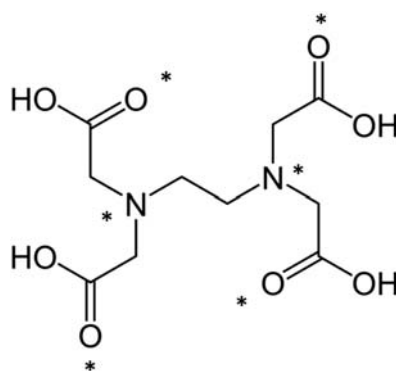
### Ethylenediaminetetraacetic Acid

Likely the most commonly recognized of all sequestrants, EDTA is widely used with great success as a chelating agent in both food and non-food applications (Yoe, 1958; Gardiner, 1976; Branen and Davidson, 2004). EDTA is colorless, water soluble solid that is typically used in the food industry in a di-sodium salt form, a di-ammonium form, a chelated ferric sodium form, or a ferric

ammonium salt form. EDTA contains six donor groups (hexadentate) and has the ability to bind six times, at two nitrogen positions and at four oxygen positions (**Fig. 1**). Examples of food applications of EDTA salts include beverages (beer and malt beverages), condiments (salad dressing and sauces), canned meats and seafoods, and many others (**Table 1**). Overall, EDTA forms very stable complexes with most metal ions (particularly calcium and magnesium), and has been proven to be safe at low inclusion levels in food products ([Lanigan and Yamarik, 2002](#); [Heimbach et al., 2000](#); [Wedeen et al., 1983](#)).

## Phosphates

Phosphates are the naturally occurring form of the mineral phosphorus and the salt-forming anion of phosphoric acid. Many different types of phosphate salts are approved for use including sodium phosphates, calcium phosphates, and potassium phosphates (**Table 2**). Common food applications include processed meat products, ice cream, and frozen dairy products. With particular inference to meat processing, phosphates offer multi-functional roles with direct effects on water retention and improved binding properties ([Sindelar, 2015](#)). The meat industry has been attempting for several years to replace phosphates with more “natural” ingredients to meet clean label requirements, yet no direct substitutes have been discovered nor are any complete substitutes being used at the present time.



**Figure 1** The chemical structure of Ethylenediaminetetraacetic Acid (EDTA) with potential binding sites depicted with an asterisk (\*).

**Table 1** List of permitted EDTA substances permitted uses according to United States Food and Drug Administration (FDA) and Canadian Food Inspection Agency (CFIA)

<i>Name of compound</i>	<i>Permitted uses</i>
Calcium disodium EDTA	<p>Ale; Beer; Malt liquor; Porter; Stout</p> <p>Unstandardized dressings; Unstandardized sauces; French dressing; Mayonnaise; Salad dressing</p> <p>Potato salad; Unstandardized sandwich spreads</p> <p>Canned shrimp; Canned tuna; Canned crabmeat; Canned lobster; Canned salmon</p> <p>Margarine</p> <p>Canned clams; Canned sea snails; Canned snails</p> <p>Canned legumes except canned green beans, canned peas and canned wax beans</p> <p>Unstandardized beverage concentrates, except unstandardized dairy beverage concentrates and unstandardized alcoholic beverage concentrates</p> <p>Pasteurized sous-vide potatoes</p> <p>Beans; Beans with pork</p>
Disodium EDTA	<p>Dressing and sauces</p> <p>Unstandardized sandwich spreads</p> <p>Canned legumes except canned green beans, canned peas and canned wax beans</p> <p>Dried banana products</p> <p>Aqueous suspensions of color lake preparations for use in coating confectionery tablets</p> <p>Pasteurized sous-vide potatoes</p> <p>Edible coating for sausages</p> <p>Beans; Beans with pork</p>

Information sourced from regulatory webpages of the United States Food and Drug Administration (FDA) and Canadian Food Inspection Agency (CFIA).

**Table 2** List of permitted phosphates with sequestration activity and their permitted uses according to United States Food and Drug Administration (FDA) and Canadian Food Inspection Agency (CFIA)

<i>Name of compound</i>	<i>Permitted uses</i>
Calcium phosphate, monobasic	Unstandardized dairy products; Ice cream mix; Ice milk mix; Sherbet
Calcium phosphate, tribasic	Ice cream mix; Ice milk mix
Potassium phosphate, monobasic	Unstandardized foods; Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers; Ice cream mix; Ice milk mix; Sherbet
Potassium phosphate, dibasic	Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers
Potassium phosphate tetrabasic	Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers; Canned seafood
Potassium tripolyphosphate	Unstandardized foods; Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers; Canned seafood
Sodium acid pyrophosphate	Unstandardized foods; Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers; Canned seafood; Ice cream mix and milk mix
Sodium hexametaphosphate	Unstandardized foods; Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers; Canned seafood
Sodium phosphate, monobasic and dibasic	Unstandardized foods; Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers; Ice cream mix; Ice milk mix; Sherbet
Sodium potassium hexametaphosphate	Unstandardized foods; Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers; Canned seafood; Ice cream mix; Ice milk mix; Liquid whey products
Sodium potassium tripolyphosphate	Unstandardized foods; Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers; Canned seafood
Sodium pyrophosphate, tetrabasic	Unstandardized foods; Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers; Ice cream mix; Ice milk mix; Sherbet
Sodium pyrophosphate, tribasic	Unstandardized foods; Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers
Sodium tripolyphosphate	Unstandardized foods; Injection or cover solution for the curing of poultry or poultry meat; Pumping pickle for the curing of pork, beef and lamb cuts; Prepared meat; Solid cut meat and poultry; Meat tenderizers
Calcium Phytate	Glazed fruit
Phosphoric acid	Mono- and di-glycerides

Information sourced from regulatory webpages of the United States Food and Drug Administration (FDA) and Canadian Food Inspection Agency (CFIA).

### Citric Acid

Citric acid and other forms of citrate (derivatives of citric acid) occur naturally in citrus fruits (lemons, limes, oranges, etc.; [Table 3](#)). In addition to the acidic properties that citric acid elicits in terms of flavor, preservation, and emulsification – citric acid and other forms of citrate are fairly effective sequestrants by binding metals into water soluble complexes ([Mehlretter et al., 1953](#); [Soccol et al., 2006](#)).

### The Role of Sequestrants in Foods

Newly formed complexes formed by sequestrants exhibit unique properties in terms of their ability to function in a food matrix. Sequestrants have been adopted as common food additives for a variety of purposes in foods; yet the major reason would be to ensure product quality after extended periods of storage and/or shelf time. Sequestrants can contribute an important part in the stabilization and sustainability of fat/oil quality. Sequestrants do not directly contribute to the inhibition of oxidation; however, play a role in the delay of lipid oxidation by chelating trace metals that are pro-oxidant catalysts. Metal chelation slows or even eliminates the ability for pro-oxidant activity of some metals in food products ([Furia and Furia, 1972](#); [Labuza and Dugan, 1971](#)). The synergistic effects of sequestrants and antioxidants have been established for a lengthy period of time in a variety of food applications ([Furia and Furia, 1972](#)). Vitamins tend to be unstable components in food products, and sequestrants have been used (along with antioxidants) for preventing and inhibiting the decomposition of oil soluble vitamins. The use of sequestrants in produce prone to enzymatic browning (i.e. fruits, vegetables, potato, and dairy products) has been well-established for decades ([Furia and Furia, 1972](#)).

**Table 3**      List of permitted citric acids and derivatives with sequestrant activity and their permitted uses according to United States Food and Drug Administration (FDA) and Canadian Food Inspection Agency (CFIA)

<i>Name of compound</i>	<i>Permitted uses</i>
Citric acid	Pumping pickle, cover pickle, and dry cure employed in the curing of preserved meat or preserved meat by-product Unstandardized foods Frozen seafood (clams, crab, crustaceans, fish, lobster, marine mammals, minced fish, molluscs, shrimp; Prepared fish or prepared meat; Preserved fish or preserved meat; Other Frozen marine invertebrates
Ammonium citrate, monobasic or dibasic	Unstandardized foods
Calcium citrate	Unstandardized foods
Sodium citrate	Unstandardized foods Ice cream mix; Ice milk mix; Pumping pickle, cover pickle and dry cure employed in the curing of preserved meat or preserved meat by-product; Sherbet Frozen clams; Frozen crab; Frozen crustaceans; Frozen fish; Frozen fish fillets; Frozen lobster; Frozen marine mammals; Frozen minced fish; Frozen molluscs; Frozen shrimp; Glaze of frozen fish; Prepared fish or prepared meat
Stearyl citrate	Margarine

Information sourced from regulatory webpages of the United States Food and Drug Administration (FDA) and Canadian Food Inspection Agency (CFIA).

Another key role that sequestrants have is their ability to aid in water retention during processing and product storage (Shults *et al.*, 1972; Hellendoorn, 1962; Thorarinsdottir *et al.*, 2001). This example may be observed to the greatest extent with the use of phosphates in processed meat products. Phosphates are a common ingredient in many processed meat products and have direct roles on sensory attributes and processing techniques that are implemented in the meat industry. Similar to the chelation in metals found in water, phosphates may also chelate divalent cations in meat. A classical theory claims that phosphates bind divalent cations away from the protein cross-bridges, allowing the protein structure to unravel and hold more water through capillary activity (Knipe, 1983). Some research even suggests that phosphates may only affect free cations with limited to no effects on cations already bound to proteins (Knipe, 1983). The cation chelation by alkaline phosphates protects cooked meats from “warmed-over” flavors, while also stabilizing cured color, and improving retention during thawing of frozen products (Knipe, 1983).

## Conclusions

Sequestrants provide food processors a valuable tool in the food additive toolbox with a wide range of purposes and uses. The integrity of whole and processed foods can be improved with sequestrants with particular inference on slowing of lipid oxidation and improvement of water retention properties. In general, sequestrants approved for use in food products are considered to be safe and effective at inclusion levels established by governing agencies such as the United States Food and Drug Administration and the Canadian Food Inspection Agency.

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## Relevant Websites

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- [https://chem.libretexts.org/Core/Inorganic\\_Chemistry/Coordination\\_Chemistry/Properties\\_of\\_Coordination\\_Compounds/Ligands/EDTA](https://chem.libretexts.org/Core/Inorganic_Chemistry/Coordination_Chemistry/Properties_of_Coordination_Compounds/Ligands/EDTA) – Inorganic Chemistry.
- <https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/food-additives/lists-permitted/12-sequestering-agents.html> – Health Canada Services.
- <https://www.fda.gov/food/ingredientspackaginglabeling/foodadditivesingredients/ucm091048.htm#abb> – Food Ingredients Packaging.
- <https://oradix.com/content/6-24-benefits-of-chelation> – Oradix.

# Starch

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## Introduction

Starch is a plant polysaccharide that is predominantly found in cereal grains, roots and tubers such as potatoes, cassava and manioc, and legumes such as peas, chickpeas and beans (Table 1). The diversity in starch granule structure and properties is appalling. Even cereal starches widely vary in properties (Tables 1 and 2). Starch occurs in intracellular granules and does not have any function as structure-building molecule in plant cells, but rather functions as a storage polysaccharide. Starch is produced in plant leaves through photosynthesis in the chloroplasts. It is stored in these green plant parts as small granules. This (poorly investigated) assimilation starch is then hydrolysed at night and transported as sucrose to those parts of the plant which either require the energy or serve as energy storage organs (*e.g.*, grains for cereals and roots for tubers). In these storage organs, starch is deposited in amyloplasts as water-insoluble granules. Starch gradually accumulates in these granules and is eventually used as energy source during germination. One amyloplast can either hold one starch granule or a cluster of starch granules. The different morphological characteristics of the starch granules, such as size, shape and architecture, depend on their botanical origin, which makes it relatively easy to microscopically gain insights in the source of the starch (Table 1). In contrast to what is the case in plants, starch does function as a major structure-building and -stabilizing carbohydrate in the human diet. In what follows, focus will be laid on starch structure, chemistry and functionality in food systems.

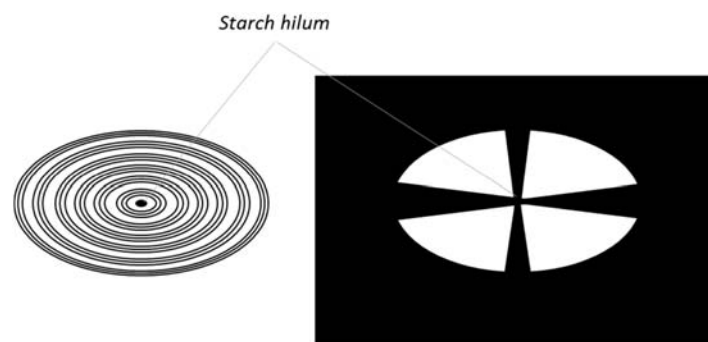
## Structure

Depending on the botanical source, the diameter of starch granules varies between a few to above 100  $\mu\text{m}$  (Table 1). When observing a starch granule under the regular light microscope, it is easy to recognize the hilum of the granule (Fig. 1). This spot is easy to find as it contains a higher amount of water than the rest of the granule. During drying or wetting of the kernels, the hilum slowly disappears. At this specific hilum site, starch biosynthesis is started and granules, hence, grow 'out of' their hilum, layer after layer of starch building blocks are then added on the outside of the growing granule. The ease with which these layers or shells can be observed is dependent on the plant source.

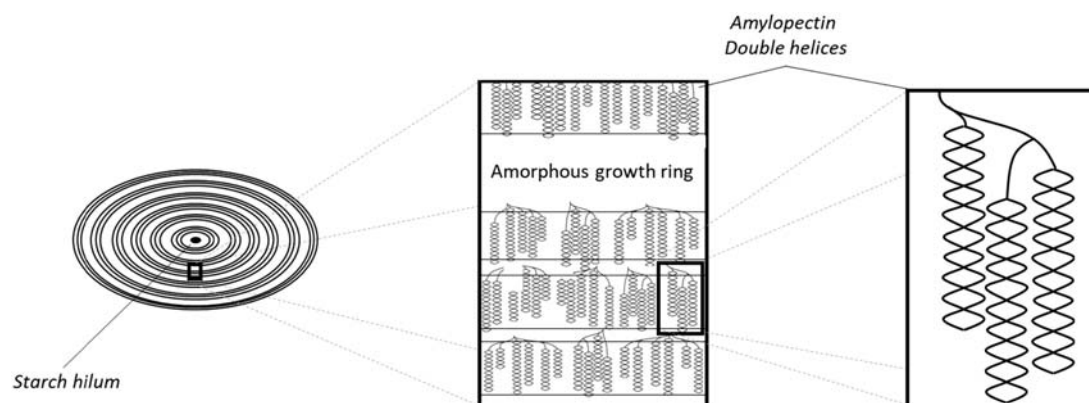
In starch granules, besides the growth rings, different levels of organization can be distinguished at different length scales: blocklets (20–400 nm), lamellae ( $\sim 10$  nm) and lattice spacings (0.1–2 nm). Growth rings can either be more amorphous or crystalline in nature. Amorphous growth rings have a radial thickness of 120 to 400 nm and are less dense and relatively rich in amylose and less ordered amylopectin. The semi-crystalline shells are composed of alternating amorphous and crystalline lamellae of about 9–10 nm. The crystalline lamellae are built of amylopectin double helices which are tightly parallel packed (Fig. 2). The amorphous lamellae are made of the amylopectin branching regions. For a more in-depth discussion on starch granule architecture, the reader is kindly referred to the review by Tester and colleagues (Tester *et al.*, 2004).

**Table 1** Characteristics of starch from different botanical sources (Hoover, 2001; Lasztity and Lasztity, 2009; Gomand *et al.*, 2010; Moorthy, 2002; Delcour and Hosney, 2010; Delcour *et al.*, 2010; Sivoli *et al.*, 2012; Santana and Meireles, 2014)

Plant source	Starch content (% dm)	Starch morphology	Granule diameter ( $\mu\text{m}$ )
Wheat	72	Lenticular Spherical	15–40 1–10
Barley	74	Lenticular Spherical	20–40 2–10
Oats	56	Polyhedral (compound starch)	2–10 (single granules)
Rye	72	Lenticular spherical	25–40 5–10
Corn	80	Polyhedral to spherical	5–30
Rice	76	Polyhedral (compound starch)	3–6 (single granules)
Triticale		Spherical	20
Sorghum	65	spherical	4–25
Potatoes	10–25	Lenticular	10–110
Cassava	25–30	Oval to round (some built as compound granules)	3–43
Peas	25–35	Kidney-shaped	5–20
Chickpeas	35	Oval to round	20
Beans (Carioca)	30	Oval to round	20



**Figure 1** Schematic representation of starch granule structure and polarization microscope image of native starch. The hilum of the starch granule is indicated. Starch granules grow out of this hilum and the hilum is at the crossover point of the Maltese cross visualized under polarization microscopy.

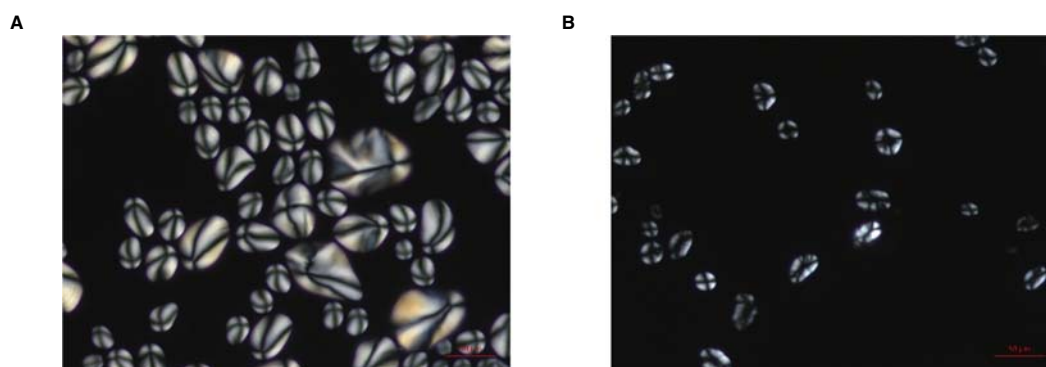


**Figure 2** Schematic representation of starch granule internal structure.

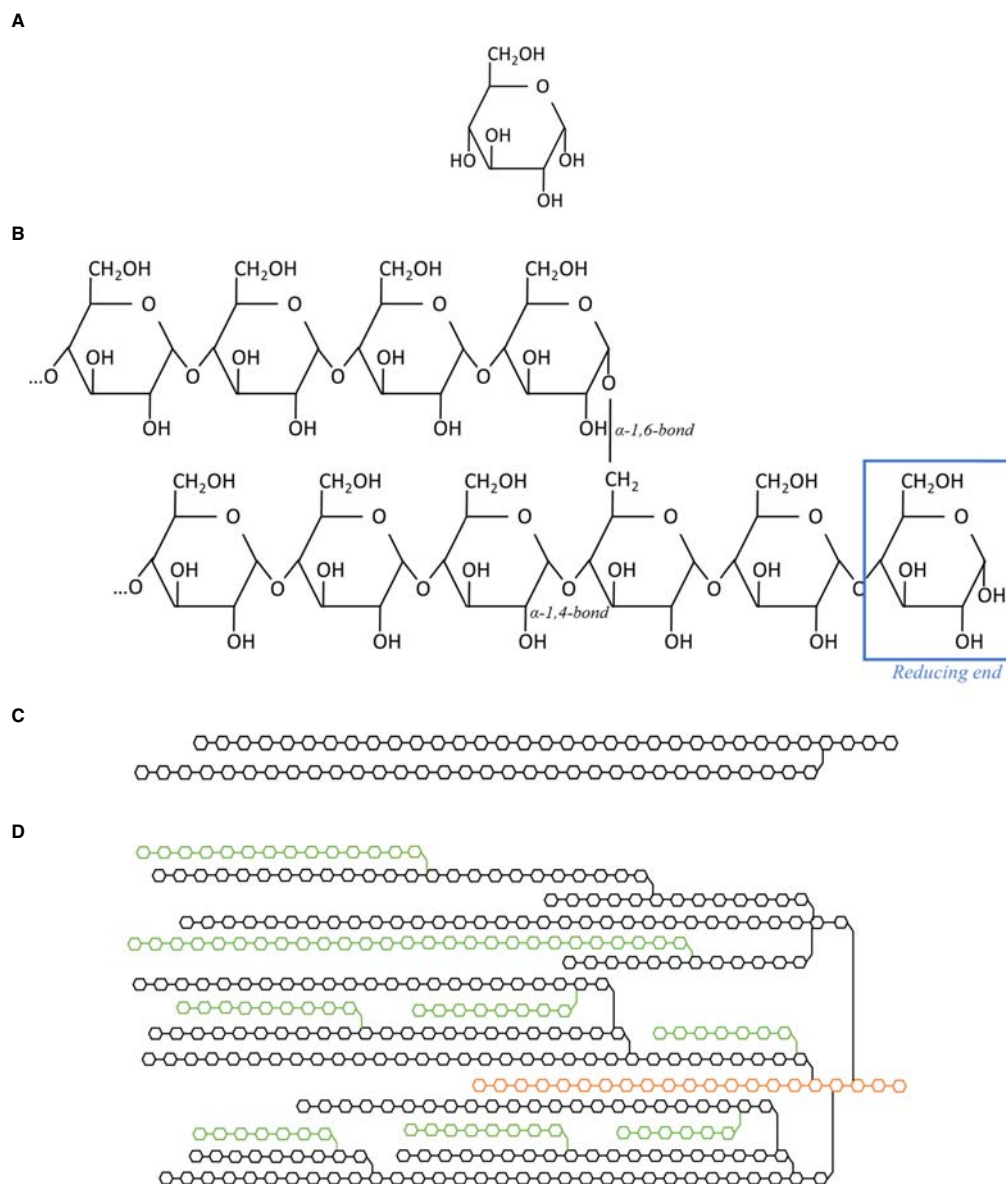
The 'crystallinity' or high degree of order in the starch granules makes them light up under a polarized light microscope as a result of their birefringent character. The typical Maltese cross that is observed for native starch granules (Fig. 3) stems from the radial orientation of the macromolecules in the granules. Amylopectin double helices are responsible for this semi-crystalline character and are organized perpendicular to the starch granule surface (Fig. 2) (Tester et al., 2004). The intersection of the two arms of the Maltese cross coincides with the hilum of the granule (Figs. 1 and 3).

## Composition

Starch granules consist of about 98% polymeric carbohydrate material. The carbohydrate is composed of  $\alpha$ -D-glucopyranosyl units (Fig. 4A). Each individual starch molecule has one hemiacetal group, *i.e.*, the reducing end (Fig. 4B). Starch granules, hence, have



**Figure 3** Polarization microscopy images of (A) potato and (B) pea starch. Image courtesy of Dr. Liu Qiang, Agriculture and Agri-Food Canada.



**Figure 4** Structure of (A)  $\alpha$ -D-glucose unit, (B)  $\alpha$ -1,4 and  $\alpha$ -1,6-linked D-glucose units, (C) amylose and (D) amylopectin. C, B and A chains in amylopectin are shown in orange, black and green, respectively.

minor reducing power. The glucopyranosyl units are connected through either  $\alpha$ -1,4 or  $\alpha$ -1,6 bonds (Fig. 4B). In starch, two main polymers are found, *i.e.*, the (predominantly) linear amylose and the highly branched amylopectin. For most starches, amylose accounts for about 18% to 33% of the starch carbohydrate weight (Table 2) (Buleon et al., 1998). Mutant genotypes exist which have much higher amylose contents (up to 70%; *e.g.*, maize, barley, rice and potato) or do not contain amylose (less than 1%; *e.g.*, corn, rice, sorghum, barley, wheat, potato and cassava). These starches are referred to as amylotypes or waxy starches, respectively.

### Amylose

Amylose is a linear polymer of D-glucose residues linked through  $\alpha$ -1,4 bonds (Fig. 4C). The molecular weight range of amylose (*i.e.*, 80000–1000000) is quite broad and varies in between plant species, varieties and maturity of the starch under study. Amylose can be considered as a linear polymer, but nevertheless contains a very low number of  $\alpha$ -1,6 branching points (less than 1.0%, Fig. 4B). The side branches in amylose are very long and of a high molecular weight, which, from a practical point of view, makes that amylose behaves and can be considered as an unbranched linear polymer. Amylose molecules tend to adopt a natural helical structure. Amylose is also known to interact with iodine, organic alcohols and fatty acids. The formed complexes are usually referred

**Table 2** Amylose content and typical gelatinization temperature range of starches of different botanical origin (Delcour and Hosney, 2010; Sivoli et al., 2012; Buleon et al., 1998; Belitz et al., 2009; Sanchez-Arteaga et al., 2015)

<i>Plant source</i>	<i>Amylose content (%)</i>	<i>Gelatinization temperature range (°C)</i>
Wheat	26–31	58–62
Barley	22–29	51–60
Oats	~27	53–59
Rye	22–28	57–70
Corn	25–28	62–87
Rice	0–33	62–79
Triticale	22–31	55–62
Sorghum	21–34	68–78
Potatoes	19–23	62–66
Cassava	18–25	60–65
Common beans	20–35	76–80

to as helical inclusion complexes. In case fatty acids are enclosed, the formed complex is referred to as an amylose–lipid complex (Putseys et al., 2010). Amylose can relatively easily be extracted using hot water.

### Amylopectin

The building blocks of amylopectin are also  $\alpha$ -D-glucose units, but in this case a considerable part of the linkages are actually  $\alpha$ -1,6 bonds (5%–6%), which gives the molecule a branched structure (Fig. 4D). Amylopectin has a very high molecular weight (up to  $10^8$ ), and clearly behaves as a branched molecule. To better describe amylopectin structure, the different chains in amylopectin have been named A, B, and C according to their position in the molecule (Fig. 4D) (Peat et al., 1952). The C-chain contains the only reducing group in the molecule and every amylopectin molecule, hence, only contains one C-chain. B-chains are side chains which themselves are carrying side chains. The outer sidechains that are not carrying branching points are referred to as A-chains. The intrinsic complexity of amylopectin structure has been stepwise resolved by using very specific hydrolytic enzymes such as  $\beta$ - and  $\alpha$ -amylases.

### Minor Constituents

As outlined above, about 98% of starch weight is carbohydrate material. However, the other 2 wt%, although minor levels, can have a big effect on the behaviour of starch in the plant and during food processing. The most prevalent minor constituents in cereal starches are lipids. These make up about 0.5 to 1.0 wt% of the starch weight. The most prevalent lipids are lysophospholipids and free fatty acids. The most abundant fatty acids in cereal starches are linoleic and palmitic acid. Starch may also be ‘contaminated’ by surface lipids, stemming from the amyloplast membrane (Tester et al., 2004). Non-cereal starches, on the other hand, contain virtually no lipids. The lipids in starch may occur as amylose–lipid complexes. In amylose–lipid complexes, free fatty acids occupy the inner apolar core of single amylose helices. These amylose–lipid complexes are naturally present in starch, but additional ones can be formed during starch gelatinization.

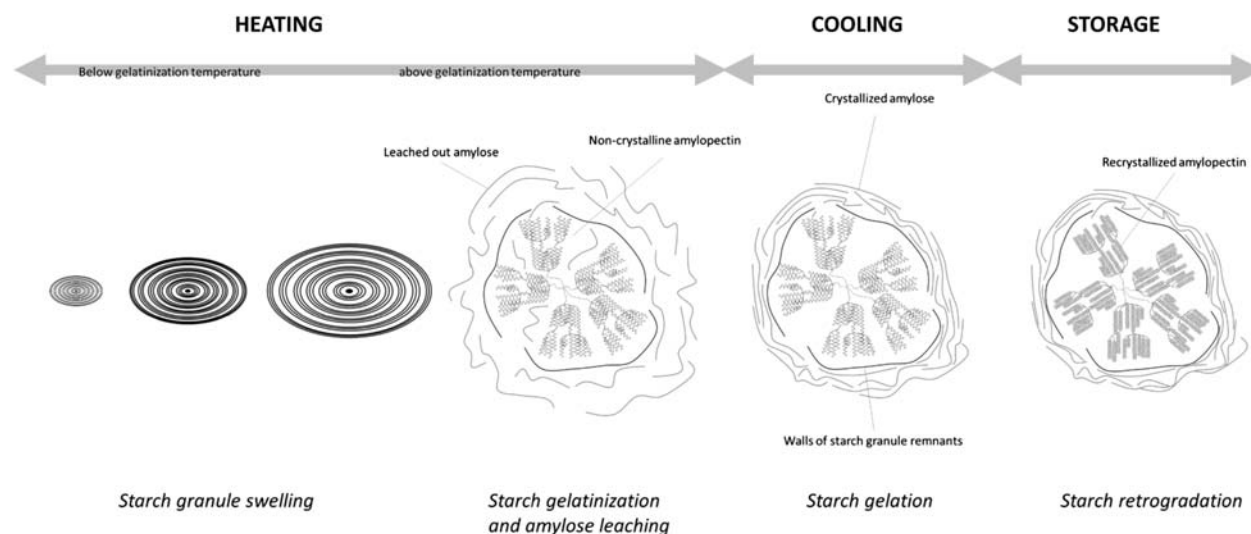
Phosphorus levels in starch can be rather high and phosphorus is either present as lysophospholipids (e.g., in cereal starches), inorganic phosphates (Tester et al., 2004) or directly esterified to the starch molecules. Potato starch is known for its high levels of phosphorus (up to 200–1000 ppm monoesterified phosphorus), while the levels in cereals and roots only go to about 0–20 ppm and 40–150 ppm, respectively. Nitrogen can also be found in starch, either originating from the lipids or stemming from proteins. Starch synthase, the starch synthesizing enzyme can often still be found associated to its product.

### Starch in Food

About 70% to 80% of the calories consumed by humans worldwide originate from starch. Apart from being inherently present in the main ingredients of a lot of food products, starch is sometimes also deliberately added for its adhesive, binding, film forming, foam strengthening, gelling, moisture holding, stabilizing, texturizing and thickening properties. Its unique properties in food processing stem from the changes discrete starch granules undergo during hydrothermal treatments.

### Hydrothermal Treatment of Starch

In what follows, the main transitions a starch suspension (starch in excess water) undergoes in function of heat will be discussed (Fig. 5). Most of these changes are non-chemical transformations.



**Figure 5** Changes in starch size and shape during hydrothermal treatment and storage.

### Starch Swelling at Low Temperatures

When starch is dispersed in water, the discrete starch granules will slightly swell (around 5 v%). The swelling rate is highly dependent on the temperature of the water. As long as this temperature does not exceed the gelatinization temperature, starch swelling is a completely reversible process. Intact starch granules will not swell as fast as their damaged counterparts (starch damage may be induced by, *e.g.*, milling of cereal grains). Without extensive heating, starch can bind up to 30% of its dry weight of water. When the starch granules are dried following this soaking step, no changes in their granule morphology or crystallinity are observed. Swelling at these low temperatures does not involve the solubilization of starch.

### Starch Gelatinization and Pasting

When starch is heated in an excess water to a temperature exceeding the gelatinization temperature, starch swelling continues, but irreversible structural changes to the granules are introduced. As a result of the higher temperature, hydrogen bonds which existed within and in between molecules weaken and the granules absorb more water and swell more obviously. A first sign of the loss of the internal structure in the starch granules is the loss of semi-crystallinity of the granules, which can be visualized through the loss of birefringence of the starch granules under polarization microscopy. The temperature at which this occurs is referred to as the gelatinization temperature and is dependent on the starch source (Table 2). In DSC measurements, gelatinization is visualized as a relatively broad gelatinization peak. Together with the loss of birefringence, the transparency of the suspension will decrease, while its viscosity will steadily increase. Gelatinization is also dependent on the availability of water. Water will first penetrate the more accessible amorphous zones in a starch granule and make these swell. The swelling of these intermittent amorphous layers puts an enormous stress on the surrounding crystalline layers (Fig. 2). These crystalline layers are believed to break under this pressure, which speeds up the loss of semi-crystallinity in the starch granules. If not enough water is present (typically less than 30% moisture content) to rip the internal starch granular structure apart, the loss of order is often just acquired through regular melting of the crystals, which would typically occur at higher temperatures. Besides the moisture content, the presence of other molecules in the aqueous phase of the starch suspension also affects the gelatinization temperature. Sucrose addition, *e.g.*, is known to, above a certain concentration (30 w/w%), drastically increase the gelatinization temperature (Kweon et al., 2012). Also, input of energy (*e.g.*, through mixing of the suspension) leads to a better and more efficient distribution of heat in the system, also resulting in a faster gelatinization process. After the loss of molecular order, the starch granules keep on swelling through further absorption of water. Crystals melt and part of the starch solubilizes. These irreversible transitions go hand in hand with a sharp increase in viscosity. The raise in viscosity is initially caused by further swelling of the granules, followed by the generation of granule remnants and leaching out of amylose molecules. Further heating leads to further opening of the starch structure and water molecules being bound at the starch chains that are freed from the structure. This viscosity increase after gelatinization is referred to as starch pasting. Under continuous stirring and when holding the starch granules suspension at a high temperature, a decrease in viscosity is observed: the starch paste displays time-dependent shear thinning or thixotropic behaviour. This shear thinning behaviour is caused by alignment of the leached out amylose molecules with the stirring direction and the diminution of the swollen starch granules (and remnants) under prolonged mixing.

### Starch Gelation

Upon cooling of a gelatinized starch suspension, an increase in viscosity is observed which is indicative of the formation of new non-covalent bonds in between the starch granules. These bonds are predominantly formed between the leached out amylose



molecules which associate with each other and hence form a matrix around the starch granule remnants, leading to an increased viscosity of the system. At high enough starch concentrations, a gel will be formed which can be visualized as a network of amylose molecules in which amylopectin enriched granules are embedded (Fig. 5).

### Starch Retrogradation

Upon storage of a gelatinized starch gel or suspension, the starch polymers associate more tightly in function of time. The reassociation and recrystallization of amylopectin is referred to as retrogradation. Strictly speaking, only amylopectin can recrystallize as amylose does not occur in crystalline form in the starch granules, and, hence, a term like retrogradation, literally meaning 'to go back', is only used to refer to the reassociation and crystallization of amylopectin. A partially crystalline system is, hence, reformed. In function of time, the association of the polymers may become more tight, eventually leading to expulsion of water entrapped in the gel structure. This is referred to as syneresis.

### Starch Analysis

The behaviour of starch during hydrothermal treatments can be monitored using visualization as well as rheological methods. Visualization methods focusing on starch swelling, loss of molecular order and granule disintegration include regular light and polarization microscopy (Fig. 3). The change in viscous properties of the starch suspension can be monitored through rapid viscoanalyzer and amylograph measurements. Gelatinization temperatures, retrogradation degree and the presence of amylose-lipid complexes can be studied through Differential Scanning Calorimetry.

### Applications in Food

As a result of the above behaviour of starch under hydrothermal treatments, starch often acts as a structuring agent in a range of food products. When starch is added as such, starch addition is often aiming at providing body and bulk to the food product. The extent of starch gelatinization in baked goods affects product properties such as storage behaviour and rate of digestion. In low moisture foods, starch usually does not gelatinize, while it does gelatinize in higher moisture food products.

Examples of food products in which structure is built by starch that is naturally present in one of the ingredients is bread, cake, pasta, cooked potatoes and vegetables. In these food products, starch is heated without shear and this results in clearly identifiable starch granules. The degree of gelatinization in these products will determine the product acceptability. Undercooked potatoes, *e.g.*, will have an undesirable texture. In cookies, characterized by high sugar levels, starch will not gelatinize during baking, while for bread and cake systems, the majority of the starch granules will be gelatinized. Initial bread crumb firmness, *e.g.*, is highly dependent on amylose gelation, while during storage crumb hardening (and bread staling) can be partially explained by starch retrogradation.

Examples of food products to which starch is added in order to build 'structure' are sauces, soups and puddings. In these products, starch plays a water binding, viscosity increasing and/or gelling role. These products, usually also undergo some shear during starch heating, resulting in granule degradation and more pronounced amylose leaching.

In most food products, instead of using unmodified 'natural' starches, modified starches are used. These starches have undergone a physical or chemical treatment to change their properties. This will be discussed in section **Starch Modification**.

### Enzymatic Hydrolysis

Starch is readily hydrolysed by a wide range of amylases once it has been gelatinized. These amylases target the hydrolysis of the  $\alpha$ -1,4 bonds connecting the glucose units and create new reducing ends upon every hydrolysis. Two well-known amylases with widely differing hydrolysis patterns are  $\alpha$ - and  $\beta$ -amylases. While the latter is an exo-amylase, *i.e.*, it cuts maltose units from the non-reducing ends of the starch granules,  $\alpha$ -amylase typically hydrolyzes  $\alpha$ -1,4 bonds that are more centrally positioned in the starch molecular structure.  $\alpha$ -amylases, hence, also have a much more pronounced effect on the molecular weight of the parent molecule containing the original reducing end and on the overall starch suspension viscosity.  $\beta$ -amylase usually does not get past  $\alpha$ -1,6 branching points and will, hence, only have a minor effect on the amylopectin molecular weight. When only  $\beta$ -amylase would be present, amylopectin would only be degraded for about 55%, generating a lot of maltose and one amylopectin residue, *i.e.*, the  $\beta$ -limit dextrin, containing the 'original' reducing end. Hydrolysis of  $\alpha$ -1,6 bonds can be achieved by pullulanase. This enzyme can release all A and B amylopectin chains and each of these released chains will have a reducing end, increasing the reducing power of the carbohydrate system. The concerted action of  $\beta$ - and  $\alpha$ -amylase and pullulanase enables the complete hydrolysis of amylose and amylopectin.

### Digestibility of Starch

After ingestion, starch is claimed to be very quickly degraded by human amylases. The hydrolysis products, small sugar molecules, serve as an important source of energy. However, it is clear that some of the starch will not be readily digested. Depending on the rate of starch digestion, starch is classified as rapidly (RDS) and slowly digestible starch (SDS), and resistant starch (RS).

### **Rapidly and Slowly Digestible Starch**

Rapidly digestible starch is readily digested as the polysaccharide chains are accessible to starch hydrolysing enzymes. This starch is usually amorphous or dispersed and can be found in high amounts in freshly cooked starch foods. This type of starch is completely converted to glucose in about 20 minutes of enzyme digestion. Conversely, slowly digestible starch is also digested completely in the small intestine, but much more slowly. This starch is usually physically inaccessible amorphous starch or starch which has a more accessible structure. This starch is defined as the starch which is converted within 100 min of enzyme digestion ([Sajilata et al., 2006](#)).

### **Resistant Starch**

Resistant starch, as it is not digested in the upper parts of the human intestinal tract, is assumed to have health benefitting physiological effects similar to those ascribed to dietary fibre. Resistant starches have been subdivided into different categories according to the underlying molecular reason for their digestion resistance:

- Resistant starch category I resists digestion because it is physically entrapped in cellular structures, making it less susceptible for hydrolytic enzymes. Products in which whole grains or shredded cereal grains appear often contain this physically inaccessible starch.
- Resistant starch category II is a native crystalline, hence, ungelatinized starch. Native starch is very poorly digested by human amylolytic enzymes due to its semi-crystalline and compact structure. Examples are starches found in raw potatoes and green bananas.
- Resistant starch category III is retrograded starch. Recrystallization of amylopectin and crystallization of amylose reduces the susceptibility of starch towards hydrolysis. Resistant starch category III is the starch in cooked and cooled potatoes and stale bread.
- Resistant starch category IV is resistant because it has been chemically or thermally modified to reduce its digestibility. During these treatments, other glycosidic bonds can be formed besides  $\alpha$ -1,4 and  $\alpha$ -1,6 bonds which are resistant against amylolytic enzymes.

The interested reader is referred to the review by [Sajilata et al. \(2006\)](#).

## **Starch Modification**

Granular starch is sometimes modified in order to change the properties and functionality. This modification can either be physical or chemical. The diversity in starch properties from different sources already offers a range of functionalities, but the degree and type of modification can further extend this functionality range.

### **Physically Modified Starches**

#### **Annealing of Starch**

Annealing is a heat treatment of starch which takes place in either an excess of water ( $>60$  w/w%) or intermediate (40–55 w/w%) water levels ([Jacobs and Delcour, 1998](#)). This hydrothermal treatment changes the physicochemical properties of starch, but does not affect and destroy its granular structure. The starch systems are heated above the glass transition temperature but kept below the gelatinization temperature. During annealing, the starch molecules are physically reorganized in the granular structure. It has been suggested that amorphous regions become more glassy during annealing and the amylopectin double helices are more ordered ([Tester and Debon, 2000](#); [Stute, 1992](#)). This would decrease the penetrability of the granular structures for water. Strictly speaking, gelatinization may not occur during annealing, which makes that the gelatinization enthalpy after annealing may not be lower than for the native starch. In practice, however, annealing is often accompanied by partial gelatinization. Annealing is intended to increase the gelatinization temperature and sharpen the gelatinization range. Annealing also leads to less pronounced starch granule swelling power. The effect of annealing on starch pasting are less clear as both lower and higher consistencies of the annealed starch paste relative to the native starch paste have been reported ([Hoover and Vasanthan, 1994](#)). On an industrial scale, starch annealing is not considered an economically viable process as there exist (cheaper) chemical modifications of starch which result in similar effects. Annealing may occur unintentionally, however, during for example the isolation of starch (e.g., wet milling of corn).

#### **Heat-Moisture Treated Starch**

This process is related to starch annealing, but is usually carried out at a lower moisture content ( $<35$  w/w%) ([Jacobs and Delcour, 1998](#)). Similar to what is the case for starch annealing, the starch systems are kept at a temperature between the glass transition temperature of starch and its gelatinization temperature. Sometimes, the term 'heat-moisture' treatment is also used for temperature treatments of starch at temperatures that exceed the gelatinization temperature. The lower moisture contents at which heat-moisture treatments of starch occur also allow for physical reorganization if the temperature is raised higher than what is customary for

annealing. Similar structural changes have been suggested for heat-moisture treated starch as were described for annealed starch (Tester and Debon, 2000; Stute, 1992). The temperature must exceed the glass transition temperature to allow for molecular mobility and the molecular reorganization. Heat-moisture treatments of starch are industrially performed to alter the quality and properties of starch.

### **Ethanol Treated Starch**

Although this treatment uses a chemical, it is, strictly speaking, not a chemical modification as there is no formation or hydrolysis of covalent bonds, but only involves a physical rearrangement within the starch granules. Native starch granules are heated in alcohol-water mixtures (Dries et al., 2016). As a result, the crystals will melt, but the granules themselves will not or only sparsely swell as there is not enough water available for swelling. After removal of the solvent and drying, the starch granules can be added to cold water and will swell (Delcour and Hoseney, 2010).

## **Chemically Modified Starches**

### **Acid Modified Starches**

Treatment of starch with acid is probably the oldest type of starch modification. Acid-treated starch is nowadays called lintnerized or Naegeli starch, after its 'inventors'. A common way to do this modification is suspending starch in a 1%–3% hydrochloric acid medium for 12 to 14 h at temperatures ranging between 25 and 55 °C. An alternative is using sulphuric acid. After the treatment, the starch slurry is neutralized and washed. Acid treatment of starch predominantly leads to hydrolysis of glycosidic bonds in the amorphous regions of the starch granules (Hoover, 2000). The crystalline regions are largely unaffected (depending on the acid hydrolysis time). The overall effect is a drastic reduction of the starch molecular weight as the connecting amorphous regions are etched away. These starches swell less and disintegrate more during hydrothermal treatments. As only the crystalline parts remain, the gelatinization temperature usually also increases and the starch becomes more soluble as a result of the molecular weight reduction. This also comes with a reduced viscosity of the starch paste (even vs. viscosity obtained for native starch suspensions). Upon cooling, a more rigid gel is formed due to the shorter chain length leading to more easy association of the chains (Delcour and Hoseney, 2010). However, reports describing a less rigid and more elastic gel can also be found (Hoover, 2000). These starches are usually used for gum candy (Hoover, 2000).

### **Cross-linked Starches**

Starch can be cross-linked by forming diesters in between the different starch chains/molecules. This can be achieved by either using phosphoryl chloride ( $\text{POCl}_3$ ) or epichlorohydrin (Majzoobi et al., 2009; Jyothi et al., 2006). Cross-linking can decrease the swelling power of starch, increase the starch gelatinization temperature and lead to lower viscosity upon pasting (Majzoobi et al., 2009). Cross-linked starches, however, are less prone to shear thinning, which may be desirable for food that needs to be pumped. It is even possible to produce starch that does not gelatinizes when boiled (Delcour and Hoseney, 2010). However, for food applications this is often not desired and moderate degrees of substitution (*i.e.*, on average there is one substituent attached to every 100 or 10 anhydroglucose residues) are aimed for. Next to viscosity control of the paste, cross-linking also increases the viscosity of starch systems in acid conditions. Cross-linking restricts starch swelling and prevents starch from falling apart, hence, maintaining a higher viscosity. Cross-linked starches usually also lead to shorter pastes, delayed crystallization and retrogradation and an increased freeze-thaw stability (Delcour and Hoseney, 2010). The reduced viscosity increase in function of heating is desirable for the production of jam, baby food, soups and salad dressing (Majzoobi et al., 2009).

### **Substituted Starches**

Starch can also be substituted by addition of monoesters of phosphoric acid. The bulky and charged phosphate groups will make the chains in the starch to repel each other. This leads to more outspoken swelling behaviour, a lower gelatinization temperature and a better solubilization during gelatinization (Liu et al., 1999). The resultant starch paste viscosity is higher (Liu et al., 1999), but displays more shear-thinning behaviour. Substitution also retards or even nullifies retrogradation and pastes turning opaque. It also improves the freeze-thaw stability of starch. In case the degree of substitution is very high, starch can be produced that gelatinizes at room temperature (Delcour and Hoseney, 2010).

### **Oxidized Starches**

Starch can be oxidized with hypochlorite (Kuakpetoon and Wang, 2001). An alternative to hypochlorite would be hydrogen peroxide (Parovuori et al., 1995). When using sodium hypochlorite, the resulting starches are referred to as 'chlorinated starches' although there is no actual incorporation of chlorine atoms in the structure. The oxidation leads to the formation of carbonyl- and carboxyl groups and a minor depolymerization. Oxidation reduces the swelling power, pasting temperature and the paste viscosity (Kuakpetoon and Wang, 2001; Zhou et al., 2016). After gelatinization of this starch, less pronounced retrogradation is usually observed (Garrido et al., 2014). Oxidized starch has only few applications in food such as increasing the adhesion to meat of bread-formulations.

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## Sugar Alcohols

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### Introduction

Sugar alcohols (polyols, polyhydric alcohols) are hydrogenated forms of mono- (erythritol, xylitol, sorbitol, mannitol), di- (lactitol, isomalt, maltitol), or polysaccharides (maltitol and sorbitol syrups). During hydrogenation process, aldehyde or ketone group is replaced by the hydroxyl one. There are seven sugar alcohols approved for use in food products in Europe but also other countries, i.e. sorbitol (E420), mannitol (E421), isomalt (E953), maltitol (E965), lactitol (E966), xylitol (E967) and erythritol (E968) ([Regulation \(EC\) No 1333/2008](#)) ([Table 1](#)).

Their presence in food products must be accompanied with the notice on the package stating that excessive consumption might result in laxative effect ([EFSA, 2011](#); [Grabitske and Slavin, 2008](#); [Livesey, 2003](#)). Such warning is due to incomplete digestion of polyols, which can undergo fermentation process in the intestines and the gastrointestinal effects such as noisy bowels, flatulence or diarrhea can be observed. However, their occurrence depends on consumer's tolerance and can be improved by regular polyols' consumption ([EFSA, 2011](#); [Grabitske and Slavin, 2008](#); [Livesey, 2003](#)). Moreover, products of their fermentation have beneficial effect on colon health as they positively influence the growth of gastrointestinal flora, which consists of glucidolytic and acidforming organisms ([Grembecka 2015, 2018](#); [Livesey, 2003](#)). Slower and incomplete absorption also influence the caloric value of sugar alcohols, which varies from 0 (erythritol) to 2.6 kcal/g (sorbitol) ([Table 1](#)) ([Evrendilek 2012](#); [de Cock 2012](#), [Grembecka, 2015](#); [Grembecka, 2018](#); [Livesey, 2003](#); [Regulation \(EC\) No 1333/2008](#)). Therefore, they can be used as sugar alternatives with low glycemic index (GI) ([Table 1](#)) in calorie-reduced foods and products suitable for diabetics ([Grembecka 2015, 2018](#)). Based on literature data, it can be concluded that mixtures of polyols, fats, starch- and protein-based foods yield lower GI than cumulative GI of the components ([Livesey, 2003](#)). They all characterize with sweet taste, which strength varies from 0.3 to 1 as compared with sucrose ([Table 1](#)). Moreover, their negative heat of solution, i.e. energy needed to dissolve crystals, creates cooling sensation in the mouth. However, such cooling effect can be observed just when the heat of solution is lower than  $-20$  cal/g ([de Cock, 2012](#)). All sugar alcohols are white odorless crystalline powders (except for syrups), and can play various technological roles, i.e. sweetening, texture, filling, preservative and retaining moisture ([Barbieri et al., 2014](#); [Evrendilek, 2012](#)). They all can be used as bulking agents that can substitute sugar in 1:1 ratio and eliminate improper taste of intense sweeteners ([Grembecka, 2015](#)). Polyols can influence spread, volume, texture, shelf life and mouthfeel of various food products due to their physicochemical properties. In general, these properties greatly depend on sugar alcohols' molecular weight, and with its decrease osmolality increases, freezing point decreases, viscosity decreases and boiling point increases ([Nabors and Hedrick, 2012](#); [Grembecka, 2018](#)) ([Table 2](#)). They characterize with high thermal, chemical and pH stability as well as do not undergo Maillard-type browning reaction. Besides, they have high microbial stability and solubility in water ([Table 2](#)).

Sugar alcohols are not fermented by mouth bacteria, thus, are non-cariogenic and non-acidogenic substances. What is more important, they were found to help in tooth mineralization as well as neutralize plaque acids and reduce tooth demineralization ([EFSA, 2011](#)). US FDA and European Commission allow on placement on a product with polyol of a health declaration "does not promote tooth decay" ([EFSA, 2011](#)). They were thoroughly investigated in trials conducted on animals and humans and no health risk was found, thus, no ADI have been specified for whole group ([WHO, Koivistoinen, 2007](#); [Lawson, 2007a](#); [Ly et al., 2006](#); [WHO](#)) ([Table 1](#)). Their usage in food industry is approved in most countries, except for maltitol, which awaits the approval of Food and Drug Administration (FDA), but it can be used as the petition regarding this compound has been already accepted ([Kearsley and Deis, 2012](#)).

### Characteristics of Particular Polyols

#### Erythritol

It is a four-carbon polyol ((2R,3S)-Butan-1,2,3,4-tetrol) ([Fig. 1](#)), which is widely found in nature in such products as wine (130–300 mg/L), soy sauce (910 mg/L), vegetables, fruits (melons – 22–47 mg/kg; pears – up to 40 mg/kg), but is also present in human body in semen, lens, cerebrospinal fluid, serum and human urine (10–30 mg/L) ([Barbieri et al., 2014](#); [den Hartog et al., 2010](#); [Evrendilek, 2012](#); [Ortiz et al., 2013](#); [Oku and Okazaki, 1996](#); [Regnat et al., 2018](#)). Due to such occurrence and levels in food products, erythritol consumption reaches 25 mg/person/day in the United States and 106 mg/person/day in Japan ([de Cock, 2012](#)).

#### Metabolism

Although, erythritol is rapidly and well absorbed (60%–90%) in the small intestine, it is not fermented, but excreted intact in urine within 24 hours ([Bernt et al., 1996](#); [de Cock, 2012](#); [den Hartog et al., 2010](#); [Friedman, 2008](#); [Grembecka, 2015](#); [Livesey, 2003](#); [Munro et al., 1998](#); [Regnat et al., 2018](#)). Therefore, the usual side effects of polyol consumption, i.e. laxative effects, are rarely observed in case of consumption of this substance ([Bernt et al., 1996](#); [de Cock, 2012](#); [den Hartog et al., 2010](#); [Friedman, 2008](#); [Livesey, 2003](#); [Munro et al., 1998](#)). However, it was found that women are less prone to suffer from diarrhea after erythritol

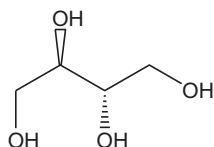
**Table 1** General characteristics of polyols (Grembecka 2015, Grembecka 2018, Livesey 2003, Regulation (EC) No 1333/2008)

Sugar alcohol	Synonyms	E number (according to EU legislation)	Functional classes	ADI	Sweetness (Sucrose = 1)	Glycemic index (Glucose = 100)	Caloric value [kcal/g]		
							EU	USA	Japan
Erythritol C <sub>4</sub> H <sub>10</sub> O <sub>4</sub>	-Erythrite -Meso-erythritol -Tetrahydroxybutane	E968	Flavor enhancer Humectant Sweetener	not specified	0.6–0.8	0	0	0	0
Isomalt C <sub>12</sub> H <sub>24</sub> O <sub>11</sub>	-Hydrogenated Isomaltulose -Isomaltitol	E953	Anticaking agent Bulking agent Glazing agent Stabilizer Sweetener Thickener	not specified	0.45–0.65	9	2.4	2	2
Lactitol C <sub>12</sub> H <sub>24</sub> O <sub>11</sub>	-Lactit -Lactobiosit -Lactositol	E966	Emulsifier Sweetener Thickener	not specified	0.3–0.4	6	2.4	2	2
Maltitol C <sub>12</sub> H <sub>24</sub> O <sub>11</sub>	-D-Maltitol -Dried Maltitol Syrup -Hydrogenated Glucose Syrup -Hydrogenated High Maltose-Content Glucose Syrup -Hydrogenated Maltose -Maltitol Syrup Powder	E965	Bulking agent Emulsifier Humectant Stabilizer Sweetener Thickener	not specified	0.9	35	2.4	2.1	2
Mannitol C <sub>6</sub> H <sub>14</sub> O <sub>6</sub>	-Mannite -D-Mannitol	E421	Anticaking agent Bulking agent Humectant Stabilizer Sweetener Thickener	not specified	0.5–0.7	0	2.4	1.6	2
Sorbitol C <sub>6</sub> H <sub>14</sub> O <sub>6</sub>	-D-Glucitol, -D-Glucitol syrup -Sorbit -D-sorbitol -Sorbol	E420	Bulking agent Humectant Sequestrant Stabilizer Sweetener Thickener	not specified	0.5–0.7	9	2.4	2.6	3
Xylitol C <sub>5</sub> H <sub>12</sub> O <sub>5</sub>	–	E967	Emulsifier Humectant Stabilizer Sweetener Thickener	not specified	1.0	13	2.4	2.4	3



**Table 2** Physicochemical properties of sugar alcohols (de Cock, 2012; Grembecka, 2018; Lawson, 2007a)

Compound	Molecular weight	Melting point [°C]	Heat of solution	Viscosity at 25 °C	Hygroscopicity	Solubility [% w/w at 25 °C]
Erythritol	122	121	−43	very low	very low	37
Isomalt	344	167	−9	high	low	25
Lactitol	344	94	−14	very low	low	57
Maltitol	344	150	−19	medium	medium	60
Mannitol	182	165	−29	low	low	22
Sorbitol	182	97	−26	medium	high	235
Xylitol	152	94	−36	very low	medium	64

**Figure 1** Chemical structure of erythritol.

consumption (up to 1000 mg/kg body) than men (Bernt et al., 1996; Oku and Okazaki, 1996). What is more, according to EFSA scientific opinion this polyol's metabolism route is independent of consumer's age (EFSA, 2015). Owing to intact excretion, erythritol is a non-caloric substance, which was attributed 0 kcal/g by international legislations (de Cock, 2012; Evrendilek, 2012) (Table 1).

### Production

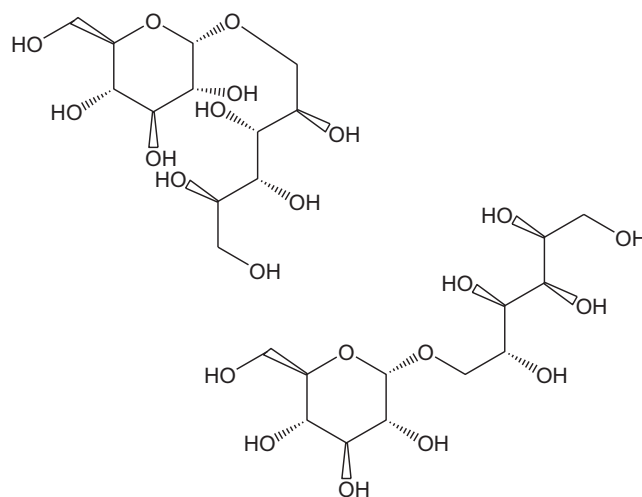
There are two methods of erythritol production, i.e. chemical synthesis (from 2-butene-1,4-diol) and biotechnological (by yeast or fungi), but only one is actually used on an industrial scale. High cost of the substrate as well as complexity of the chemical procedure, resulted in application of various microorganisms that produce this polyol by fermentation in quite large quantities. Most useful are osmophilic species of yeast as *Moniliella pollinis* (Barbieri et al., 2014; Chattopadhyay et al., 2014; de Cock, 2012; Embuscado and Patil, 2001; Evrendilek, 2012; Lin et al., 2010), but the most productive according to literature data are *Trichosporonoides megachiliensis* and *Pseudozyma tsukubaensis* (Park et al., 2016). Besides these microorganisms, there are also applications of *Oenococcus oeni*, *Leuconostoc mesenteroides*, *Lactobacillus sanfranciscensis* and *Yarrowia lipolytica* MK1 (Carly et al., 2017; Liu et al., 2017; Mironczuk et al., 2015; Rakicka et al., 2017; Regnat et al., 2018; Richter et al., 2001; Stolz et al., 1995; Tomaszewska et al., 2014; Veiga-da-Cunha et al., 1993; Yang et al., 2014). The starting material is usually starch or sucrose, but glycerol was also found useful (Carly et al., 2017; Mironczuk et al., 2015; Tomaszewska et al., 2014; Yang et al., 2014).

### Properties and Applications

It is a white non-hygroscopic powder that have sweetness lower than sucrose (0.6%–0.8%, Table 1), which can be increased (up to 30%) when blended with other polyols or intense sweeteners (Barbieri et al., 2014; Boileau et al., 2012; de Cock, 2012). Erythritol is mainly used as a sweetener, flavor enhancer and humectant. Among its advantages, there is also inability to take part in the Maillard-type browning reactions, stability in various pH conditions and high temperatures (up to 160 °C) (de Cock, 2012). Besides giving a strong cooling effect when dissolving, it also has no aftertaste and is able to mask the taste of intense sweeteners (Barbieri et al., 2014; Boileau et al., 2012; Chattopadhyay et al., 2014; de Cock, 2012; INCHEM, Friedman, 2008; Oku and Okazaki, 1996; Regnat et al., 2018). As erythritol is not metabolized and freely circulates in the body, it can act as a radical scavenger with membrane-protecting properties (de Cock, 2012; den Hartog et al., 2010). With the lowest among polyols molecular weight, it provides higher osmotic pressure and lower activity in solution (Lawson, 2007a). Moreover, it supports microbiological safety as it inhibits the growth of *mutans streptococci*, which results in reduced plaque growth (Runnel et al., 2013). That makes it a very desirable low-calorie, tooth-friendly, bulk sweetener, which finds its application in such products as tooth-friendly chewing gums, candy products, ice creams, ipocaloric beverages, tabletop sweeteners and calorie reduced beverages (Barbieri et al., 2014; de Cock, 2012). When mixed with maltitol it can be useful in production of bakery products (Grembecka, 2015). It is also suitable for use in foods for diabetics as it does not influence blood glucose or insulin levels (Lawson, 2007a). It has very high digestive tolerance and its safety has been comprehensively tested all around the world.

### Isomalt

It is composed of equimolar amounts of two disaccharides (Fig. 2), i.e. 6-*O*- $\alpha$ -*D*-glucopyranosyl-*D*-sorbitol (1,6-GPS) and 1-*O*- $\alpha$ -*D*-glucopyranosyl-*D*-mannitol dihydrate (1,1-GMP dihydrate) and produced exclusively from sucrose (Joint Expert Committee on



**Figure 2** Chemical structure of isomalt.

Food Additives, [Sentko and Bernard, 2012](#)). It is an odorless, non-hygroscopic, crystalline substance, which finds its application as anticaking, bulking and glazing agent as well as stabilizer, sweetener and thickener ([Grembecka, 2015](#)) ([Table 1](#)).

### Metabolism

Due to its stability, isomalt is hardly hydrolyzed and absorbed in the intestine (10%) ([Grembecka, 2015](#); [Livesey, 2003](#); [Sentko and Bernard, 2012](#)). The remaining unabsorbed part is transported to the large intestine, where is fermented to volatile short-chain fatty acids, CO<sub>2</sub>, CH<sub>4</sub> and H<sub>2</sub> ([Dehghan et al., 2013](#); [Livesey, 2003](#); [Sentko and Willibald-Ettle, 2012](#)). As one of the products is butyrate, thus, this polyol exhibit prebiotic effects (increase of the *Bifidobacteria* growth) ([Sentko and Bernard, 2012](#)). However, when consumed in large quantities, and especially in liquid form, the gastrointestinal effects occur, which also depend on the moment and frequency of consumption ([Sentko and Bernard, 2012](#)).

### Production

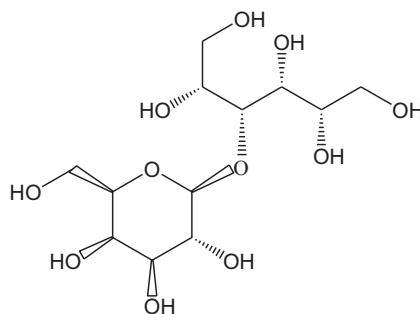
Isomalt is not found in the nature but was developed and patented by BENEOPalatin GmbH, which is owned by Südzucker AG (Germany) ([Sentko and Bernard, 2012](#)). The substrate is sucrose, which undergoes two-stage process that is enzymatic transformation into more stable isomaltulose (6-*O*- $\alpha$ -*D*-glucopyranosyl-*D*-fructose) and then hydrogenation in the presence of metal catalyst ([Sentko and Bernard, 2012](#); [Sentko and Willibald-Ettle, 2012](#)).

### Properties and Applications

Isomalt characterizes only with about the half sweetening power of sucrose ([Table 1](#)), but it resembles disaccharide in a similar sweetness profile. The intensity of the sweetness depends of isomalt type, concentration and temperature, but can be also increased though blending with intense sweeteners ([Ferguson et al., 2007](#); [Sentko and Willibald-Ettle, 2012](#)). Isomalt also blends with any flavor and has no cooling effects (the highest heat of solution, [Table 2](#)) so it can enhance flavor transfer ([Ferguson et al., 2007](#); [Sentko and Willibald-Ettle, 2012](#)). Isomalt is also used as decorative material as it crystallizes slowly. Its high temperature of melting results in resistance to boiling, baking and extrusion process, while very low hygroscopicity in ability to be stored for long periods ([Table 2](#)) ([Sentko and Bernard, 2012](#)). These properties allow on isomalt usage for coatings (chewing gum pellets) and chocolate applications as a glazing agent. Moreover, it can act as a sweetener, bulking agent and anticaking agent (FAO-WHO Food Standards). Isomalt is also anti-cariogenic and enhance teeth remineralization so can be used in products safe for teeth (EFSA, 2011; [Takatsuka et al., 2008](#)). Moreover, it can be found in hard candies, toffees, chewing gum, chocolates, baked goods, nutritional supplements, cough drops and throat lozenges (FAO-WHO Food Standards, [Grembecka, 2015](#)). It can be also recommended for diabetic people as it characterizes with a low glycemic index ([Table 1](#)) ([Grembecka, 2015](#)).

### Lactitol

This disaccharide polyol with a systematic name 4-*O*- $\beta$ -*D*-galactopyranosyl-*L*-glucitol, consists of sorbitol and galactose ([Fig. 3](#)). Lactitol is an odorless white crystalline powder with a mild sweet taste. It has similar to sucrose technical and handling properties and can substitute sugar in almost every application ([Koivistoinen, 2007](#)). Anhydrous form is especially applicable in case of moisture-sensitive products. Moreover, it can be used as an emulsifier, sweetener and thickener ([Table 1](#)) ([Grembecka, 2015](#)). Commercially, it is sold in two forms, i.e. monohydrate and anhydrous ones ([Koivistoinen, 2007](#); [Zacharis, 2012a](#); [Zacharis and Stowell, 2012](#)).



**Figure 3** Chemical structure of lactitol.

### Metabolism

Lactitol is hardly absorbed in the small intestine (only in 2% by passive diffusion) and mainly is fermented in the large intestine, where it is transformed into biomass, short-chain fatty acids, carbon dioxide, a small amount of hydrogen and organic acids (Chattopadhyay et al., 2014; Koivistoinen, 2007; Zacharis, 2012a; Zacharis and Stowell, 2012). Short-chain fatty acids are then utilized by human organism to produce energy (2–2.4 calories per gram) (Table 1). Lactitol acts as a prebiotic stimulating *Lactobacilli* and *Bifidobacteria* growth (Ballongue et al., 1997; Koivistoinen, 2007; Zacharis and Stowell, 2012). The gastrointestinal effects, i.e. laxative ones are dependent on the person's diet, age, general gut health and the mode and frequency of digestion, but they are unlikely to observe when less than 20 g of lactitol is consumed per day (Grembecka, 2015; Zacharis, 2012a; Zacharis and Stowell, 2012).

### Production

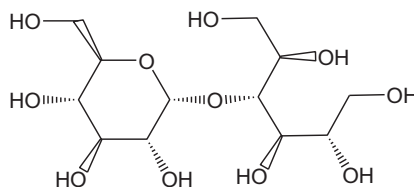
It is a polyol that cannot be found in nature and was firstly synthesized in 1920 (Zacharis and Stowell, 2012). The chemical process is based on a catalytic hydrogenation of 30%–40% lactose solution using Raney nickel as the catalyst (Koivistoinen, 2007; Zacharis, 2012a; Zacharis and Stowell, 2012).

### Properties and Applications

Although it characterizes with a relatively low mild sweetness with no aftertaste (Table 1), it can be increased through blending with intense sweeteners such as alitame, aspartame or acesulfame K (Zacharis and Stowell, 2012). Lower melting temperature of lactitol results in lower costs of production of food products (Koivistoinen, 2007; Zacharis, 2012a). This polyol can be also used as a sucrose 1:1 substitute and characterizes with high stability in various conditions (pH and temperature), good solubility and low hygroscopicity (Zacharis, 2012a). Due to the low heat of solution (Table 2) it has a very small cooling effect, which is useful for particular applications (i.e. in chocolates). Similarly to sucrose, it can be also used as a cryoprotectant for surimi. Lactitol is also a substance known for its positive prebiotic effects on the intestinal microflora, i.e. *Bifidobacteria* and *Lactobacilli* (Ballongue et al., 1997; EFSA, 2011; Finney et al., 2007; Koivistoinen, 2007; Ouwenhand et al., 2009; Zacharis, 2012a). Simultaneously, it inhibits the growth of intestinal putrefactive bacteria, lowers pH, production and absorption of ammonia (Ballongue et al., 1997; Finney et al., 2007; Koivistoinen, 2007; Ouwenhand et al., 2009; Zacharis, 2012a). Being not fermented in the mouth it does not contribute towards dental caries (EFSA, 2011; Koivistoinen, 2007). Besides, it characterizes with very low glycemic index, so it is useful for diabetic people (Table 2). This substance was found to be helpful in treatment of encephalopathy and constipation (Cammà et al., 1993; Chen et al., 2007; Kummel and Brokx, 2001; Mas et al., 2003; Morillas et al., 2014; Salerno et al., 1994).

### Maltitol

This disaccharide polyol (4-O- $\alpha$ -D-glucopyranosyl-D-glucitol) is composed of glucose and sorbitol in equal parts (Fig. 4) (Grembecka, 2015; Kearsley and Boghani, 2012; Lawson, 2007b; Livesey, 2003). Maltitol is a white crystalline powder that acts as a bulking agent, an emulsifier, a humectant, a stabilizer, a sweetener and a thickener (Table 1) (Grembecka, 2015).



**Figure 4** Chemical structure of maltitol.

### Metabolism

There is no active transport mechanism of this compound, thus, before absorption, this polyol must be enzymatically hydrolyzed to glucose and sorbitol as maltitol molecules are too large to be absorbed by diffusion (Lawson, 2007b; Livesey, 2003). As the process is quite slow, so the absorption rate varies between 5% and 80% (Livesey, 2003). The intact molecules of maltitol are moved to lower gut, where undergoes fermentation, which can result in osmotic laxation (Grabitske and Slavin, 2008; Kearsley and Deis, 2012). However, the laxative effects were noted for doses higher than 25–30 g/kg body weight (Lawson, 2007b).

### Production

The industrial process of maltitol production is mainly based on a catalytic hydrogenation (100–150 °C; 100–150 bar) of glucose syrup with high maltose levels, obtained by enzymatic hydrolysis of starch (Kearsley and Boghani, 2012). However, the reaction product is maltitol syrup of different grades, i.e. with 50%–55%, 72%–77% and 80%–90% of maltitol, which can be subjected to crystallization process, thus, leading to maltitol powder. European Union legislation requires the maltitol powder to contain at least 98% of this polyol (on a dry basis), whereas according to American regulations maltitol concentration should be within 92% and 100.5% (on a dry basis) (Kearsley and Boghani, 2012).

### Properties and Applications

Maltitol properties such as sweetness and taste resemble those of sugar (Kearsley and Boghani, 2012; Kearsley and Deis, 2012). It can be used as a fat substitute as it gives food creamy structure and has very small cooling effect (Kearsley and Boghani, 2012; Kearsley and Deis, 2012; Lawson, 2007b). It characterizes with high melting point as well as medium hygroscopicity (Table 2). Moreover, the insulin response after its consumption is reduced as compared to sucrose (EFSA, 2011; Livesey, 2003). Decrease in postprandial glycemic response was observed when maltitol was used with shortchain fructo-oligosaccharides (Respondek et al., 2014). Its non-cariogenic properties allow on its application in many sugar-free foods (Evrendilek, 2012; Kearsley and Boghani, 2012; Lawson, 2007b), as well as a variety of reduced-calorie, reduced-fat foods (Evrendilek, 2012; Kearsley and Boghani, 2012; Lawson, 2007b).

### Mannitol

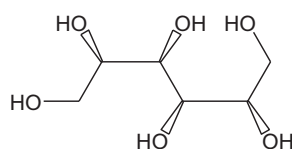
This 6-carbon polyol can be found in nature, i.e. bacteria, fungi, brown seaweeds and some higher plants (Fig. 5) (Jacobsen and Frigaard, 2014; Ruperez and Toledano, 2003). It is a white, crystalline, odorless, non-hygroscopic substance that is stable in high temperatures (>160 °C) (Table 2). Mannitol is an isomer of sorbitol, with the difference in the planar orientation of the OH group. It can be used as an anticaking and bulking agent, humectant, stabilizer, sweetener and thickener (Table 1) (Grembecka, 2015).

### Metabolism

Only 25% of consumed mannitol is absorbed in the small intestine by passive diffusion. This part of this sugar alcohol is converted by mannitol dehydrogenase to D-fructose, which enters the normal fructose pathway. The unabsorbed mannitol reaches the colon, where is fermented to produce short chain fatty acids (Ghoreishi and Shahrestani, 2009; Livesey, 2003; Wisselink et al., 2002). It is one of the least well-tolerated polyols, which should be consumed in amounts less than 20 g per day in order to prevent side effects (Friedman, 2008).

### Production

Industrial production of mannitol is based on the catalytic hydrogenation of glucose/fructose (1:1) mixture, obtained from invert sugar or starch. The process is carried out at high temperatures and pressure and yields both sorbitol and mannitol, which are later separated based on their solubility (Ghoreishi and Shahrestani, 2009; Makkee et al., 1985; Song and Vieille, 2009). As hydrogenation characterizes with rather low efficiency (25%) and purity of the products, thus, fermentative processes are researched. Application of heterofermentative lactic acid bacteria as well as cyanobacteria is being considered (Akinterinwa et al., 2008; Jacobsen and Frigaard, 2014; Soetaert et al., 1995; von Weymarn et al., 2002; Wisselink et al., 2002). Extraction from natural sources is also investigated as mannitol can be found in many natural sources such as exudates of certain trees (Manna ash, *Fraxinus ornus*), figs, olives, larches, edible fungi, yeasts and seaweed (Deis and Kearsley, 2012; Ghoreishi and Shahrestani, 2009; Ruperez and Toledano, 2003; Song and Vieille, 2009; Wang et al., 2013; Wilson, 2007). Such approach is applied commercially in China, where mannitol is extracted from certain species of seaweed (Deis and Kearsley, 2012).



**Figure 5** Chemical structure of mannitol.

### Properties and Applications

Mannitol can positively modify taste, texture and keeping properties of food products. Its low solubility affects the strength of cooling effects (Table 2) (Ghoreishi and Shahrestani, 2009; Livesey, 2003; Ortiz et al., 2013). Mannitol characterizes with lower sweetness than sucrose, thus, can be combined with other sweeteners in order to increase sweetness and create better taste (Grembecka, 2015). Owing to its very low hygroscopicity, it can be applied as a dusting powder, preventing gums from sticking to wrappers and equipment (Table 2) (Ghoreishi and Shahrestani, 2009; Grembecka, 2015; Gombás et al., 2003; Jamieson, 2012). Its physicochemical properties and non-cariogenicity allow on its usage in production of nutritional tablets, chocolate-flavored coating agents for ice cream and confections or "breath-freshening" and "sugar-free" products (Grembecka 2015, 2018). Mannitol also characterizes with glycemic and insulinemic indexes amounting to 0, which allows on its consumption by diabetic people (Livesey 2003, 2012; Song and Vieille, 2009; Wisselink et al., 2002). Besides food and pharmaceutical industry, it is also known for its medical activities, such as prebiotic, antioxidant and diuretic effects (Gaspar et al., 2004; Ghoreishi and Shahrestani, 2009; Jagannatha et al., 2016; Kassim and Esmat, 2016; Monedero et al., 2010; Saha and Racine, 2010; Shawkat et al., 2012; Wisselink et al., 2002). Moreover, it helps to get rid of mucus and cough in asthmatics and other hypersecretory diseases (Daviskas et al. 2010a, 2010b). It was found that combined with hydration during endovascular aortic aneurysm repair might improve renal function (Kalimeris et al., 2014).

### Sorbitol

This sugar alcohol, called d-glucitol, is an isomer of mannitol (Fig. 6), but characterizes with different properties. It can be found in nature in various fresh and dried fruits, i.e. apples, pears, peaches, prunes, dates and raisins, as well as in some vegetables (Barbieri et al., 2014; Budavari et al., 1996; Deis and Kearsley, 2012; Gutierrez and Gaudillere, 1996; Lawson, 2007c; Milala et al., 2013; Silveira and Jonas, 2002). It is a white, hygroscopic, well soluble, crystalline powder that is stable in high temperatures (Grembecka, 2015).

### Metabolism

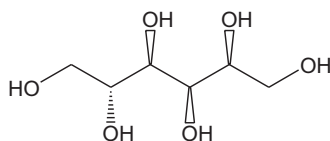
Sorbitol is slowly absorbed (25% to 80% of the consumed dose) in the gut by facilitated diffusion and later metabolized in the liver (Livesey, 2003). The unabsorbed part is moved to colon, where fermentation takes place (Deis and Kearsley, 2012; Ghoreishi and Shahrestani, 2009; Jamieson, 2012; Livesey, 2003; Wisselink et al., 2002). The side effects such as laxation can occur as a result of an osmotic imbalance in the gut, when sorbitol is consumed in doses exceeding 50 g per day or 10 g per single serving (Jamieson, 2012).

### Production

Although it is found in plants, i.e. stone and berries from trees of the genus *Sorbus*, it is produced on an industrial scale by catalytic hydrogenation. This process requires glucose or sucrose substrates which are hydrogenated at elevated temperatures using nickel catalyst (Barbieri et al., 2014; Evrendilek, 2012; Grembecka, 2015; Kusserow et al., 2003; Ortiz et al., 2013). Sorbitol can be obtained both in liquid and crystalline form (Deis and Kearsley, 2012; Lawson, 2007c), while the latter is produced through evaporation of the solution and crystallization. There are also other routes of sorbitol production such as electrochemical reduction of dextrose in alkaline conditions (Barbieri et al., 2014; Evrendilek, 2012; Grembecka, 2015) or biotechnological using *Zymomonas mobilis* and *Candida boidini* that are able to produce this polyol (Akinterinwa et al., 2008; Jonas and Silveira, 2004; Ladero et al., 2007; Ortiz et al., 2013; Silveira and Jonas, 2002; Silveira et al., 1999).

### Properties and Applications

Although sorbitol is less sweet than sucrose (Table 1), it characterizes with a pleasant taste, which can mask the unwanted one of other substances. Moreover, its sweetness can be increased by blending with other sweeteners. Its solubility is slightly higher than sucrose and high negative heat of solution results in marked cooling effect, which is important for certain applications (Table 2) (Basu and Shivhare, 2013; Deis and Kearsley, 2012; Le and Mulderrig, 2001). Due to high hygroscopicity, easy compression and other physicochemical properties such as thermal stability and chemical inertness, it can be used as a humectant, softener, texturizing and anti-crystallizing agent (Basu and Shivhare, 2013; Deis and Kearsley, 2012; Gombás et al., 2003; Grembecka, 2015; Jamieson, 2012; Jonas and Silveira, 2004; Le and Mulderrig, 2001; Ortiz et al., 2013). It is also an important precursor of the vitamin C production as well as sorbose and surfactants (ADA Reports 2004; Ladero et al., 2007; Silveira and Jonas, 2002). Sorbitol is not digested by mouth bacteria, thus, preventing from cavities and erosion of tooth enamel (EFSA, 2011). Therefore, it can be used as a tooth-friendly substance in foods and pharmaceuticals. This sugar alcohol is also suitable for use in products for diabetics



**Figure 6** Chemical structure of sorbitol.

as well as in sugar-free candies, chewing gums, and sugar free foods such as frozen desserts and baked goods as well as cosmetics (Grembecka, 2015).

### Xylitol

Five-carbon monosaccharide polyhydric alcohol, d-erythro-pentitol (Fig. 7), is usually called birch sugar, as initially birch wood was used for its production. It can be found in nature in fruits (yellow plums - 935 mg/100 g d s.; strawberries - 362 mg/100 g d s.), and vegetables (cauliflower - 300 mg/100 g d s.; endives - 258 mg/100 g d s.), berries, oats and mushrooms and is produced in small quantities in human organism (5 to 15 g/day in carbohydrate metabolism) (Barbieri et al., 2014; Bond, 2007; Evrendilek, 2012; Granström et al., 2007; Ly et al., 2006; Lee and Park, 2014; Nigam and Singh, 1995; Zacharis, 2012b). It has been used as a sweetener since the 1960s (Zacharis and Stowell, 2012).

### Metabolism

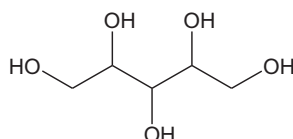
Absorption of xylitol amounts up to 50% and takes place in the small intestine. There are two routes of xylitol metabolism, i.e. direct in the liver via the glucuronic acid–pentose phosphate shunt of the pentose phosphate pathway or indirect by fermentation conducted by intestinal flora (Sheet et al., 2014). The unabsorbed part undergoes fermentation in the large bowel, where minor amounts of gas ( $H_2$ ,  $CH_4$ ,  $CO_2$ ) as well as short-chain volatile fatty acids are produced. Xylitol can be consumed even up to 100 g per day (Lee and Park, 2014).

### Production

Commercially, xylitol is produced by a catalytic hydrogenation of xylose, which sources are birch trees, hardwood or lignocellulosic biomass (Chattopadhyay et al., 2014; Granström et al., 2007; Ly et al., 2006; Nigam and Singh, 1995; Park et al., 2016; Zakaria, 2001; Zhang et al., 2012). The whole process consists of xylan hydrolysis, chromatographic purification, and hydrogenation with nickel catalyst (Evrendilek, 2012). Due to the cost of this approach, there are researched other ways of this polyol production including biotechnological ones with the use of such microorganisms as *Candida tropicalis* and *Candida guillermundii* (Akinterinwa et al., 2008; Ortiz et al., 2013; Park et al., 2016). However, these species are not suitable for food industry as these are pathogenic species (Grembecka, 2015, 74, Ortiz et al., 2013). There are also data indicating that recombinant microalgae or fungi might produce xylitol (Hirabayashi et al., 2015; Park et al., 2016; Pourmir et al., 2013).

### Properties and Applications

Xylitol resembles sugar in view of sweetness (Table 1) and pleasant taste. Moreover, it characterizes with the greatest cooling sensation (Table 2) in the mouth and is readily soluble in aqueous solutions (Granström et al., 2007; Mäkinen, 2011). Therefore, it can be used to enhance and complement mint flavor or providing products with refreshing cooling sensation. Moreover, it has non-insulin stimulant properties, thus, is safe for diabetic people (Chattopadhyay et al., 2014; Grembecka, 2015; Nigam and Singh, 1995; Zacharis, 2012b). It is also known for anti-cariogenic effects and is responsible for a decrease in plaque formation as it inhibits the growth and metabolism of *Streptococcus mutans* and *Streptococcus sobrinus*, species responsible for caries and dental plaque acid production (ElSalhy et al., 2012; Lee and Park, 2014; Lee et al., 2012; Misra et al., 2012; Salli and Forssten, 2016; Söderling et al., 2008). What is more, it increases oral pH and forms complexes with  $Ca(II)$  which allows on teeth remineralization (Bahador et al., 2012; Cardoso et al., 2016; Mäkinen, 2011; Tanzer, 1995). Both frequency and amounts of consumption are important and it was found that xylitol works most efficiently when is applied 3 to 5 times a day and the total daily dose ranges from 6 to 10 g (Ly et al., 2006). Due to its properties, xylitol fights unpleasant mouth odor and stimulates saliva production, which is of great importance for people with xerostomia (Grembecka, 2015; Mäkinen, 2011; Ship et al., 2007). Due to its fermentation in the colon, it can be used as prebiotic that increase the growth of *Bifidobacterium*, thus resulting in short-chain fatty acids production and lowering of intestinal pH (Bond, 2007). Xylitol also exhibits antifungal and antibacterial effects and can reduce ear infections incidence and pneumococcal nasal colonization (Lee and Park, 2014; Mäkinen, 2011; Nyssölä et al., 2005; Ortiz et al., 2013; Vernacchio et al., 2007; Uhari et al., 2000). It was also noted that xylitol solution inhibits the growth of *Streptococcus pneumoniae*, *Haemophilus influenza*, *Candida albicans* and *Helicobacter pylori* (Grembecka, 2015; Lee and Park, 2014). There is also data that xylitol is helpful in bone mineralization, through stimulation of calcium absorption, and increases minerals bioavailability (Kaivosoja et al., 2008; Xiao et al., 2015).



**Figure 7** Chemical structure of xylitol.



## Summary

Today we encounter a great variety of readily available sugary foods, which are often of very low nutritional value, but with high energy value, thus, their preference over others can cause many health problems. Sugar alcohols are natural sugar alternatives but are also referred to as semi-synthetic sweeteners. This is due to the fact that they are present in small amounts in plants, fruits, vegetables and mushrooms, but are produced synthetically for technological purposes. They characterize not only with lower caloric content and glycemic index, but with beneficial anti-caries, prebiotic, antioxidant and antibacterial effects. Therefore, polyols are bulk sweeteners that constitute excellent substitutes to sugar in food and pharmaceutical industry. As these substances do not influence insulin level in blood, they can be recommended for people with diabetes but also for everyone who want to lead a healthy lifestyle.

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## Surfactants

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### Glossary

**Coalescence** Is the formation of one droplet from two or more droplets.

**Emulsifier** Are surface-active agents that are amphiphilic compounds containing both polar and non-polar residues allowing them to reside at the oil-water interface and reduce the interfacial tension.

**Flocculation** Is the process by which particulates are caused to clump together into a floc while the individual droplets retain their original interface.

**HLB Value** It is calculated from the weight percentage of the hydrophilic groups to the hydrophobic groups in a molecule, with values ranging from 1–20.

**Ostwald's Ripening** Is the process where droplets (i.e., the discontinuous phase) diffuse from smaller to larger droplets through the continuous phase.

### Introduction to Food Emulsions

An emulsion consists of two liquid immiscible phases: oil and water. Either of these two phases can be the continuous or the dispersed phase and as such there are two primary types of emulsions: oil-in-water (o/w) or water-in-oil (w/o). An oil-in-water emulsion consists of an oil phase dispersed into a continuous water (aqueous) phase. Common food examples include: mayonnaise, salad dressing, whipped toppings, and ice cream. In contrast, a water-in-oil emulsion consists of an aqueous phase dispersed in a continuous oil phase, such as butter and margarine (Kralova and Sjöblom, 2009). In addition to an oil phase and an aqueous phase, the formation of an emulsion requires energy input and addition of surfactants. The resulting emulsion that is formed (o/w or w/o) is greatly influenced by the chemistry of surfactant (Walstra, 1993). Surfactants are used to reduce the work required to overcome surface free energy and disperse one phase into the other and to stabilize the final emulsion. Properties of o/w emulsions rely more heavily on the type of surfactant used and the composition of the aqueous phase; while, properties of w/o emulsions are more dependent on the type of fat (Kralova and Sjöblom, 2009).

Food emulsions exist in a pseudo-equilibrium or 'frozen' kinetic state making them "shelf-stable" only for a limited time. The stability of the emulsion is dependent on the crystallinity of oil/fat droplets, the interfacial material, and the chemistry of the aqueous phase. The degree of crystallinity of the oil phase influences its melting properties, which is important for partial coalescence in ice cream and whipped products. The quality of the interfacial layer depends on the mixture of macromolecules and small molecule weight surfactants. The quantity and structure of the adsorbed surface-active compounds affects interfacial properties. Small molecular weight surfactants often displace adsorbed proteins or bind/interact with proteins (ie. lecithin), which modifies emulsion droplet stability. Therefore, even small changes in interfacial composition can significantly influence overall stability. The interactions between emulsion droplets and the aqueous phase also impacts emulsion stability (Dalgleish, 2006).

The terms emulsifier, surface-active agents and surfactant are often used interchangeably to describe amphiphilic compounds that contain both polar and non-polar residues (Lauridsen, 1976; Weiss, 1971). This characteristic allows them to reside at the oil-water interface and reduce the interfacial tension (Lauridsen, 1976). This general review will focus on the mechanisms of emulsification, prevention of emulsion destabilization, common surfactants, and their applications in food.

### Mechanisms of Emulsification

Emulsions are *thermodynamically unstable* because they have a positive free energy of formation ( $\Delta G_f$ ) and are considered non-equilibrium systems as emulsified droplet sizes increase with time (Taylor, 1998; Bibette et al., 1992). This instability is due to the large interfacial area of droplets, represented by surface area ( $A$ ) & interfacial tension ( $\gamma$ ):

$$\Delta G_f = \gamma A - T\Delta S_f$$

The entropy of formation ( $\Delta S_f$ ) is relatively small because only a small number of droplets are being formed in entropic terms. Hence, the larger interfacial area variable results in an overall  $\Delta G_f \geq 0$ . Nonetheless, emulsions are *kinetically stable* due to the presence of an adsorbed interfacial layer which may include ionic surfactants (electrostatic forces) or non-ionic surfactants (steric forces) (Taylor, 1998).

To reduce the emulsion droplet size, energy is required to overcome its *Laplace pressure* which is the pressure differential between the convex and concave side of the interface:

$$\text{Laplace pressure}(p_L) = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right)$$

where  $p_L$  is the Laplace pressure,  $R_1$  and  $R_2$  are the radii. Accordingly, a smaller droplet radius corresponds to a greater pressure needed to be overcome to achieve further particle size reduction; however, by lowering the surface tension, via the addition of surfactants, the Laplace pressure also decreases. The process of emulsification involves: droplet deformation and breakdown, transportation and adsorption of surfactants to the newly formed droplet interface, and droplets possibly colliding and coalescing. As these processes occur simultaneously a steady-state is not necessarily achieved. Ultimately, the main functions of surfactants are to (1) lower interfacial tension to facilitate a droplet size reduction, and (2) prevent recoalescence of droplets (Walstra, 1993).

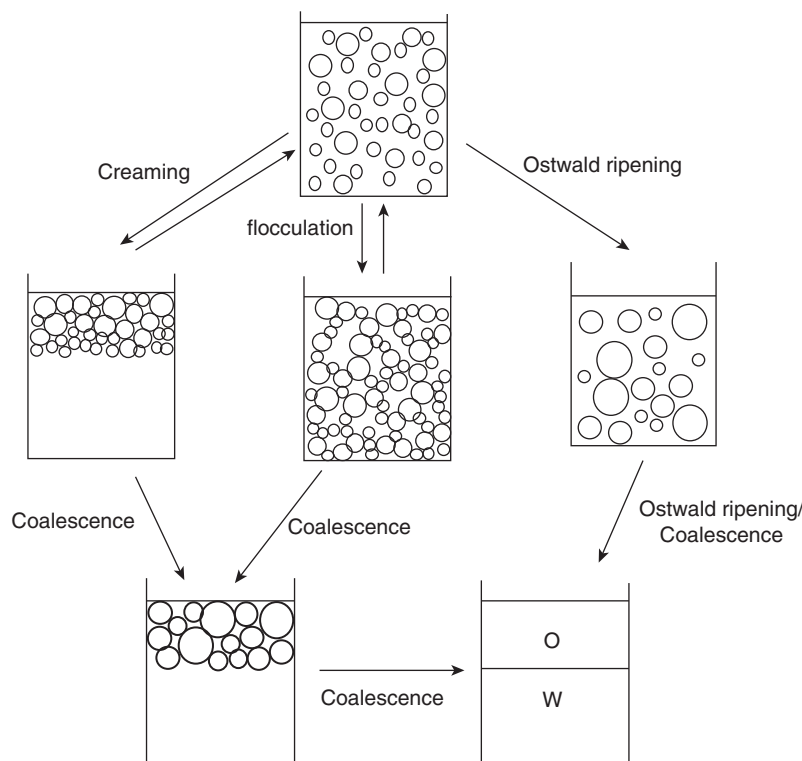
### Prevention of Emulsion Destabilization

Without appropriate controls or after a prolonged duration, an emulsion will eventually separate into its two distinct phases. There are four primary mechanisms which destabilize an emulsion: creaming (gravitational separation), aggregation, coalescence, and Ostwald ripening. Typically, these mechanisms occur in combination (Fig. 1) (Fredrick et al., 2010).

Perhaps the most straightforward destabilization mechanism, creaming of an o/w emulsion involves the gravitational separation of the dispersed oil and continuous aqueous phases due to differences in density (Rousseau, 2000). For example, after some time, milk fat droplets in unprocessed raw milk will rise and gather together, forming a cream layer at the top (Fredrick et al., 2010). An effective method of stabilizing against creaming is to simply reduce average droplet size and increase the uniformity of the droplets via homogenization (Rousseau, 2000).

Aggregation occurs when droplets come into contact, but whether they remain in contact depends on internal (attraction/repulsion) and external forces (ie. stirring/agitation) (Fredrick et al., 2010). Flocculation is the aggregation of particles due to weak attractive forces, in which droplets retain their structural integrity. It is also described by the DLVO (Derjaguin-Landau, Verwey-Overbeek) theory as a function of interparticle distance and the balance between attraction (London-van der Waals forces) and repulsion (electrostatic) (Rousseau, 2000). Emulsions stabilized by globular proteins are stable against flocculation if no salt is present – due to electrostatic screening, but this is not usually the case in foods (Zhai et al., 2013). As flocculation involves weaker associations, it is easier to disrupt the aggregates and may be reversible. However, when stronger forces are required to disrupt aggregates, this form of aggregation is referred to as coagulation (Fredrick et al., 2010).

If two droplets remain in close contact for long enough, the film separating them may rupture (Fredrick et al., 2010). Creaming and flocculation are both precursors to coalescence as they bring droplets close together (Taylor, 1998). The likelihood of film



**Figure 1** The relationships between different o/w emulsion destabilization mechanisms. Reprinted with permission from Meinders, M.B.J., Kloek, W., Van Vliet, T., 2001. Effect of surface elasticity on Ostwald ripening in emulsions. *Langmuir*, 17, 3923–3929. Copyright 2001 American Chemical Society.



rupture increases with larger droplet size, thinner film (greater attraction between droplets), and lower interfacial tension (which depends on the surfactant used). Coalescence results in the formation of one larger droplet from two smaller droplets, which assumes a spherical shape as this has the lowest Laplace pressure (Fredrick et al., 2010). To prevent coalescence, surfactants must form a viscous and rigid film at interface that acts as a physical barrier. While complete coalescence occurs between liquid droplets; the presence of crystalline matter within droplets results in *partial* coalescence (Rousseau, 2000). A partly-crystalline oil phase allows for the formation of a crystal network within oil droplets, with some crystal protrusion into the aqueous phase (Fredrick et al., 2010).

Ostwald ripening and coalescence are both examples of “coarsening phenomena”. Coalescence can be prevented by using certain surfactants, Ostwald ripening may occur whenever curved interfaces are present (Meinders et al., 2001). Ostwald ripening does not require droplets to be in close proximity, as it is based on diffusion (Taylor, 1998). It involves the preferential diffusion of small droplets into larger droplets, due to their greater Laplace pressure (Bibette et al., 1992). The solubility of dispersed droplets increases with decreasing radius of curvature, smaller droplets tend to dissolve into the bulk phase and preferentially diffuse to larger droplets. This is because by growing larger to minimize interfacial area, emulsions droplets can reduce their free energy (Taylor, 1998). A critical radius ( $C_r$ ) determines which droplets shrink (radius <  $C_r$ ) and which droplets grow (radius >  $C_r$ ) (Meinders et al., 2001).

### Hydrophilic-Lipophilic Balance of Surfactants

The hydrophilic-lipophilic balance (HLB), often used to describe surfactants, is calculated from the weight percentage of the hydrophilic groups to the hydrophobic groups in a molecule, with values ranging from 1–20 (Kralova and Sjöblom, 2009). The HLB value of a surfactant should match the HLB value of the oil phase based on the notion of “like dissolves like”. It is not a simple linear relationship in which functionality increases with higher hydrophobicity (Nakai, 1983).

As mentioned previously, the type of emulsion formed (o/w or w/o) is mainly determined by the surfactant present (Walstra, 1993). Surfactants with an HLB value between 3–6 (i.e., glycerol esters, propylene glycol fatty acid esters, polyglycerol esters, and sorbitol fatty acid esters) promote formation of w/o emulsions. Surfactants with HLB values between 8–16 tend to form o/w emulsions. Surfactants between 8–16 include: proteins, phospholipids, potassium and sodium salts, and alginates (Kralova and Sjöblom, 2009).

Nevertheless, there are limitations to the HLB concept. Depending on temperature, a single emulsifier can produce either an o/w or w/o emulsion. At high oil concentrations, a single emulsifier can produce an o/w at low emulsifier concentration, or a w/o emulsion at high emulsifier concentration. Furthermore, o/w emulsions can be prepared with emulsifiers across the entire HLB range. The stability of an emulsion prepared with a blend of opposite emulsifiers (ie. Spans and Tweens) will depend on how the emulsifiers are positioned at the interface. Lastly, the theoretical calculation of HLB values does not apply to blends of emulsifiers that associate at the oil-water interface (Boyd et al., 1972).

### Gibbs-Marangoni Effect

The Gibbs-Marangoni effect describes the mechanism that prevents recoalescence of newly formed emulsion droplets (Fig. 2). When two droplets that are insufficiently coated with surfactant move towards each other, they will accumulate additional surfactants as they approach each other (Fig. 2(1)). But, the amount of surfactant available for adsorption will be lowest at the point where the droplets are closest (Fig. 2(2)). This creates an interfacial tension ( $\gamma$ ) gradient, where  $\gamma$  is highest at this point. Due to this gradient, surfactants will move in the direction of highest  $\gamma$ , where there is the least amount surfactant coverage (Fig. 2(3)). This streaming of liquid along the surface is known as the Marangoni effect, a self-stabilizing mechanism in which droplets are driven away from each other. However, this only occurs if the surfactant is present in the continuous phase and before the system reaches equilibrium. According to Bancroft’s Rule, when forming an emulsion, the phase in which the surfactant is most soluble becomes the continuous phase. The efficacy of the Gibbs-Marangoni mechanism depends on Gibbs elasticity ( $\epsilon$ ) of film, where ( $A$ ) represents area of the interface.

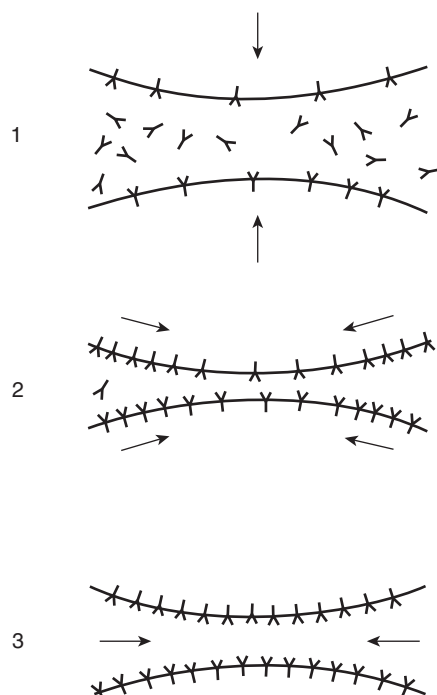
$$\epsilon = \frac{2d\gamma}{d\ln A}$$

If  $\epsilon$  is high, there is a strong interfacial gradient and stabilization occurs (Walstra, 1993). Interfacial layers formed by non-protein surfactants are stabilized by the Gibbs-Marangoni effect. This mechanism relies on rapid diffusion of surfactants to the interface to prevent surface concentration gradients (Wilde et al., 2004).

### Critical Micelle Concentration

As surfactants are amphiphilic structures with a non-polar hydrocarbon tail and polar (usually ionic) head group, they arrange themselves into micelles in polar solvents, such as water. This is due to the hydrophobic effect, in which surfactants arrange themselves into a micellar structure with a hydrophobic core and hydrophilic head groups at the interface. The critical micelle concentration (CMC) refers to the concentration of surfactant above which micelles will form. There are three common methods of CMC determination: (1) measure the change in UV-vis spectrum of benzoylacetone, (2) measure the fluorescence emission spectrum of pyrene monomers, or (3) measure the electrical conductivity of an ionic surfactant solution as concentration increases. To





**Figure 2** Portrayal of the Gibbs-Marangoni effect on two approaching emulsion droplets, with surfactants represented by Y. One represents the two emulsion droplet approaching one and other, 2 illustrates the movement of surfactant molecules along the interface and 3 signifies the water flowing between droplets to dilute the high surfactant concentration. Reprinted with permission from Walstra, P., 1993. Principles of emulsion formation. Chem. Eng. Sci. 48 (2), 333–349. Copyright 1993 Elsevier.

determine CMC, simply plot the physical property as a function of surfactant concentration and the CMC will be the point in which the slope changes. CMC decreases with increasing hydrocarbon chain length of non-polar groups, or addition of electrolytes for ionic surfactants (Domínguez et al., 1997).

## Major Food Surfactants

### Low MW Surfactants

Low MW surfactants are very mobile at the interface making them efficient at lowering interfacial tension (Kralova and Sjöblom, 2009). Low MW emulsifiers do not form a viscoelastic interfacial layer, but rather form a compact interfacial layer. Competitive destabilization may occur when both proteins and surfactants are present at the interface. Surfactants weaken protein interactions, while proteins hinder surfactant mobility (Gibbs-Marangoni effect) (Wilde et al., 2004). As low MW surfactants are smaller than proteins, they tend to displace proteins from the interface (Rousseau, 2000). At low surfactant concentration, protein-surfactant complexes form. While, high surfactant concentration results in protein displacement from the interface. Therefore, low MW surfactants are commonly used to intentionally destabilize food emulsions, such as ice cream allowing for partial coalescence (Dickinson et al., 1989).

Lecithin used in foods are a mixture of phospholipids and can be modified to obtain various HLB values (Van Nieuwenhuyzen and Szuhaj, 1998). Methods of modification include: fractionation in alcohol, hydrolysis (enzymatic, acid or alkali), acetylation or hydroxylation. Lecithin can be derived from egg yolk or various oilseeds (soybean, flax, cottonseed, corn, rapeseed, sunflower). However, soybean is the most common source due to its availability, emulsifying properties, color and taste. There are many food applications of soy lecithin, such as: viscosity lowering in chocolate, protein interaction in baked products, retardation of fat crystallization in chocolate, and dispersing effect in instant drinks. Furthermore, its stability increases when used together with other surfactants, such as monoglycerides or proteins (Van Nieuwenhuyzen, 1976).

Mono- and di-glycerides are glycerol esters of fatty acids. They are most commonly used in the food industry for bakery products (bread, cake, cake mixes) and frozen desserts (Weiss, 1971). Monoglycerides have two isomeric forms (1-form and 2-form) based on a temperature-dependent equilibrium (Lauridsen, 1976). Sorbitan esters, also known as “Spans”, are products of a reaction between sorbitol and fatty acids. Polysorbates, also known as “Tweens” are polyoxyethylene derivatives of sorbitan esters. Spans and Tweens are often used together in cakes, cake mixes, whipped toppings, icings, and coffee whiteners (Weiss, 1971). According to Boyd et al. (1972), optimum stability in o/w emulsions is achieved when using a 1:1 ratio of Spans and Tweens.

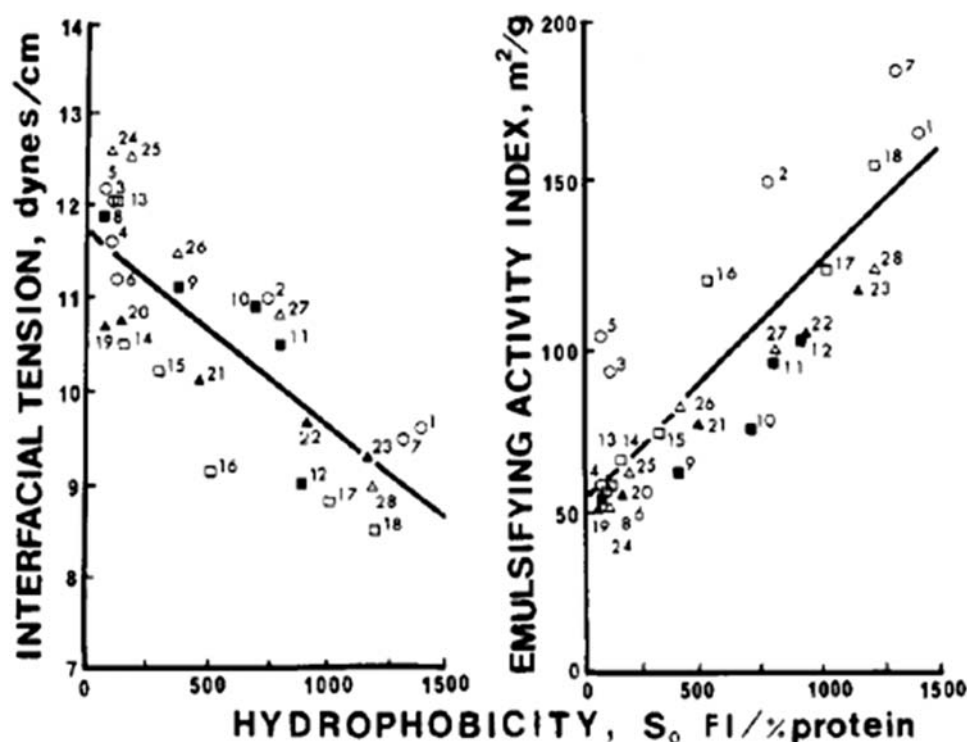
## Proteins

The main emulsion stabilization mechanism of proteins is through the formation of a viscoelastic adsorbed layer. However, the extent of adsorption depends on protein surface hydrophobicity and charge (Wilde et al., 2004). As non-polar amino acids reside within protein molecules to avoid contact with aqueous surroundings, only some of them partake in the emulsification process. Thus, the term protein hydrophobicity typically refers to *surface* hydrophobicity. As emulsifiers, proteins adsorb to the interface due to their amphiphilic nature and form a thick coating around oil droplets. The emulsifying ability of proteins depends on a balance between hydrophilic and lipophilic portions (Nakai, 1983). As shown in Fig. 3, when protein hydrophobicity increases, interfacial tension decreases and emulsifying activity increases. Thus, greater exposure of hydrophobic residues on proteins improves emulsifying and foaming ability. Additionally, the effectiveness of a protein as an emulsifier is also attributed to its molecular flexibility. For example, casein molecules are better emulsifiers than whey proteins as they have a looser conformation (Nakai, 1983).

## Applications in Food Systems

Cream liqueurs are o/w emulsions that contain ethanol in its aqueous continuous phase. The presence of ethanol (up to 20% wt.) improves emulsification and produces finer droplets, as it lowers interfacial tension. However, increasing alcohol content may eventually lead to protein aggregation, with precipitation observed at > 30% wt. ethanol (Dickinson et al., 1989). Sodium caseinate is the primary stabilizer in cream liqueurs (Kralova and Sjöblom, 2009). Sodium caseinate forms an interfacial layer of caseinate submicelles around fat droplets to stabilize emulsions (Rousseau, 2000). If low MW surfactants are added to cream liqueurs, they reside in the primary interfacial layer with ethanol; while, the secondary interfacial layers contain adsorbed caseinates. But due to the intrinsic emulsifying ability of cream liqueurs, the addition of low MW surfactants does not significantly influence droplet size distribution. Ethanol content also lowers the surface viscosity of adsorbed caseinate film at the interface. This is beneficial for the consumer acceptance of cream liqueurs which should not be too viscous or viscoelastic (Dickinson et al., 1989).

Ice cream and whipped toppings are both examples of partially-coalesced emulsions that undergo shear and incorporation of small air bubbles. Whipped products use surfactants to displace proteins and weaken the interfacial layer, allowing oil from emulsion droplets to spread into the air–water interface (Dalglish, 2006). Compared to whipped products, ice cream has a lower fat volume, presence of ice crystals, and also utilizes a different whipping process (Petrut et al., 2016). For these products, it is important that the fat phase is semi-liquid during whipping, but crystallizes afterward (Dalglish, 2006). The purpose of fat network formation is to entrap air bubbles and stabilize the foam structure (Petrut et al., 2016).



**Figure 3** Relationship between interfacial tension, surface hydrophobicity and emulsifying activity of various proteins. Reprinted with permission from Nakai, S., 1983. Structure-function relationships of food proteins with an emphasis on the importance of protein hydrophobicity. J. Agric. Food Chem. 31 (4), 676–683. Copyright 1983 American Chemical Society.

In ice cream, the destabilization of fat emulsions during whipping and subsequent freezing is necessary to impart dryness during extrusion, smooth mouthfeel/texture, and resistance to meltdown. Ice cream has an aqueous phase composed of sugar, salt, and a colloidal casein suspension. Its discrete globular phase is partially-crystalline and contains triglycerides, diglycerides and monoglycerides. The interface contains milk proteins, phospholipids, lipoprotein particles and any added surfactants (Goff and Jordan, 1989).

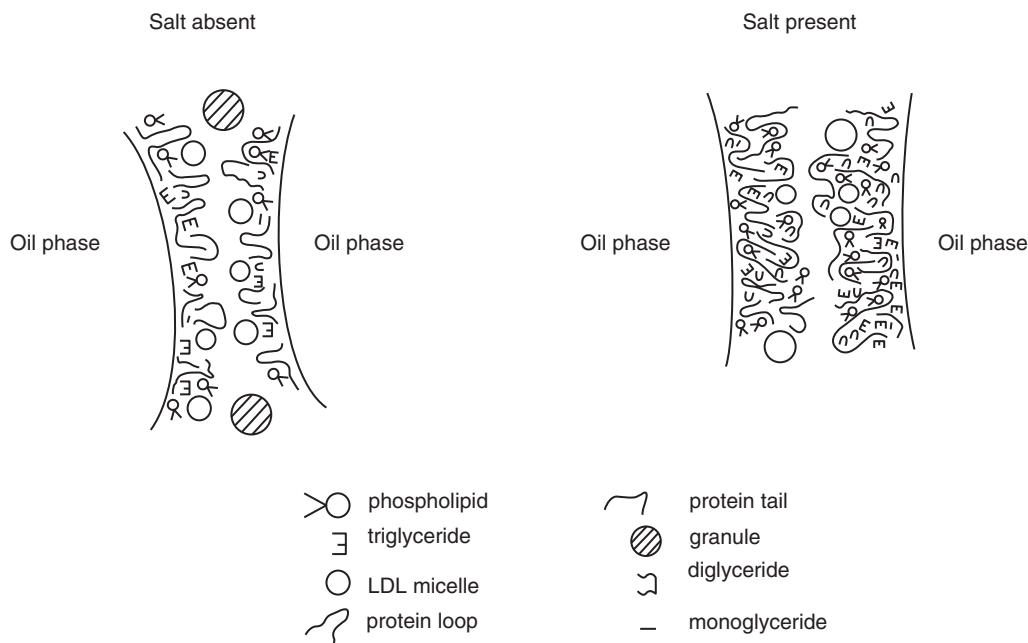
Low HLB surfactants are not effective destabilizers as they adsorb to the fat globules, which helps retain their structural integrity. High HLB emulsifiers do not associate with fat globules as strongly and are easily displaced during agitation. This exposes the fat globules and promotes coalescence. For example, Tweens (HLB = 15) are very effective destabilizers, while Spans (HLB = 4.3–8.6) are not (Goff and Jordan, 1989). Casein stabilized emulsions are more stable against partial coalescence than whey proteins; however, caseins are more easily displaced by low MW surfactants. This protein displacement mechanism is important in lowering emulsion stability to reduce viscoelasticity and thickness of interface (Fredrick et al., 2010). Typically, monoglycerides and diglycerides are used to induce partial coalescence, while polysaccharides (such as carrageenan and gums) are used as stabilizers in ice cream (Kralova and Sjöblom, 2009).

Mayonnaise contains up to 70%–80% fat, and is considered an acidic o/w emulsion. The low pH and high fat content of mayonnaise makes it highly resistant to microbial spoilage (Depree and Savage, 2001). To maintain emulsion stability, there must not be fat crystals within the dispersed oil phase. The aqueous continuous phase typically consists of eggs, sugar, vinegar, salt and spices. Egg yolk contains lecithin that function as the emulsifier and stabilizer (Van Nieuwenhuyzen and Szuhaj, 1998). Salt is also an important ingredient as it helps disperse egg yolk granules and neutralizes the charges proteins. This allows proteins to strongly adsorb to the interface and better interact with adjacent droplets, as shown below in Fig. 4 (Depree and Savage, 2001).

To make mayonnaise, the first step is to mix together the components with the aqueous phase. Next, oil must be *gradually* added while continuously blending. This is an important step as the mixing of both phases together all at once will result in the formation of a w/o emulsion. Oil droplets within mayonnaise are distorted from their normal spherical shape as they are tightly associated with each other. This flocculation is important for mayonnaise viscosity and texture. Consequently, the pH of mayonnaise should be near the isoelectric point of the egg yolk proteins ( $\sim 3.9$ ) to allow for maximum viscoelasticity and stability (Depree and Savage, 2001). This partial-phase inversion results in a heat stable emulsion that can be pasteurized. Heating up mayonnaise to 60 °C denatures the egg proteins, followed by hydrolysis to produce phosphatidylcholine which is an effective o/w surfactant. Alternatively, hydrolyzed soy lecithin is also commonly used in mayonnaise (Van Nieuwenhuyzen and Szuhaj, 1998).

## Conclusion

Overall, surfactants have a multitude of applications within the food processing industry. As emulsions are thermodynamically unstable, these surface-active agents are employed to improve kinetic stability and increase product shelf-life. Emulsions may be



**Figure 4** The interfacial components and affect of salts on mayonnaise o/w emulsion. Reprinted with permission from Depree, J.A., Savage, G.P., 2001. Physical and flavour stability of mayonnaise. Trends Food Sci. Technol., 12 (5–6), 157–163. Copyright 2001 Elsevier Science Ltd.

destabilized via creaming (gravitational separation), aggregation, coalescence, and/or Ostwald ripening. However, surfactants may combat these processes through several means. Electrostatic interactions involve the surface charge of emulsions droplets which is influenced by the presence of ionic surfactants, proteins and other charged compounds. Low MW surfactants have the ability to displace proteins to induce partial coalescence which is desired in promoting foam stability of ice cream and whipped toppings. Lastly, dispersed emulsion droplets may also be stabilized by the steric repulsive forces of mono- and di-glycerides or proteins.

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# Artificial Antioxidants

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## Nomenclature

ADI Acceptable daily intake

ANS Food additives and nutrient sources added to food

BHA Butylated hydroxyanisole

BHT Butylated hydroxytoluene

BMDL<sub>10</sub> Lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence

CoAH Co-antioxidant

EFSA European Food Safety Authority

EDTA Ethylene diaminetetraacetic acid

IONOX-100 Di-*tert* butyl-4-hydroxymethylphenol

MPAOs Multipotent antioxidants

NOAEL No-observed-adverse-effect level

PG Propyl gallate

TBHQ *tert*-Butylhydroquinone

THBP 2,4,5-Trihydroxybutyrophenone

## Introduction

It is common sense that the shelf-life of food is a key factor to ensure its perfect state and integral nutritional value. Among major food components, fats are particularly sensitive to different deterioration processes, mainly based in oxidation reactions. These reactions, along with the subsequent decomposition of oxidation products, cause the major losses in the nutritional value and sensory qualities of food products. Therefore, preventing or delaying these oxidation processes is essential for food producers and all intervenient involved in the food chain. Actually, there are different methods employed to inhibit oxidation, such as preventing oxygen access or reducing its pressure, using low temperatures, inactivating specific enzymes, or using suitable packaging. Obviously, another strategy employed against oxidation is the use of specific additives, which inhibit or delay the associated reactions (Yehye et al., 2015). These antioxidant compounds are usually classified as having natural, synthetic or artificial origin.

Since communication in science is highly dependent on precise language, it is mandatory to clarify the differences among those three concepts. Artificial refers to any compound that cannot be found in nature, while synthetic would generally describe any man-made compound. Accordingly, and just to establish an example, chemically synthesized  $\alpha$ -tocopherol should not be considered artificial, since it is chemically, analytically and functionally indistinguishable from  $\alpha$ -tocopherol isolated from natural sources. Likewise, chemically synthesized  $\alpha$ -tocopherol is not a natural compound, as it was not directly purified from natural sources, resulting instead from a synthesis procedure conducted by man (Nielsen, 2010).

Considering the previous concepts, it is easy to conclude that most antioxidants used in Food Industry are either synthetic or artificial, except for some particular cases such as tocopherol-rich or rosemary extracts, identified in the European Union as E306 and E392, respectively (Official Journal of the European Union, 2011a,b).

In addition to their origin-based classification, and owing to their structural diversity and different mechanisms of action, other classification systems were established. In general, antioxidants may be compounds or systems with the ability to inhibit the autoxidation by preventing the formation of free radicals or interrupting their propagation according to different mechanism. One of the most well-accepted defines five types of antioxidant compounds: primary antioxidants, chelators, O<sub>2</sub><sup>-•</sup> quenchers, oxygen scavengers and antioxidant regenerators (Carocho et al., 2015).

In addition to the previous introductory explanations, it should be clarified that the present article will be fully dedicated to aspects related to the application of antioxidants in food products, without detailing their multiple physiological effects or specific laboratorial assays employed in their detection. It is specifically intended to revise antioxidant compounds with top use in food products, their potential interaction with other food components, absorption dynamics, potential toxicological issues and, of course, recent legal aspects.

## Antioxidants in Food Industry

The main reason supporting the incorporation of antioxidants in food products is preventing the oxidation of fats, an undesired process associated with the production of pungent off-flavors (Shahidi et al., 2010). In fact, lipid oxidation causes detrimental changes in taste, odor, texture, flavor and appearance of foods, likewise reducing the levels of fat-soluble vitamins (McClements and Decker, 2000; Zeb and Murkovic, 2013).

In recent decades, different antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), propyl gallate (PG), 2,4,5-trihydroxybutyrophenone (THBP) and di-*tert* butyl-4-hydroxymethylphenol (IONOX-100) have been widely employed at industrial level with the main purpose of delaying the oxidative degradation of lipids, taking advantage of their high oxidative stability, low cost and good performance (Guo et al., 2006). Besides the indicated compounds, several others are legally recognized as being safe food additives (please check Regulation No 1333/2008 of the European Parliament and of the Council and its further amendments represented by Commission Regulation No 1129/2011 and Commission Regulation No 1130/2011, as indicated in the References section), such as exemplified in Table 1.

Other important antioxidants, despite primarily classified as colorizing agents, are represented by the E160a-f and E161a-j series (which includes mainly carotenoids), as also by E163 (which is composed by different anthocyanins).

Pertaining their use in food products, there is an intense discussion about the pros and cons of artificial antioxidants, particularly regarding BHA (E320), BHT (E321), TBHQ (E319) and propyl gallate (E310), which are the most commonly used (Makahleh et al., 2015; Xiu-Qin et al., 2009). Many studies have been carried out regarding these compounds, but no unequivocal results were obtained on their potentially hazardous effects over human health or lack of acute toxicity. In fact, while some reports point out hazardous effects, such as different toxicity levels or carcinogenicity, others describe opposite effects, classifying them as tumor suppressant (Bauer et al., 2001; Botterweck et al., 2000; Moon and Park, 2011; Saito et al., 2003; Vandeghanooni et al., 2013). Regarding these particular topics, and owing to their immense use, BHA and BHT will be analyzed in detail in further sections.

In either case, antioxidants should be tested for safety and must be approved for use in food, despite the allowable limits for their use vary greatly from country to country (Reische et al., 2008). Their usage in food industry, either as direct or indirect additives, should be made taking into account that all antioxidants have points of strength and weakness. Furthermore, factors, such as, effective concentration, thermal stability and synergism, should be taken into consideration when selecting antioxidants (Saso and Firuzi, 2014).

## Mechanism of Action

Regarding their functional properties, antioxidants might be generally divided into two major groups: primary and secondary antioxidants. The first group includes radical terminators, oxygen scavengers and chelating agents. The majority of antioxidants used as food preservatives, which include BHA, BHT, TBHQ, PG and other gallic acid esters, are all examples of radical terminators, specifically preventing lipid oxidation by terminating the free radical chains (Carocho and Ferreira, 2013).

Still regarding their mode of action, five different types of antioxidants (previously indicated in Introduction) might be considered:

- i) primary antioxidants: also known as radical scavengers or chain-breaking antioxidants, which scavenge species that initiate peroxidation (Muralikrishna Adibhatla and Hatcher, 2006);
- ii) chelators: bind to metallic elements,  $\text{Fe}^{2+}$  in particular, preventing the initiation of radical formation (Brewer, 2011);
- iii)  $\text{O}_2^{\cdot-}$  quenchers: have the ability to deactivate high-energy oxidant species, preventing formation of peroxides (Brewer, 2011);
- iv) oxygen scavengers: act by removing oxygen from the system, avoiding their destabilization;
- v) antioxidant regenerators: regenerate other antioxidants when these are converted into radical species (Carocho et al., 2015).

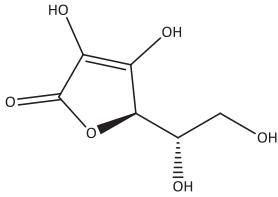
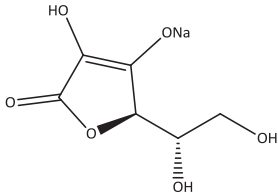
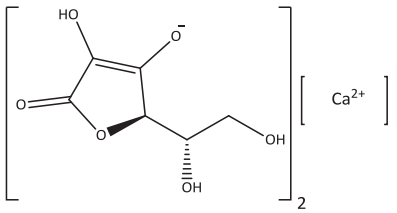
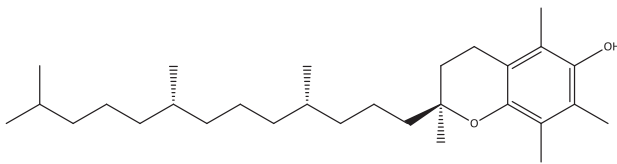
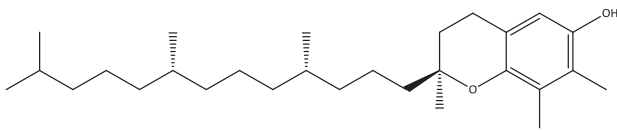
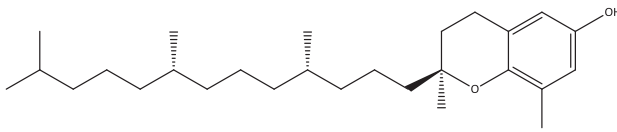
Some specific antioxidants, such as sulphites, glucose oxidase or ascorbyl palmitate, act as oxygen scavengers, operating essentially as reducing agents. In addition, chelating agents might also prevent lipid oxidation by binding specific catalysts (e.g., iron or copper). Their action may occur by either precipitating metal elements or by occupying all their coordination sites. The most well-known examples of these agents include polyphosphatases and ethylene diaminetetraacetic acid (EDTA, which is classified as E385) (Makahleh et al., 2015; Thorat et al., 2013).

On the other hand, secondary antioxidants act by breaking down hydroperoxides formed during lipid oxidation, ending up by forming stable products. Thiodipropionic acid (E388) and dilauryl thiodipropionate (E389) are among the most used secondary antioxidants (Berdahl et al., 2010).

The antioxidant activity is also dependent on structural variations within each compound; for instance, among the two isomers of BHA (3-*tert*-butyl-4-methoxy phenol and 2-*tert*-butyl-4-methoxy phenol), 2-*tert*-butyl-4-methoxy phenol is generally considered to be a better antioxidant.

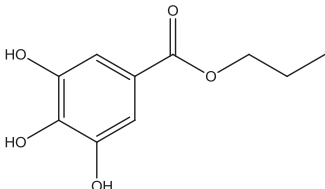
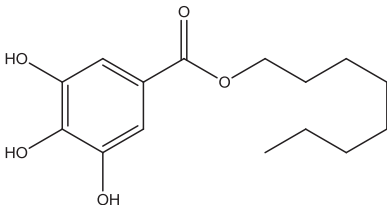
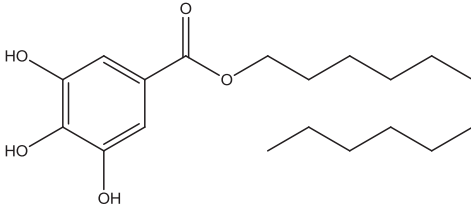
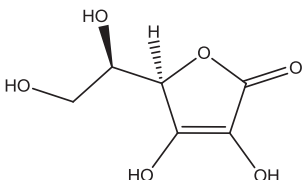
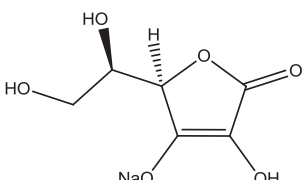


**Table 1** Main antioxidant compounds employed in food industry

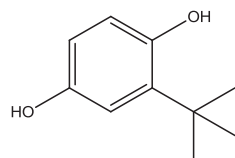
Compound name	Molecular structure	Molecular formula	E number	CAS number
Ascorbic acid		$C_6H_8O_6$	E300	50-81-7
Sodium ascorbate		$C_6H_7NaO_6$	E301	143-03-2
Calcium ascorbate		$Ca(C_6H_7O_6)_2$	E302	5743-27-1
$\alpha$ -Tocopherol		$C_{29}H_{50}O_2$	E307	59-02-9
$\gamma$ -Tocopherol		$C_{28}H_{48}O_2$	E308	54-28-4
$\delta$ -Tocopherol		$C_{27}H_{46}O_2$	E309	119-13-1

(Continued)

**Table 1** Main antioxidant compounds employed in food industry—cont'd

Compound name	Molecular structure	Molecular formula	E number	CAS number
Propyl gallate		$C_{10}H_{12}O_5$	E310	121-79-9
Octyl gallate		$C_{15}H_{22}O_5$	E311	1034-01-1
Dodecyl gallate		$C_{19}H_{30}O_5$	E312	
Erythorbic acid		$C_6H_8O_6$	E315	89-65-6
Sodium erythorbate		$C_6H_7NaO_6$	E316	6381-77-7

TBHQ

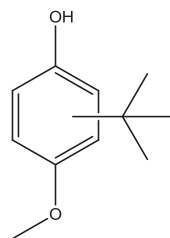


$C_{10}H_{14}O_2$

E319

1948-33-0

BHA

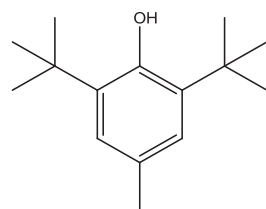


$C_{11}H_{16}O_2$

E320

25013-16-5

BHT

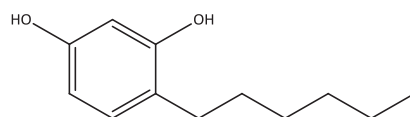


$C_{15}H_{24}O$

E321

128-37-0

4-Hexylresorcinol



$C_{12}H_{18}O_2$

E586

136-77-6

## Butylated Hydroxyanisole

BHA (like BHT) is a hindered phenol with di-*tert*-butyl groups in the phenolic ring, representing an extremely effective primary antioxidant (Reische et al., 2008). Owing to its high fat-solubility, BHA is extensively used in bulk oils as well as oil-in-water emulsions. Some of its main applications include the preservation of soybean and palm oil in cereal and confectionery products (nevertheless, TBHQ is more effective in vegetable oils than BHA). Likewise, it is widely employed in baking products, owing to its thermal stability and mild alkaline conditions. However, it is not equally suitable to be applied in frying due to its volatility (Yehye et al., 2015). Furthermore, BHA might act as a co-antioxidant (CoAH) by regenerating other antioxidants such as BHT or  $\alpha$ -tocopherol (De Guzman et al., 2009).

From a metabolic point of view, BHA is rapidly absorbed from the gastrointestinal tract, metabolized and excreted mainly in urine and/or feces. The major metabolites of BHA are glucuronides, sulphates and free phenols (including TBHQ) in proportions that vary from species to species. BHA and its metabolite TBHQ were reported as inducing chromosomal aberrations *in vitro*, but this clastogenic activity was recognized as being an indirect effect resulting from the formation of reactive oxygen species via pro-oxidant chemistry (EFSA, 2011).

In terms of toxicity, BHA present low acute toxicity (with LD<sub>50</sub> in mouse and rat higher than 2000 mg/kg bw/day), having also been reported as having no potential to induce point mutations or to interact with or damage DNA, as reported by different genotoxicity studies. However, after a large number of long-term toxicity and carcinogenicity studies, BHA was demonstrated to induce proliferative changes in the forestomach, including epithelial hyperplasia, papilloma and carcinomas. Nevertheless, and since humans don't have a forestomach, the forestomach hyperplasia in rodents is hardly considered relevant for human risk assessment (EFSA, 2011).

From a legal standpoint, an European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources added to Food (ANS) re-evaluated recently the safety of BHA (E320). Based on a no-observed-adverse-effect level (NOAEL) of 100 mg/kg bw/day for growth retardation, increased mortality and behavioral effects in rat pups at higher dose levels, and using an uncertainty factor of 100, the Panel established an acceptable daily intake (ADI) of 1.0 mg/kg bw/day. This NOAEL also covers the lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence (BMDL<sub>10</sub>) values for forestomach hyperplasia observed in rat, which was established as 0.5 mg/kg bw/day, based on proliferative changes in the rat forestomach. The Panel also concluded that, at the current levels of use, refined intake estimates are generally below the ADI of 1.0 mg/kg bw/day and that BHA does not represent a major threat with respect to genotoxicity, as indicated by a large number of long-term toxicity and carcinogenicity studies, demonstrating proliferative changes in the rat forestomach with values of 115 mg/kg bw/day (EFSA, 2011).

In related studies, a NOAEL of 0.125% BHA in the diet (equivalent to a dose of at least 100 mg/kg bw/day) was reported for reproductive and developmental toxicity in rats, while a NOAEL of 400 mg/kg bw/day was calculated for reproductive and teratogenic parameters in rabbits (EFSA, 2011).

## Butylated Hydroxytoluene

This compound is one of the antioxidants with higher use in food industry, especially in low-fat foods, fish products, packaging materials, paraffin, and mineral oils (in all food products in which BHT, BHA or TBHQ are not appropriate, the usual alternative is PG). BHT is a white crystalline solid with properties similar to BHA. Despite being resistant to thermal treatment, it is not as stable as BHA. BHT has been documented in thousands of scientific journals, patents, general reviews and conferences, and has been approved for use in foods and food packaging in low concentrations by FDA since 1954, being also recognized as safe for use in foods (Yehye et al., 2015).

In view of its widespread use as a food preservative, its biochemical properties have been studied extensively, mainly to obtain unequivocal information about its possible toxicity. Among other relevant data, it has been reported that large doses of BHT produce centrilobular necrosis, increased serum transaminase activities, hemorrhage in the liver, increased mitotic activity of hepatocytes in rats and cats, potentially promoting hepatocarcinogenesis (Yehye et al., 2015).

However, and despite having been described as a potential liver carcinogen in rat, there are no significant evidences indicating hepatocarcinogenic potential for BHT in either rats or mice. In addition, BHT showed capacity to inhibit chemical carcinogenesis in various organs when fed before or concurrently with specific carcinogens, being also a potent inhibitor of lipid enveloped viruses (Yehye et al., 2015).

Similarly to BHA, BHT was recently re-evaluated by an EFSA ANS Panel. Based on previously established ADI values of 0–0.05 mg/kg bw/day, and considering a NOAEL of 25 mg/kg bw/day and a 100-fold safety margin, the Panel derived an ADI of 0.25 mg/kg bw/day for BHT based on thyroid, reproduction and hematological effects in the rat (EFSA, 2012).

In addition, the established NOAEL (25 mg/kg bw/day) is much lower than 247 mg/kg bw/day, the BMDL<sub>10</sub> derived from data for the incidence of hepatocellular carcinomas in male rats (also observed in long term studies with BHT). Likewise, the exposure of adults to BHT is unlikely to exceed the recently established derived ADI, either at the mean or the 95th percentile. Even using a worst-case scenario of combined exposure to BHT, the estimated dose for adults would be 0.01–0.03 mg/kg bw/day, on average, and 0.03–0.17 mg/kg bw/day at the 95th percentile. However, regarding the exposure of children to BHT (mainly as a food additive), and despite it is also unlikely that the ADI is exceeded at the mean (considering an estimated potential average exposure in the

range of 0.01–0.09 mg/kg bw/day), it surpasses the 95th percentile in some European countries, like Finland or The Netherlands, as an exposure in the range of 0.05–0.30 mg/kg bw/day was estimated at the 95th percentile (EFSA, 2012).

Regarding the acute toxicity, BHT presents relatively low levels, with oral LD<sub>50</sub> values of 1700–1970 mg BHT/kg bw in rats, 2100–3200 mg BHT/kg bw in rabbits, 10700 mg BHT/kg bw in guinea pigs, 940–2100 mg BHT/kg bw in cats, and 2000 mg BHT/kg bw in mice. In general, BHT is not of concern with respect to genotoxicity and any carcinogenicity should be thresholded. In fact, the genotoxicity studies on BHT indicate no potential to induce point mutations, chromosomal aberrations, nor damage or interact with DNA (EFSA, 2012).

Considering the high dissemination of BHT as a food additive, different studies pertaining its absorption, distribution, metabolism and excretion have been performed in mice, rats, rabbits, chickens, monkeys and humans. Overall, the obtained results showed that BHT is rapidly absorbed from the gastrointestinal tract and further metabolized in a complex way, which might be different among species. It is not known, for example, if human organism forms the quinone methides that were found in rats and mice as BHT metabolites (EFSA, 2012).

These conclusions are certainly relevant to maintain BHT as one of the most popular antioxidants, being also used in combination with other antioxidants such as BHA, propyl gallate, and citric acid for the stabilization of oils and high-fat foods (Yehye et al., 2015).

## Conclusions and Future Tendencies

The advantages of using antioxidant compounds in food products are obvious, and their employment seems to be indispensable, but the total absence of potential toxic effect is yet to be validated, thereby requiring careful studies on their direct and side-effects.

Despite the necessity to adequate each antioxidant to its specific use, it might be generally stated that the antioxidants to be used in foods should be economic (or at least representing a minor impact on the product's cost), non-toxic, efficient at low concentrations, easy to incorporate, able to maintain their integrity throughout food processing, stable at least as long as the product shelf-life and capable of exert their action without causing undesirable changes in the organoleptic properties of food. However, there is no antioxidant compound that fulfil all the previous criteria. Even BHA and BHT, which are the most used antioxidants and recognized as being highly effective, present some limitations. For instance, both compounds have a slightly unpleasant odor, especially under high processing temperatures.

Therefore, there is an intensive search for completely effective compounds, aiming to combine multiple functions in a synergistic way. Considering all presented data, it seems quite reasonable to expect that the field of antioxidant research will remain rather active in next years.

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## Further Reading

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# Synthetic Food Colors

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## Introduction

Color plays an essential factor in a food's acceptability and palatability. Color additives are routinely added to foods to enhance their appearance, compensate for natural variations in raw materials or color loss during processing, and assign color/flavor identity to otherwise colorless food products. Since the discovery of the first synthetic color, a purple aniline derivative dubbed "mauveine" or "mauve", by William Henry Perkin in 1856, the food industry has historically favored synthetic food colors over natural colorants. This preference is based on synthetic colors' low production cost, vibrancy, no or limited contribution to flavor and/or aroma in the finished product, high efficacy at low concentrations, and superior chemical stability to processing, distribution, and storage conditions such as heat, low pH, light or oxygen. Despite the technical advantages that synthetic colors provide, concerns about adverse health effects, consumer growing demands for natural alternatives, and misuse and adulteration have driven the strict regulation of their use in foods and their on-going replacement or elimination from the food supply.

## Main Synthetic Food Colors in Foods

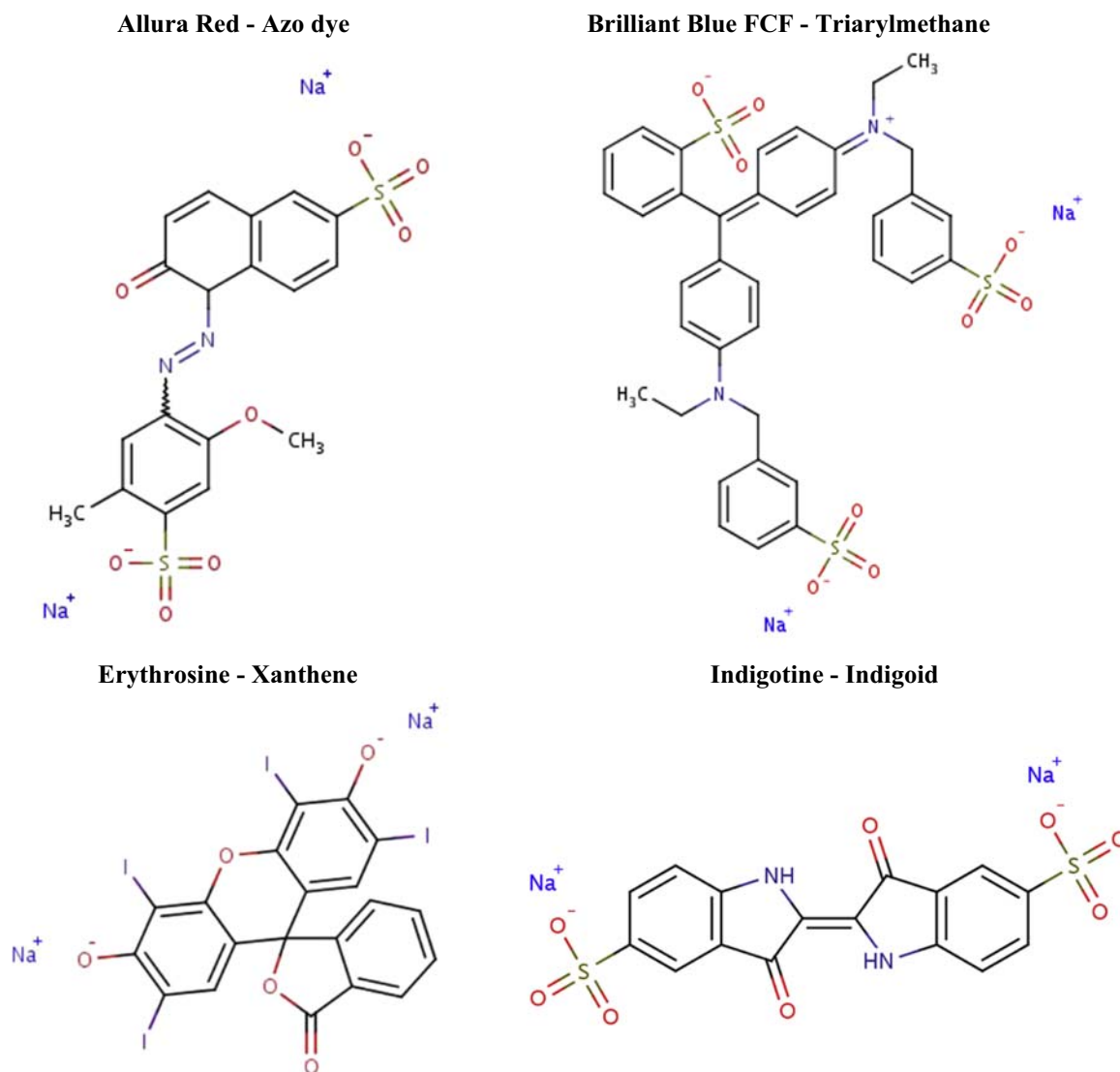
The term "synthetic" or "artificial color" is applied to coloring agents that are not nature-identical and have been obtained by chemical synthesis. Chemical modified natural colors, e.g., copper chlorophyllin, or natural colors homologues obtained using chemical synthesis, e.g., nature-identical beta carotenes, are not typically included within the synthetic colors category. The most commonly used synthetic food colorants are mono- and di-azo dyes. More than half of the synthetic dyes approved for food use in the United States (US) and the European Union (EU) belong to this category. Azo food dyes are obtained from the diazotization of an aromatic primary amine and the subsequent coupling of the diazonium salt with a phenol or an aromatic amine. They contain at least one functional azo group with the structure  $R-N=N-R'$  where the pendant R groups are bulky aromatic rings functionalized with large charged groups, which contribute to their coloration and solubility (See Fig. 1). Triarylmethane derivatives, xanthene dyes, quinophthalones and indigoid compounds constitute the rest of the synthetic food colorants approved for foods. Erythrosine, the only xanthene dye accepted for use in food in the US and the EU, and triarylmethane dyes are prepared by condensing a substituted phenol, e.g., resorcinol in the case of erythrosine, and phthalic anhydride. To obtain erythrosine, a halogenation reaction follows. Similarly, Quinoline Yellow, the principal quinophthalone dye used in food products, is synthesized from phthalic anhydride and quinaldine. In the case of the indigoid compounds, the Heumann-Pfleger process is the most important synthetic pathway to obtain indigotine from N-phenylglycine and sodium amide. The extensive  $\pi$ -conjugated structures that are obtained from these procedures confers all these compounds with a vivid coloration as well as photophysical properties that allow for their detection. Currently, 9 and 15 artificial colors belonging to these groups are approved for use in the US and the EU, respectively. Table 1 summarizes their chemical characteristics, common designations, stability, and applications.

The selection of these colors for specific food applications relies on their compatibility and solubility within the target food product. Approved azo dyes are typically commercialized as their sodium salts and are hydrosoluble, except for Citrus Red 2 that is liposoluble and Litholrubine, which disperses in oils. The permitted triarylmethane, xanthene, and indigoid derived compounds are also water soluble. The complexation of these hydrosoluble dyes with an inorganic substrate, e.g., a metal cation such as aluminum, produces lakes with enhanced light and chemical stability, as well as dispersibility in oils. Quinoline Yellow is soluble in water and slightly soluble in organic solvents.

## Uses and Functionality

Among the azo food dyes, Allura red AC, Azorubine, Brilliant Blue FCF, Sunset Yellow FCF and Tartrazine are the most commonly found in edible goods. Due to restricted functionality and/or regulatory requirements, the use of other azo dyes such as Amaranth, Citrus Red 2, Litholrubine BK, Ponceau 4R, and Orange B have been limited to specific applications (see Table 1). For example, Citrus Red 2 is only applied to enhance the external appearance of fresh oranges. Among the triarylmethane dyes, Fast Green FCF is the least used, since similar hues can be obtained using a combination of other colors. The extensive use of the only xanthene compound approved in the US and the EU, erythrosine, has progressively decreased in recent years due to its lower stability in comparison to other available red synthetic food dyes and health concerns associated with its use.

While several natural color additives, such as riboflavin and anthocyanins, contribute the food with desirable functional properties, e.g., antioxidant and bacteriostatic activity, or potential health benefits, synthetic colors do not exhibit any nutritional value or health attribute. Most of them are poorly absorbed in the intestine, and their consumption has been potentially associated with adverse health effects, such as allergenicity and behavioral disorders in children. Additionally, azo and triarylmethane color compounds can electrostatically interact with proteins, which might reduce protein functionality in foods and in the body.



**Figure 1** Molecular Structures of Selected Synthetic Dyes.

Despite their lack of nutritional value, these colors are gaining popularity as stains, markers or intrinsic sensors. Their comparative innocuity makes them adequate replacements of toxic dyes in diagnostic and analytical tools, such as Fast Green FCF as a safe stain for tumor detection, azo dyes as low-cost tracing dyes for the analysis of nucleic acids and proteins, and Allura Red AC as an effective scatterer in luminescence spectroscopy. Additionally, the environmental sensitivity of the photophysical properties, i.e., luminescence intensity, quantum yield and lifetime, of approved food azo dyes and triarylmethane compounds have been recently reported, and their potential application as intrinsic sensors of food quality attributes, e.g., micro and bulk viscosity of liquid and semi-solid foods has been described. Similarly, the use of xanthene dyes as sensors of molecular mobility, dynamic heterogeneity, and air permeability has been extensively explored and used in food model systems to evaluate and characterize them.

### Analysis of Synthetic Colors in Foods

Several analytical methods have been developed to determine the purity of synthetic colors and detect their presence in food matrices. The extraction of the dyes from the food matrices is crucial to their appropriate assessment, which can be accomplished using liquid-liquid, solid-phase, ultrasound-assisted and cloud-point extraction methods, for example. Spectrophotometry has been traditionally used to determine the dye content and the authenticity of food additives based in their characteristic absorbance and extinction coefficients, for example, absorbances at 526, 540, and 625 nm are used for erythrosine, Allura Red AC, and Fast

**Table 1** Commons designations, color provided, stability, and main uses of synthetic food colors

Type of compound	Common designations & Molecular formula	Color provided	Stability	Main applications
Azo dyes	Allura Red AC FD&C Red # 40, E129 $C_{18}H_{14}N_2Na_2O_8S_2$	Orange/Red	Highly stable to light, heat, acid. Degraded by reducing and oxidizing agents	Baked goods, beverages, candies, desserts, extruded foods, salad dressings
	Amaranth FD&C Red # 2, E123 $C_{20}H_{11}N_2Na_3O_{10}S$	Bluish Red	Highly stable to light, heat, and acid. Ascorbic acid, $SO_2$ , and alkali reduce its stability	Beverages, confectionary products, preserves
	Azorubine Carmoisine, E 122 $C_{20}H_{12}N_2Na_2O_7S_2$	Magenta Red	Highly stable to light, heat, and acid. Degraded by reducing agents	Chocolates, confectionary products, salad dressings, beverages
	Brilliant Black BN Food Black 1, E151 $C_{28}H_{17}N_5Na_4O_{14}S_4$	Purple/Deep Blue	Medium stability to light. Low stability to heat. Ascorbic acid and $SO_2$ reduce its stability	Desserts, dairy and confectionary products, fish roe
	Brown HT Food Brown 3, E155 $C_{27}H_{20}N_4O_9S_2$	Reddish Brown	Highly stable to light and heat. Stability reduced by oxygen and $SO_2$	Confectionery and baked goods
	Citrus Red 2 E121 $C_{18}H_{16}N_2O_3$	Orange/Red	Highly stable to heat.	In the US, limited to fresh orange surfaces (<2 ppm)
	Litholrubine BK D&C Red # 6, E180 $C_{18}H_{12}N_2Na_2O_6S$	Orange/Red	Highly stable to light and heat.	In the EU, limited to rind of hard cheeses
	Orange B C.I. Acid Red 137 $C_{22}H_{16}N_4Na_2O_9S_2$	Orange/Red	–	In the US, limited to hot dogs and sausages casings (<150 ppm)
	Ponceau 4R C.I. Acid Red 18, E124 $C_{20}H_{11}N_2Na_3O_{10}S_3$	Bright Red	Medium stability to light and acid. Ascorbic acid and $SO_2$ reduce its stability	Confectionery products, beverages, canned fruits, jellies, soups
	Sunset Yellow FCF FD&C Yellow #6, E110 $C_{16}H_{10}N_2Na_2O_7S_2$	Yellow/Orange	Highly stable to light, heat, and acid. Ascorbic acid and $SO_2$ reduce its stability	Baked goods, beverages, desserts, extruded foods, confectionary products
	Tartrazine FD&C Yellow # 5, E102 $C_{16}H_9N_4Na_3O_9S_2$	Greenish Yellow	Highly stable to light, heat, and acid. Ascorbic acid and $SO_2$ reduce its stability	Baked goods, candy, desserts, jellies, pickles, sauces, ice creams
Xanthene dye	Erythrosine, FD&C Red # 3, E127 $C_{20}H_{6}I_4Na_2O_5H_2O$	Bright Pink/Red	Medium stability to heat. Susceptible to photodegradation. Precipitates at pH < 4.5	Canned foods, cocktail cherries, dairy products, desserts
Triaryl methane derivatives	Brilliant Blue FCF FD&C Blue # 1, E133 $C_{37}H_{34}N_2Na_2O_9S_3$	Blue Greenish blue (pH < 3)	Medium stability to heat, light, and acid. Sensitive to oxygen	Beverages, candies, dairy products, jellies, condiments, syrups
	Fast Green FCF FD&C Green # 3, E143 $C_{37}H_{34}N_2Na_2O_{10}S_3$	Teal to Blue (low to high pH)	Medium stability to light and acid. Sensitive to oxygen	Beverages, candy, dairy products, puddings
	Green S FD&C Green # 4, E142 $C_{27}H_{25}N_2NaO_7S_2$	Green/Blue	Highly stable to heat and acid. Medium stability to light	Canned vegetables, desserts, sauces
	Patent Blue V Food Blue 5, E131 $C_{27}H_{31}N_2O_7S_2Ca_{1/2}$	Turquoise Blue	Highly stable to light and heat. Acids, ascorbic acid, and $SO_2$ reduce its stability	Beverages, canned products, baked goods, jellies
	Indigotine FD&C Blue # 2, E132 $C_{16}H_8N_2Na_2O_8S_2$	Blue (pH < 11), Yellow (pH > 11)	Low stability to heat and acid. Oxygen, $SO_2$ and ascorbic acid reduce its stability	Baked goods, desserts, candies, confectionary products
Quinophthalone	Quinoline Yellow D&C Yellow # 11, E104 $C_{18}H_9N Na_2O_8S_2$	Greenish Yellow	Medium stability to heat, light, and acid.	Beverages, juices, ice cream

Reproduced from Emerton (2008), Griffiths (2005), Carocho et al. (2014), and Martins et al. (2016).

Green FCF, respectively. Currently, the use of chemometric tools has expanded the use of spectrophotometric methods permitting the simultaneous determination of numerous artificial dyes. Due to their sensitivity and reliability, chromatographic techniques, particularly, thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), ion-pair liquid chromatography, and reversed-phase high performance liquid chromatography (RP-HPLC), equipped with adequate detection systems, e.g. diode-

array (DAD), are routinely used to quantify the levels of permitted food colors, verify their authenticity and potential adulteration, and identify the presence of banned food dyes. The utilization of liquid chromatography-tandem mass spectrometry (LC-MS/MS) allows for multiple identification of food dyes with increasing accuracy. Adsorptive voltammetry, differential pulse polarography, and capillary electrophoresis techniques have also been reported as adequate tools to monitor food dyes content and quality. Enzyme-linked immunosorbent assays (ELISA) provide convenience for this kind of tests. ELISA kits are available to detect approved synthetic dyes such as Sunset Yellow FCF and Tartrazine and banned artificial colors such as Sudan I, a frequent adulterant in chili powders, and rhodamine B. Electrochemical methods, for example, detection of Tartrazine with a boron-doped diamond electrode, provide reasonable sensitivity at a low cost. The development of fluorescence-based sensors and surface enhanced Raman spectroscopy (SERS) techniques have received attention due to their high sensitivity, convenience, rapid response, and low cost. It should be noted that the development of in-situ, fast, and economic multi-colorant screening and confirmatory techniques has been identified as a priority by regulatory organisms around the world to ensure compliance with current specifications regarding the application of synthetic food dyes in approved foods, quantity used, purity, and potential adulteration.

## Regulatory Status and Labeling

Although the use of synthetic colors is highly regulated, and their permitted uses and doses are continually being re-evaluated, their status, acceptable daily intake (ADI), applications, and labeling requirements differ significantly among countries. Despite these discrepancies, most regulations list the color additives that are approved and to which extent and form they can be incorporated into foods. The specifications of the Codex Alimentarius or the Joint FAO (Food and Agricultural Organization)/WHO (World Health Organization) Expert Committee on Food Additives (JECFA) are used by most countries to guide their use and set allowable doses in foods.

In the United States, the Food and Drug Administration (FDA) determines their regulatory status. The FDA regulations allow for the classification of food colors into two categories, namely certified and exempt from certification. All permitted synthetic food colors fall within the former categorization, and as such, when produced, they should be assessed for identity and purity prior to their commercialization. All certified food colors, i.e., synthetic colors, should also be listed on the label of the food product and in the case of Tartrazine, since it is considered a potential allergen, it should be declared as such on the label. The safety evaluation of these dyes is based on toxicological data, chemical structure and potential exposure.

In the EU, the European Food Safety Authority (EFSA) is responsible for the evaluation of the safety of synthetic colors and establishes their ADI. It also requires that the synthetic color name or its corresponding additive identification number, i.e., E number, is stated in the label within the ingredient list. Additionally, if any of the six synthetic colors identified as potential causative agents of behavioral problems in children, i.e., Allura Red AC, Azorubine, Ponceau 4R, Quinoline Yellow, Sunset Yellow FCF, and Tartrazine, is present in the food product, its label should include a warning that indicates that adverse effects on activity and attention may arise. Within the EU, the Rapid Alert System for Food and Feed (RASFF) facilitates the exchange of information on risks associated to foods. Regarding food colors, this system issues notifications about the detection of illegal colors, banned or unauthorized use of these components, and failure to declare a food dye in the label in inspected food products. This system also keeps records of the frequency of each infraction and provides an additional level of safety to the consumer. Recently, in the United Kingdom, a voluntary action of the food industry has been instrumental in the removal of these colors from foods and their replacement with natural colors by most major food manufacturers.

The acceptable daily intakes (ADI) for synthetic colors currently authorized in United States (US) and the European Union (EU) are summarized in [Table 2](#).

## Health Concerns

Allergenicity, behavioral problems such as hyperactivity syndrome in children, neurotoxicity, genotoxicity, and carcinogenicity are some of the potential adverse health effects that have been linked to synthetic food colors. Concerns about the safety of these additives from the general public, food additive regulatory organisms, and the food industry currently drive the continuous re-evaluation of the potential risks and the exposure to colorants for specific populations such as children.

Genotoxic and carcinogenic effects have not been consistently reported for approved food synthetic dyes, however the similarity of their metabolic products to potentially carcinogenic or genotoxic compounds has fueled extensive, although for some dyes inconclusive, evaluations. The *in vivo* reduction of azo-dyes by the intestinal flora results in the formation of sulfonated aromatic amines such as 1-amino-2-naphthol-6- sulfonic acid and sulfanilic acid. The genotoxicity and carcinogenicity of the unsulfonated analogues of these compounds have been demonstrated and consequently has caused severe scrutiny of the potential effects of the ingestion of food dyes that can be metabolized into aromatic amines. A recent study on the genotoxicity of Allura Red AC and their metabolic products, i.e., sulfonated naphthylamines, was required in 2016 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The lack of genotoxicity of the sulfonates metabolites of Allura Red AC was corroborated, and it was proposed that due to the molecular structural resemblance of Allura Red AC to other permitted azo dyes, this conclusion could be extended to the whole category. Similarly, no genotoxic activity was found for Sunset Yellow in *in vivo* and *in vitro* studies. However, in the case of Tartrazine, its metabolic products, i.e., sulfanilic acid and aminopyrazolone, have been linked to the formation of reactive oxygen species (ROS) which can affect renal and hepatic tissues.

**Table 2** Acceptable daily intake (ADI)<sup>a</sup> and regulatory status of commonly used synthetic food colors in the United States (US) and The European Union (EU)

Synthetic Color	Acceptable daily intake (mg kg bw <sup>-1</sup> per day) <sup>a</sup>	Regulatory status	
		US	EU
Allura Red AC	0–7	Approved	Approved
Amaranth	0–0.5	Not Approved	Approved
Azorubine	0–4	Not Approved	Approved
Brilliant Black BN	0–1	Not Approved	Approved
Brilliant Blue FCF	0–6	Approved	Approved
Brown HT	0–1.5	Not Approved	Approved
Citrus Red	Not to be used	Approved- Limited applications	Not Approved
Erythrosine	0–0.1, 0–2.5 (US)	Approved	Approved
Fast Green FCF	0–25, 0–2.5 (US)	Approved	Not Approved
Green S	No ADI allocated, 0–5 (EU)	Not Approved	Approved
Indigotine	0–5, 0–2.5 (US)	Approved	Approved
Litholrubine BK	No ADI allocated, 0–1.5 (EU)	Not Approved	Approved - Limited Applications
Orange B	–	Approved - Limited Applications	Not Approved
Patent Blue V	No ADI allocated, 0–15 (EU)	Not Approved	Approved
Ponceau 4R	0–4	Not Approved	Approved
Quinoline Yellow	0–3	Not Approved	Approved
Sunset Yellow FCF	0–4, 0–3.75 (US)	Approved	Approved
Tartrazine	0–10, 0–5 (US), 0–7.5 (EU)	Approved	Approved

<sup>a</sup>Values reported as established by the Joint FAO (Food and Agricultural Organization)/WHO (World Health Organization) Expert Committee on Food Additives (JEFCA, <http://apps.who.int/food-additives-contaminants-jecfa-database/search.aspx?fc=31>). Alternative values used by US or EU are specified between brackets when needed.

Compiled from Letho et al. (2017), Feketea and Tsabouri (2017), US FDA ([www.fda.gov](http://www.fda.gov)) and EFSA ([www.efsa.europa.eu](http://www.efsa.europa.eu))

Allergic and pseudo-allergic adverse reactions to synthetic dyes are non-immunoglobulin E (non-IgE) mediated. These reactions are rare in the general population; however, they are a frequent cause of mild and moderate clinical manifestations, e.g., urticaria, facial flushing, itching, and angioedema, in hypersensitive individuals. Tartrazine has been consistently correlated with intolerance and allergic reactions in atopic populations and Patent Blue has been documented as a causative agent of allergies in adults.

Since the 1970s, the potential adverse effects of synthetic food dyes and behavioral disorders in children, i.e., hyperactivity, sleep disorders, irritability, and learning disabilities, have been studied. In 2007, the Southampton study conducted by McCann and others revived the attention to this topic. Links between a significant reduction in attention deficit and hyperactivity disorder (ADHD) symptoms due to the withdrawal of synthetic dyes from the diet have been reported and required further investigation. Both the EFSA and the FDA consider that the present evidence does not conclusively sustain the relation between consumption of food dyes and behavioral disorders. However, the EFSA has modified its labeling requirements to include a warning on potential effects of synthetic dye consumption on children behavior (see section **Regulatory Status and Labeling**). The FDA did not revise its labeling requirements but requested an exposure assessment of the population to the seven permitted synthetic dyes in the US. Estimations of additive intakes suggest that the average intake for all permitted synthetic food colors are below their corresponding ADI, however, for high consumers of artificially colored products, the estimations indicate that the intake for Tartrazine, Sunset Yellow, and Erythrosine might exceed their corresponding acceptable limits.

## Concluding Remarks

The replacement of synthetic colors in food products by natural alternatives, guided by consumer demands for cleaner labels and the public perception that natural compounds are safer than artificial ones, will continue. Attaining the same attributes conferred by synthetic colors to foods using natural colors is challenging and slows down this inevitable process. This trend will shift the use of synthetic colors from being the most prevalent in foods to the least desirable in the future.

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# Encyclopedia of Food Chemistry: Water

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## Glossary

**Bound Water** Bound or vicinal water interacts very closely with ions, solutes, proteins and carbohydrates and does not significantly contribute to the water activity.

**Capillary Water** Capillary water is more mobile than bound water and aids in deleterious reactions in foods supporting microbial growth and aiding in oxidative and enzymatic pathways.

**Bulk Water** Free or bulk water has no hindrance of molecular mobility and is readily available to partake in deleterious microbial and enzymatic reactions.

**Chaotropes** Chaotropes are solutes that interfere with the tetrahedral hydrogen bonding networks of water.

**Kosmotropes** Kosmotropes are solutes that increase the tetrahedral hydrogen bonding networks of Water–Water interactions.

**Glass transitions** A glass is a non-equilibrium, metastable, amorphous solid with an apparent viscosity greater than  $10^{12}$  Pas.

## Introduction to Water

The bent configuration and dipole moment causes water molecules to attract each other due to the presence of the partial charges on the oxygen and hydrogen atoms leading to intermolecular hydrogen bonding (average bond energies between 5 and 40 kJ/mol). The interaction between a positively charged hydrogen atom on one molecule of water with a negatively charged atom on a different water molecule neutralizes the charges and is the basis of hydrogen bond between two molecules of water (Pauling, 1945).

Water exists in numerous states, if water is present as an isotope it can vary the interactions between water molecules. For example, if an isotope of oxygen (i.e.,  $^{17}\text{O}$  or  $^{18}\text{O}$ ) is present than the molecule becomes more electronegative and the hydrogen bonding is stronger. If deuterated water ( $^2\text{H}$ ) is present the hydrogen bonding is weaker because more positive protons are present in the hydrogen atom to attract the electrons, reducing the dipole moment. When two water molecules hydrogen bond, one of the water molecules partially donates a hydrogen atom to the other. For the molecule which donates its hydrogen, the lone pair of electrons, on the oxygen atom, becomes increasingly electronegative. This increased electronegativity amplifies the strength of the dipole of the water molecule which is donating its hydrogen, causing a cooperative interaction between the next water molecule which hydrogen bonds (i.e., a stronger hydrogen bond) with the next incoming water molecule (Bartha et al., 2003). The other water molecule which accepts the hydrogen atom attains a greater positive charge, which results in the lone pair of electron being less electronegative causing the next water molecule to interact less strongly with this water molecule. This type of hydrogen bond is termed anti-cooperative hydrogen bonding (Bartha et al., 2003). Hence, in bulk water, there is a distribution in hydrogen bond distance, strength and angle due to the complex interactions between molecules.

## Ionization of Water

Typically, water exists with a molecular formula,  $\text{H}_2\text{O}$ , however it is capable of endothermically ionizing to form  $\text{OH}^-$  and  $\text{H}_3\text{O}^+$ . The generation of ions is due to an excited O–H stretching overtone vibration (Bakker and Nienhuys, 2002). Once these ions are generated, typically they are unstable and recombine to form  $\text{H}_2\text{O}$  in a few femtoseconds. Hence, water reaches equilibrium with an autoionization constant  $K_w$ :



$$K_w = [\text{OH}^-][\text{H}_3\text{O}^+]$$

$K_w$  represents an equilibrium constant between the formation of water and hydroxyl ions ( $\text{OH}^-$ ) and hydronium ions ( $\text{H}_3\text{O}^+$  which is often simplified to  $\text{H}^+$ ). Any changes to the system, including addition of acid products and changes in temperature, adjusts the balance between water and the hydroxyl and hydronium ions according to Le Chatelier's principle. It is possible that the  $\text{OH}^-$  and  $\text{H}^+$  interact independently with other water molecules and form stable hydrates. This has effects on the native structure of water because they are capable of forming hydrogen-ionic bonds which are stronger than typical hydrogen–hydrogen bonds which increase the order of liquid water. The ability of water to dissociate is a function of the temperature (Table 1).

Although we often assume that the pH of pure water is always 7, this is not true because the autoionization constant is a function of temperature. This does not mean that the solution is basic at low temperatures and acidic at elevated temperatures. For a system

**Table 1** Autoionization constants for water at different temperatures

Temperature (K)	$K_w$	pH
273	$0.11 \times 10^{-14}$	7.47
283	$0.68 \times 10^{-14}$	7.27
288	$1.00 \times 10^{-14}$	7.00
313	$2.92 \times 10^{-14}$	6.77
373	$51.3 \times 10^{-14}$	6.14

to be acidic, there has to be an excess of hydronium ions compared to hydroxyl ions. In pure water, these are always equal. Commonly, we refer to basic systems as having  $\text{pH} > 7$  and acidic systems having  $\text{pH} < 7$ . This is only true at 25 °C. Hence, if the system is at 100 °C, an acidic solution would have  $\text{pH} < 6.14$ . If pure water is at 100 °C with a  $\text{pH} = 7$ , it would be considered slightly basic. Using  $\text{pH} = 7$  as the distinction between acidic and basic properties for pure water only applies when water is at 25 °C.

## Physical Properties of Water

It is the bent configuration and dipole moment of water that gives rise to the numerous very interesting and unique properties of water. For example, water takes up a smaller volume and is lighter than other atmospheric molecules including  $\text{O}_2$ ,  $\text{N}_2$ , Ar,  $\text{CO}_2$ . If molecules are symmetrical (i.e., group IV hydrides  $\text{CH}_4$ ,  $\text{SiH}_4$ ,  $\text{GeH}_4$ ,  $\text{SnH}_4$ ), they are nonpolar and rely on van der Waals forces for adhesion forces. This results in significantly lower melting and boiling points. The group VI hydrides (i.e.,  $\text{H}_2\text{O}$ ,  $\text{H}_2\text{S}$ ,  $\text{H}_2\text{Se}$ , and  $\text{H}_2\text{Te}$ ) lack symmetry due to the two lone electron pairs in their valence shells and each should be capable of hydrogen bonding. Group VI hydrides demonstrate some interesting properties, if the boiling point was a function of only London dispersion forces,  $\text{H}_2\text{Te}$  would have the highest boiling point when in fact it has the lowest boiling point.  $\text{H}_2\text{Te}$  boils at  $-2^\circ\text{C}$ ,  $\text{H}_2\text{Se}$  at  $-42^\circ\text{C}$ ,  $\text{H}_2\text{S}$  at  $-60^\circ\text{C}$  and  $\text{H}_2\text{O}$  would be predicted to boil below  $-60^\circ\text{C}$ . However, the unusual ability of  $\text{H}_2\text{O}$  to hydrogen bond causes water to boil at 100 °C.

Changing the temperature modifies the dipole moment of the molecule which results in changes to the length of the hydrogen bond which is directly correlated with the strength of the hydrogen bond (Dougherty, 1998). An increase in hydrogen bonding leads to increased cohesion, dielectric constant, specific heat, and viscosity; while a decrease in hydrogen bonding leads to a decrease in adhesion forces, density, diffusion coefficient, and thermal conductivity (Table 2). As the temperature is reduced to approximately 4 °C there is a maximum in hydrogen bonding strength resulting in the highest possible density for water. As a food product is frozen, water turns to ice at the surface and then continues to freeze inwards. As the layer of ice builds at the surface of the material it begins to freeze quicker, this is due to an increase in the thermal diffusivity and thermal conductivity. However, as a food product is thawed, the ice turns to water at the surface which then acts as an insulator resulting in significantly longer thawing than freezing times.

## Types of Water

Water is typically subdivided into three categories which include bound, capillary and bulk water. Each of these types of water behaves and influences the product very differently. Typically bound or vicinal water interacts very closely with ions, solutes, proteins and carbohydrates. This water is required for proteins to fold correctly and is tightly bound to the macromolecule or solute. When drying foods or when measuring water content, this water is not removed or accounted for because it is bound so tightly via hydrogen bonding or dipole–dipole interactions with ions that it does not behave as free water. Bound water is unable to partake in chemical reactions and no added molecular mobility is attained from the presences of this water.

Capillary water is far more mobile than bound water and aids in deleterious reactions in foods supporting microbial growth and aiding in oxidative and enzymatic pathways. Although this water does have limited molecular mobility due to the confinement induced by the capillary walls, there is sufficient mobility to support diffusion of waste and nutrients, respiratory gases and enzymes.

**Table 2** Physical properties of water

Temperature (°C)	Density ( $\text{kg/m}^3$ )	Thermal conductivity ( $\text{W/m}^2\text{C}$ )	Thermal diffusivity ( $\times 10^{-6} \text{ m}^2/\text{s}$ )	Absolute viscosity ( $\times 10^{-6} \text{ Pas}$ )
20	998.2	0.597	0.143	993.414
5	1000	0.568	0.135	1534.74
0 (water)	999.9	0.558	0.131	1793.63
0 (ice)	916.2	2.22	11.82	N/A
−7	917.8	2.27	12.23	N/A
−18	919.4	2.32	12.91	N/A

Adapted from Dickerson (1969) and Raznjevic (1976).

During drying, capillary water may be removed via vigorous processing and occurs during the falling rate period which is controlled by the rate of removal of moisture from the interior and is often referred to as being “mass-transfer limited” (Onwude et al., 2016).

Finally, free or bulk water has no hindrance of molecular mobility and is readily available to partake in deleterious microbial and enzymatic reactions. This water is very volatile and is rapidly removed during processing and is removed during the constant rate period.

## Water – Ion Interactions

Addition of small amount of ions (i.e., salts) to water affects the interactions between water molecules. Since the strength of hydrogen – ionic bonds is stronger than hydrogen – hydrogen bonds, water will associate with ions before they interact with another water molecule. The native tetrahedral structure of water is disrupted by the addition of salt. When NaCl is hydrated water molecules interact such that the slight positive charge on hydrogen atom in water non-covalently interacts with the negatively charged chlorine and the negatively charged oxygen atom in water interacts with the sodium. Regardless of the ion added the native structure of water is always altered.

There are two possible outcomes when different salts with different polarizing powers are added to water. The polarizing potential is a function of the charge versus the molecular radius. Small and/or highly charged ions (i.e.,  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{H}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , etc.) have a high charge density (strong electric field for a small atomic volume) and are therefore “structure formers” or kosmotropes. The high charge density of these ions causes water molecules to become highly structured upon solvation of the ion, creating multi-layers of up to six mono-layers of water. Structure formers modify the structure of water such that the viscosity of the ion solution is greater than pure water. On the other hand, if low charge density ions (i.e., an ion with a low charge and large atomic volume (i.e.,  $\text{K}^+$ ,  $\text{NH}_4^+$ ,  $\text{Cl}^-$ ,  $\text{I}^-$ , etc.) are added to pure water they tend to break, or disrupt the structure of water, and are thus called “structure breakers” or chaotropes. The disruption the water–water interactions is not compensated by the new interactions with the ion in solution such that a solution has a lower resulting compared to pure water.

## Water – Solute Interactions

Addition of non-ionic kosmotropes solutes can structure water (i.e., trehalose, maltose, sucrose, glucose, galactose, glycine, proline and numerous zwitterions) in a similar manner as observed with the addition of ions while other, non-ionic chaotropes solutes (i.e., urea), can disrupt the structure of pure water. Generally, non-ionic kosmotropes are very soluble and are well hydrated by water. The hydration of these compounds depends on the ability of water to interact with the hydrogen bonding groups of the solute. These molecules efficiently hydrogen bond to water and have very little tendency to aggregate or hydrogen bond internally. Compounds such as sugars can replace an all-*gauche* chair-form hexamer from an icosahedral water cluster. The counter clockwise equatorial arrangement of hydroxyl groups is likely due to the lone oxygen pair repulsion rather than the very weak hydrogen bonds. Such weak interactions are easily overcome by hydration forces (Ramadugu et al., 2009). Hence the complimentary structure of the chair configuration of sugars does not disrupt the native structure of water.

The ability for sugars to hydrate in water is dependent on the equatorial/axial configuration of the hydroxyl groups on the sugar which promotes intramolecular interactions. When sugars form intramolecular bonds, it increases the hydrophobic nature of the carbohydrate and decreases the ability to be hydrated (Dashnau et al., 2005). Trehalose, has a larger hydrated volume than other sugars and is often excluded from the hydration shell of macromolecules decreasing the solvation of proteins and hence stabilizing their ternary structure (Sola-Penna and Meyer-Fernandes, 1998). This is crucial in enzymatic reactions in aqueous medium to preserve the native structure of a protein.

Chaotropes interfere with the tetrahedral hydrogen bonding networks of water. Therefore, water is more likely to interact with proteins and other macromolecules which often induces denaturation of the ternary structure. These solutes reduce the order of water, leading to weaker hydrogen bonds, as well as reductions in viscosity and surface tension. Urea, at high concentrations, disrupt the structure of water because it has a tendency to hydrogen bond with other urea molecules forming long linear chains, up to 60 molecules long. The urea–urea interactions limit the amount of water capable of interacting with water and it is observed that only one molecule of water can hydrogen bond two times to one urea molecule (Stumpe and Grubmüller, 2007). The urea–urea interaction limits the amount of water–urea interactions leading to a disrupted structure of water.

## Water – Hydrophobic Structure Interactions

Hydrophobic compounds (i.e., hydrocarbons, fats and oils) are molecule which “hate water” and interact via van der Waals interactions. When hydrophobic compounds mix with water generally they aggregate to minimize their surface contact with the water. When apolar compounds are added water, water restructures itself forming clathrates to minimize its interactions with the apolar compounds. Clathrates may be thought of as an ice-like inclusion of highly ordered water entrapping the apolar compounds.

## Water Activity

It is often believed that the water content of foods is directly related to food perishability. Foods with the exact same amounts of water may perish very different. Hence, it is the free water which determines the shelf-life of foods. The term water activity ( $a_w$ ) refers to the amount of water, in equilibrium, available to hydrate material, or the water which is not bound by solutes or macromolecules such as proteins and carbohydrates.  $a_w$  is a measure of the energy status of water in the system. The addition of solutes and macromolecules to water results in a decrease in the entropy of the solution relative to pure water, resulting in a decrease in the vapour pressure. Materials have an  $a_w$  between 1 and 0 (100% and 0% relative humidity) indicating pure water and no water; respectively.  $a_w$  effects microbial growth, deleterious reactions, oxidations, and vitamin and color degradation. The addition of solutes and macromolecules effect  $a_w$  via colligative effects. When salts, sugars, and other soluble components are added to water they interact via non covalent interactions (i.e., dipole–dipole, ionic and hydrogen bonds). In complex systems, such as bread, water exists in capillaries which affects the vapour pressure differently than the addition of solutes because in bread water is affected by surface confinement within the capillaries. As well, macromolecules, even though they are not soluble, are able to interact via non-covalent interactions. The addition of solutes, macromolecules and interfaces all interact with water thereby reducing the entropy of the water and lowering the  $a_w$ .

The concept of water activity is derived from laws of thermodynamics. In the equilibrium state, the chemical potential,  $\mu$ , is the same everywhere in the entire system, the temperature must be defined and pure water must have a water activity equal to 1:

$$\mu = \mu_o + RT \ln \frac{f}{f_o}$$

where  $\mu$  (J/mol) is the chemical potential of the system,  $\mu_o$  is the chemical potential of the pure material at temperature,  $T$  (K),  $R$  is the ideal gas coefficient (J/mol K),  $f$  is the fugacity and  $f_o$  is the fugacity of the pure material (Berg and Bruin, 1981). Fugacity is a measure of the tendency of the substance to escape from the system. When dealing with water,  $f/f_o = a_w$  which represents the escaping tendency of water in the system. For water the fugacity is closely related to the vapour pressure and hence water activity may be rewritten as:

$$a_w = \frac{f}{f_o} \sim \frac{p}{p_o}$$

where  $p$  is the vapour pressure of water in the material and  $p_o$  is the vapour pressure of pure water at the same temperature. The equilibrium relative humidity is the water activity multiplied by 100.

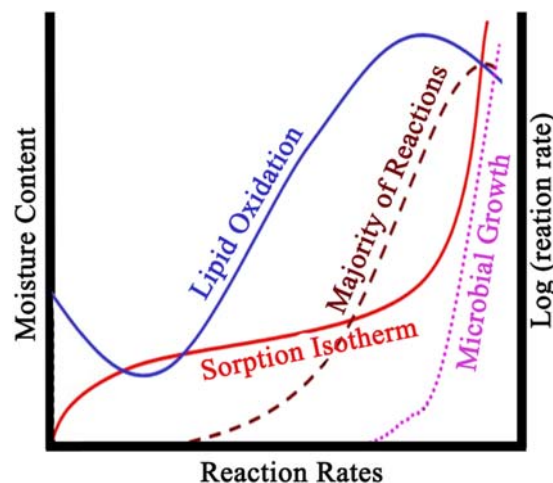
Water activity affects many aspects of food stability including microbial growth, enzymatic and non-enzymatic spoilage. The  $a_w$  of a food may be reduced by adding humectants or by the removal of water during drying. Processed foods with water contents below 15%, with lower  $a_w$ , have their water associated in monolayers and multilayers and are not capable of participating in hydration of microbes or partaking in mobility of enzymes. Therefore, foods with low  $a_w$  are stable and the shelf-life is not affected by microbial degradation. Intermediate moisture foods, between 20% and 40% moisture, contain monolayer and multilayer water and also capillary water. These foods are highly susceptible to enzymatic reactions and to a lesser extent microbial spoilage. And high moisture foods above 40% moisture will undergo rapid spoilage if there are no alternative processes which limit deleterious reactions from occurring.  $a_w$  can be used to predict the occurrence of different types of food spoilage. For example, when the  $a_w$  is reduced below 0.65 very little to no spoilage occurs via microbial populations. Lipid oxidation shows interesting behaviour where a minimum in oxidation is observed at  $a_w$  of 0.2. This is attributed to small amounts of water binding hydroperoxides and when more water is added to the system it aids in the solubility of oxygen into the food matrix thereby increasing the rate of oxidation (Karel and Yong, 1981). Vitamin degradation, Maillard reactions and microbial growth each exhibit a rate maximum at a specific  $a_w$  (Fig. 1). Rate maxima may be due to feedback inhibition when water is an end product or enhanced solubilization of substrates.

## Hysteresis

Hysteresis has important applications and challenges in food science when it pertains to food chemistry, processing and microbiology. Hysteresis occurs when the addition of water to a sample, also known as resorption, does not superimpose onto the isotherm generated by removal of water or desorption. The deviation of the two isotherms is referred to as hysteresis. Typically, the resorption isotherm is lower than the desorption isotherm. A physical description for hysteresis is based on principals of capillary condensation for water. The Kelvin equation was developed for capillary condensation (Kapsalis, 1981).

$$\ln \frac{P_v}{P_{sat}} = \frac{-2H\gamma V_l}{RT}$$

where  $P_v$  is the equilibrium vapour pressure above the curved meniscus,  $P_{sat}$  is the saturation vapour pressure of water,  $\gamma$  is the liquid/vapor surface tension,  $V_l$  is the molar volume of water,  $H$  is the mean radius of curvature for the meniscus,  $T$  is the absolute temperature and  $R$  is the ideal gas constant. The ink bottle theory is used to describe water being added and removed from capillaries. The ink bottle is comprised of a thin neck ( $r_1$ ) and a large well ( $r_2$ ). Upon addition of water the entry into the capillary is restricted until it has first condensed in the well ( $r_2$ ) at:



**Figure 1** Reaction rates related to the moisture content of the food.

$$\frac{P}{P_o} = e^{\left(\frac{-2\sigma V}{r_2 RT}\right)}$$

Now we can substitute  $r_2$  for the Kelvin equation. This suggests that water desorption (i.e., during drying) is governed by the small capillary radius and undergoes evaporation immediately only when the desorption pressure ( $P_d$ ) is equal to:

$$P_d = e^{\left(\frac{-2V}{r_1 RT}\right)}$$

## States of Water

The physical state of water may influence aspects of food quality including microbial growth, physical disruptions of cellular components, rates of chemical reactions and sensory properties. Typically in literature when discussing state diagrams, as they pertain to foods, they are referred as phase diagrams. However, a phase is under equilibrium conditions and since foods contains complex interfaces and meta-stable forms the term state diagram is more suitable. There are many important aspects which will be discussed regarding a simplified water state diagrams.

Water can exist in three states, solid, liquid and/or vapour. Within the ice and liquid phases there are sub-phases which relate to how the molecules interact and how water molecules pack into crystal lattices. We typically think of water boiling at 100 °C; however this is only true at 101 kPa or atmospheric pressure whereas water would boil at 71 °C on the top of Mt. Everest. If we increase the pressure, water boils at higher temperatures; conversely, if we pull a vacuum (i.e., decreased the pressure) water will boil at lower temperatures. The ability to modify the boiling point of water is often overlooked when discussing water chemistry but it has tremendous consequences as it pertains to food processing. Water is only capable of boiling when the vapour pressure of the liquid is equal to the pressure of the atmosphere or the headspace of the package. This is often used during thermal processing and drying of heat sensitive foods. For example, when sterilizing a can of soup, the cans are placed into a pressurized retort and steam is injected into the headspace of the retort to increase the pressure while the product is being sterilized in the can. If the cans were not heated in a pressurized environment, the water would boil in the can. Since, water vapour is far less dense than liquid water, the can would expand and in some cases explode. On the other hand, when drying heat sensitive foods a vacuum is applied reducing the headspace pressure causing water to boil at lower temperatures. Rising film evaporators, used to produce orange juice concentrate from fresh orange juice, allow water to boil at ambient temperature by reducing the pressure from 101 kPa to 2.9 kPa. This allows water to be removed without developing cooked flavours in the final product.

The second important phase boundary is the ice-liquid boundary. The slope of this boundary is far less sensitive to pressure compared to the vapour-liquid or solid-vapour boundaries because the density of liquid and ice are very similar compared to the density of the vapour. Hence, there are no food processing techniques which use pressure to modify the ice liquid boundary. The triple point is where all three phase boundaries meet. Here the three states (i.e. ice, water, vapour) all exist in equilibrium. Hence, a water molecule in the vapour phase can condense into a liquid or a solid. Similarly, a water molecule in the ice phase may either melt into a liquid or sublime into the vapour phase. Below the triple point, ice may directly sublime into the vapour phase and vapour may condense directly to the ice phase. Freeze drying is an important application to food scientists where a food is frozen placed into a chamber and the pressure is reduced allowing the ice to sublime into a vapour. This has tremendous benefits when drying because it prevents cooked flavours and prevents case hardening.

## Crystalline Nature of Water

The formation of ice is a complex event which involves nucleation followed by crystal growth. Depending on the nature of nucleation and crystal growth the number, size and shape of crystal depend on the cooling regime, storage temperature and fluctuations in storage temperature. In aqueous system, water and solute may crystallize. Lactose is prone to crystallization during scraped surface freezing of ice creams which produces long thin crystals inducing a grainy texture. Nucleation results in a new phase developing such that the chemical potential of the new phase is smaller than the existing phase. This results in small clusters of water associating and orientating together in a manner similar to ice. This small cluster of molecules is referred to as an unstable crystal embryo. During the early stages of nucleation, they are unstable because the embryo has excess free energy which is associated with the creation of the new surface. Embryos and nuclei form in a spherical geometry to reduce the surface area to volume ratio. Hence, the free energy of the embryo with radius  $r$  is a combination of surface (positive) and volume changes (negative):

$$\Delta G_n = A_n \delta - V_n \frac{\Delta \mu}{V_m^s}$$

where  $A_n$  is the surface area of the nuclei,  $\delta$  is the surface free energy per unit area,  $V_n$  is the volume of a nuclei,  $\Delta \mu$  is the chemical potential difference between the solid and liquid and  $V_m^s$  is the molar volume of the solid.

For nucleation to occur, the free energy associated with the phase change must be negative. This does not indicate the rate of the reaction; just that in time the reaction will occur. Examining the free energy associated with a new phase, when a small embryo forms there is a critical size in which it becomes stable. Unstable nuclei result in the area and interfacial term dominating over the volume and chemical potential thus  $\Delta G_n$  is positive indicating that the system is not thermodynamically stable. At early stages of nucleation, the nuclei form and break down because there is a large free energy association with their surface. Upon further cooling, the nuclei grow and the embryo reaches a critical radius and becomes stable. This occurs when the volume term becomes larger than the surface area term and the change in the free energy is negative. Since there is a critical size, pure water with no impurities may not freeze until  $-40^\circ\text{C}$ . Impurities include dissolved gasses, solutes, ions or surfaces.

Following nucleation, crystal growth occurs where liquid molecules diffuse to the surface of the nuclei and adsorb onto the growing surface. Crystals have highly ordered structures which are ordered into Bravais lattices. The unit cell of hexagonal ice contains 4 molecules held together via hydrogen bonding. This results in an open structure which is less dense than water. Irregular crystal growth often forms as thin dendrites which is very undesirable in food systems because it results in a graining texture (Raza et al., 2011).

Follow nucleation and crystal growth, the shape of the crystal may change in time due to recrystallization. Recrystallization changes the number, shape and size of the ice crystals. During storage small crystals disappear and result in larger crystals growing to even larger crystals. This type of recrystallization is referred to as Ostwald ripening where large crystals grow and small crystals disappear at a constant temperature and pressure. This occurs because of differences in the surface free energy between different size crystals. Temperature fluctuations accelerate recrystallization resulting in all the depletion of small crystal and the increase in the size of larger crystals. The outcome results in grainy texture and consumer rejection of products. Typically, crystals that are not spherical do not have an optimal surface area to volume ratio and attempt to minimize this surface area to volume ratio via iso-mass recrystallization which results in crystals becoming spherical.

## Rubbery and Glass States of Water

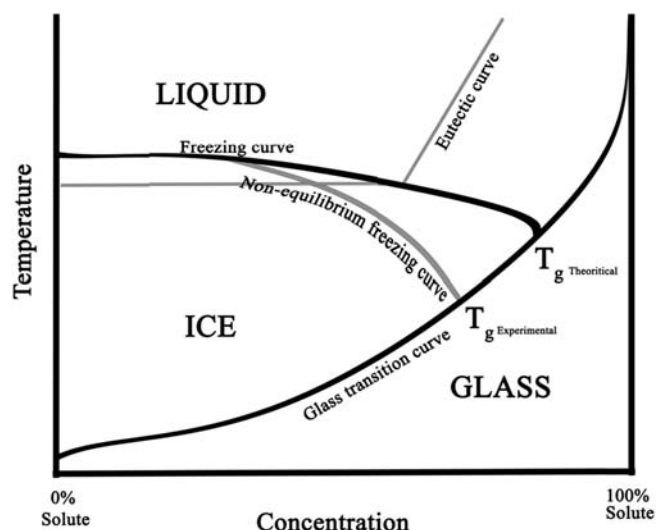
A glass is a non-equilibrium, metastable, amorphous solid with an apparent viscosity greater than  $10^{12}$  Pas. Although both glasses and crystalline materials appear solid, a glass demonstrates no regular periodicity and the molecules do not orient onto Bravais lattices. X-ray diffraction is the simplest method to determine if a glass or a crystalline solid is present. Crystalline solids have sharp peaks which correspond to reflections from crystalline planes while a glass demonstrates no peaks. When a food becomes a glass the molecular mobility decreases. Molecular mobility of molecules is related to the diffusion coefficient ( $D$ ):

$$D = \frac{k_B T}{6\pi\eta r}$$

where  $k_B$  is boltzman constant,  $T$  is the absolute temperature,  $\eta$  is the viscosity, and  $r$  is the molecular radius. The diffusion coefficient is  $D \sim 10^{-24} \text{ m}^2 \text{ s}^{-1}$  if we assume a viscosity of  $10^{12}$  Pas. The advantage of using glasses to preserve foods is that any deleterious reaction relying on diffusion cannot occur. Typically, enzymatic reactions cannot occur because the enzyme and substrate cannot diffuse preventing contact with each other. Another major advantage occurs due to the lack of ice crystals. Therefore, there is no cellular disruption due to crystals rupturing plant and animal cells.

Theoretically, it is possible to reach a glass transition of pure water (Fig. 2) by rapidly cooling to  $-134^\circ\text{C}$  to avoid the formation of ice. From a food processing perspective, this does not occur because the material will crystallize before the glass transition is reached. Typically, during the freezing of foods, water undergoes a phase change from liquid to ice via nucleation and crystal growth. When water is removed from the unfrozen phase via the conversion to ice the unfrozen phase becomes more concentrated with solutes. At every temperature, a thermodynamic equilibrium is reached between the unfrozen phase and ice. This ideal cooling regime follows the equilibrium freezing line to the eutectic point. From a thermodynamic standpoint, at the eutectic point the solute





**Figure 2** Solid/liquid coexistence boundaries and glass transition profile for a binary sucrose/water system. Below and to the right of the glass transition line, the solution is in the amorphous glass state, with or without ice present depending on temperature and freezing path followed, while above and to the left of the glass transition line, the solution is in the liquid state, with or without ice depending on temperature.

should begin to crystallize out of solution. However, in the time frame of food processing conditions this often does not occur due to the kinetic conditions including low temperatures and a very high viscosity of the unfrozen phase. Hence beyond the eutectic point freeze concentration continues to occur until the glass transition is reached with further reduces the molecular motion.

Although we have focused on glasses produced with small solutes, there is also the possibility to form a glass with polymers. When a polymer chain, such as starch or gelatine, undergoes a glass transition they are completely immobile causing the final product to become very brittle. Near the glass transition is the rubbery transition which still has a high viscosity however the product becomes malleable. Increasing water content in these products, at a constant temperature, will significantly reduce the viscosity. Hence it is said that water acts as a plasticizer to polymer glasses. From a food perspective, generally they contain several components which may pass through the glass transition, such as starches and sugars, in the same material allowing the food to attain the physical properties of a glass much more readily.

## Edible Glass Structure

Many food products get their desirable final physical properties, microbial stability and sensory characteristics from removing water from the systems and creating a new state. When complex food systems are baked often their stability and structure is obtained by removing sufficient water to reach the glass transition. Crackers, cookies, milk powders and hard candies all obtain their structure by creating an edible glass. The simplest system is hard candies. Typically, a sucrose solution containing small amounts of colours and flavours is boiled to remove water. By doing so, we continually concentrate the sugar until it is approximately 82% of the final weight. This is sufficient to produce a sucrose glass. The end result is the same as the unfrozen phase glasses we discussed earlier. Hard candies seem like a crystalline solid however they have no crystal lattices. One major problem hard candies encounter is a limited shelf-life. Once the package is opened the sugar attracts water to absorb since it is very hygroscopic. Upon absorbing atmospheric water, the concentration of sucrose decreases and eventually a state transition occurs from the glassy state to the rubbery state which causes the product to become sticky. This is why when candies are placed into a dish they stick together.

Crackers and milk powders are also glasses however in this case, they rely on creating a glass due to the presence of polymers including proteins and long chain carbohydrates, such as starches. Water is removed from these foods either through baking or spray drying, and a glass of the macromolecule is produced. This is why crackers are crispy. However, if crackers absorb water they become soft and malleable which makes them far less desirable and more susceptible to microbial growth. When milk powders absorb water they also become very soft and sticky as well the dramatic decrease in the viscosity allows for the porous structure to collapse making them much harder to re-solubilize.

## Conclusions

Water plays an essential role not only in food structure but also in limiting the spoilage microorganisms. It dictates stability of foods, textural properties, microbial stability and consumer acceptance. By controlling the amount and state of water in foods we can control the structure of food which gives rise to the desirable organoleptic properties of the food as well as its safety.

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# Water-Soluble Vitamins

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## Overview

A vitamin is an organic compound that is required in small quantities for normal functions of the body including growth and metabolism (Table 1). It cannot be synthesised in humans to meet the required needs of the individual and deficiency symptoms will occur if a single vitamin is omitted from the diet of a species which requires it. Many vitamins act as coenzymes, whereas others have no single role but to perform certain essential functions. Vitamins are grouped by function rather than their chemical characteristics. Certain substances that are considered vitamins can be synthesized in small amounts by gastrointestinal tract bacteria (e.g. B-groups). Vitamins are generally grouped into two groups: water- and fat-soluble, based on their solubility characteristics. Water-soluble vitamins are found in all living tissues, whereas fat-soluble vitamins are completely absent from some tissues. Water-soluble vitamins generally contain one or more polar or ionizable groups (carboxyl, keto, hydroxyl, amino, phosphate) (Combs and Mcclung, 2017).

## Classification and Properties

Identifying and classifying vitamins can be difficult due to a number of reasons: (1) a vitamin can exist in a number of different structures with similar, or different, biological activities; (2) most vitamins are only active when converted to another form or when attached to a specific protein; (3) the biological activity is organism-dependent; (4) vitamins can be biologically active in different forms (forms that are active in humans may not be the form that is found in food e.g. B vitamins are active in coenzyme forms); (5) vitamins can change into different forms during extraction (e.g. pyridoxine, pyridoxamine, pyridoxal, and their phosphorylated derivatives); and (6) the man made version of a vitamin can be more stable than the natural form.

Multiple biosynthetic pathways are involved in the synthesis of the water-soluble vitamins. Some compounds are classified as 'vitamin-like' compounds and are related to a vitamin in activity. In general, they can be synthesised by the body in required amounts. These include vitamins F (essential fatty acids), L (*o*-aminobenzoic acid), P (bioflavonoids), U (S-methylmethionine sulfonium chloride), and B<sub>13</sub> (orotic acid); choline;  $\alpha$ -lipoic acid; myoinositol; plastoquinone; pyrroloquinoline quinone; and ubiquinones.

During the early years of vitamin discovery, their chemical composition was largely unknown and vitamins were each assigned a letter of the alphabet for convenience. This was further complicated when it was found that the activity attributed to a single vitamin was, in some instances, the result of a combination of several vitamins, for example, vitamin B complex. To simplify the system, letters were sometimes replaced with names, for example, thiamin (vitamin B1) and biotin (vitamin H), based on the chemical structure of a specific function or source. 'Thiamin' contains the prefix 'thi', which is derived from the Greek word for sulfur and refers to its sulfur content.

Vitamins work in metabolism by the following ways: as coenzymes, as substances that can be oxidised or reduced in metabolism, as antioxidants, as hormones, and as gene transcription effectors (Combs and Mcclung, 2017).

## Occurrence in Food and Methods of Analysis

Water-soluble vitamins are found in all living tissues. The major vitamin forms found in foods and some examples of rich food sources are given in Table 2.

The UK Composition of Foods Dataset contains 3291 foods each of which contain up to 185 nutrient values. Around 90% of the data are analytical values; the methods that have been used for the analysis of foods can be found in McCance and Widdowson's, The Composition of Foods book (Finglas et al., 2015). The nutrient values quoted in the tables have been determined by a variety of methods and in many cases laboratories would have used modified versions of the methods referenced in the book. It should be noted that although most give results of the same order of accuracy, with new methods merely improving the efficiency of analysis, some methods may give different results because they only measure certain forms of the vitamin. It is important that all biologically active vitamins of a certain nutrient are included in the nutrient analysis, otherwise the results may not reflect the nutritional value of the food.

Recent reviews into analytical methods have suggested there may be some advances in vitamin analysis (Wang et al., 2018). Microbiological assays are still used to quantify certain vitamins, such as biotin and niacin, however HPLC is most commonly used in vitamin analysis as it is highly selective and sensitive. A large volume of sample is required when analysing samples by HPLC. Capillary electrophoresis is a newer alternative to HPLC and produces results faster from smaller amounts of sample at a lower cost, however it uses a short light path so its sensitivity may be a problem for some vitamins. Capillary electrophoresis can be coupled with other techniques such as UV absorbance, laser-induced fluorescence, electrochemical and MS detection to allow for different needs. Chemiluminescence is a very sensitive method that doesn't require a bulk light source but it is expensive. It can be coupled with electrochemical detection to produce a very effective method for electroactive analytes such as vitamin C.

**Table 1** Water-soluble vitamins: forms, functions, deficiency symptoms and potential health benefits

<i>Vitamin</i>	<i>Vitamins<sup>a</sup> and related forms</i>	<i>Functions</i>	<i>Deficiency symptoms</i>	<i>Potential health benefits</i>
B <sub>1</sub> /Thiamin	Thiamin, thiamin monophosphate (TMP), thiamin pyrophosphate (cocarboxylase, TTP) complexed to proteins	Helps convert carbohydrates into energy metabolism; required by the brain, nervous system and heart	Beriberi	Important role in energy metabolism
B <sub>2</sub> /Riboflavin	Flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD)	Involved in carbohydrate, protein and fat metabolism; helps release energy into cells; part of the blue-sensitive pigments in the eye	Legions on mouth, magenta tongue, seborrheic dermatitis	Important role in energy metabolism; healthy skin
Niacin	Nicotinic acid, niacinamide (nicotinamide), nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide diphosphate (NADP)	Involved in carbohydrate, protein and fat metabolism	Pellagra, nervous manifestations	Reduces risk of atherosclerosis
B <sub>6</sub>	Pyridoxine, pyridoxal, pyridoxamine, and related phosphorylated forms	Essential for proper protein utilisation; involved in homocysteine metabolism	Retarded growth, acrodynia, alopecia, skeletal changes, anaemia, seizures	Reduces risk of cardiovascular disease (with folate and B <sub>12</sub> ) and osteoporosis
B <sub>12</sub>	Cyanocobalamin, methyl- and hydroxocobalamins, adenosylcobalamin	Needed for red blood cell formation and DNA and RNA synthesis; involved in homocysteine metabolism	Macrocytic megaloblastic anaemia, paraesthesia, leg weakness, memory loss	Prevents certain anaemias and reduces risk of cardiovascular disease
Folic acid (folacin); folate	Pteroylmonoglutamic acid, 5-methyl-tetrahydrofolic acid as both mono- and polyglutamates, tetrahydrofolic acid, 5-formyltetrahydrofolic acid	Involved in amino acid interconversions and methylation reactions, needed for red blood cell formation and DNA and RNA syntheses, involved in homocysteine metabolism and protection against neural tube defects	Megaloblastic anaemia, increased homocysteine level, depression, irritability, neural tube defects in foetuses	Prevents certain anaemias and birth defects, reduces risk of cardiovascular disease (with B <sub>6</sub> and B <sub>12</sub> ) and certain cancers
Pantothenic acid	Coenzyme A, pantotheine, and acyl carrier protein (ACP)	Active in protein, carbohydrate and fat metabolism	Burning feet syndrome, headache, fatigue, insomnia, paresthesia, increased insulin sensitivity	General health and well-being
Biotin	Biotin, desthiobiotin	Active in protein, carbohydrate and fat metabolism	Alopecia, depression, muscular pain, paresthesia	Helps develop healthy nails
C	Ascorbic and dehydroascorbic acids, isoascorbic acid	Helps form/maintain collagen; important for healthy tissues and wound healing; enhances iron absorption; protects against free-radical damage and infection	Scurvy, fatigue, weakness, anaemia, aching joints and muscles, bleeding gums, delayed wound healing	Protects against cancer and cardiovascular and eye diseases; reduces symptoms of colds and flu and risks of osteoporosis

<sup>a</sup>The term 'vitamins' relates to the nutritionally active chemical species.

Reproduced from Francis, F.J. (Ed.), 2000. Vitamins: Survey. In: Encyclopedia of Food Science and Technology, second ed. vol. 4. Wiley, New York, pp. 2440–2449.

**Table 2** Water-soluble vitamins – main food forms and rich dietary sources

<i>Vitamin</i>	<i>Main food forms</i>	<i>Rich dietary sources</i>
B <sub>1</sub>	Thiamin, thiamin monophosphate, thiamin pyrophosphate complexed to proteins	Yeast and beef extract, wheat germ, pork, peanuts
B <sub>2</sub>	Riboflavin, riboflavin mononucleotide, flavin adenine dinucleotide complexed to proteins	Yeast extract, liver, kidney, brown crab meat
Niacin	Nicotinic acid, nicotinamide adenine dinucleotide (NAD) and its phosphate form (NADPH) complexed to proteins	Yeast and beef extract, wheat bran, tuna, liver, chicken
B <sub>6</sub>	Pyridoxine, pyridoxal, and pyridoxamine and their phosphorylated forms bound to proteins	Wheat germ, yeast extract, turbot, plantain
B <sub>12</sub>	Methyl-, adenosyl-, hydroxy-, and sulfitecobalamins	Liver, kidney, cockles, brown crab meat, mussels, oysters, yeast extract
Folic acid (only in fortified foods), folates	Folic acid, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, 10-formylfolate, tetrahydrofolate as mono- and polyglutamates	Yeast and beef extract, liver, wheat germ, blackeye beans, broccoli, asparagus, spinach, fortified foods (bread, breakfast cereals)
Pantothenic acid	Pantothenic acid, coenzyme A and acyl carrier protein (ACP)	Liver, kidney, broad beans, heart, egg yolk, mushrooms, peanuts
Biotin	Biotin and biotin bound to lysine and proteins	Widely distributed, especially liver, kidney, nuts, egg yolk, pangasius, plaice
C	Ascorbic acid, dehydroascorbic acid, isoascorbic acid	Guava, blackcurrants, spring greens, capsicum peppers, watercress, papaya, citrus fruits

Reproduced from Francis, F.J. (Ed.), 2000. Vitamins: Survey. In: Encyclopedia of Food Science and Technology, second ed. vol. 4. John Wiley, New York, pp. 2440–2449; Finglas, P., Roe, M., Pinchen, H., Berry, R., Church, S., Dodhia, S., Farron-Wilson, M., Swan, G., 2015. McCance and Widdowson's The Composition of Foods, seventh summary ed. Royal Society of Chemistry, Cambridge.

LC-MS/MS is a fast method that is used to profile and quantify folate vitamers. LC-MS/MS differs to GC-MS as GC-MS cannot distinguish all folate forms within a sample (Vishnumohan et al., 2011).

## Nutritional Aspects

Nutritional requirement values represent the daily amount of a nutrient that a person would need to ensure that deficiencies are prevented and all their requirements are met. They are determined by each country based on statistical distributions of individual nutrient requirements which are determined from primary research including nutrient balance studies, measures of tissue saturation, normal vitamin intakes in the general population, and extrapolation from animal studies. Therefore, recommended nutrient values vary between countries. With new research and policies, there is a need to review the values, for example, the UK dietary reference values are monitored by the Scientific Advisory Committee on Nutrition (SACN) and are reviewed periodically. In a 2017 report by EFSA, the 1993 DRVs for the European population was updated. The report contains population reference intakes, which relate to the level of nutrient intake that is adequate for virtually all people in a population group; these values are given in Table 3 for adult males and females.

Some vitamins have been shown to have additional health benefits when given via diet or by supplementation, at levels above those required for eliminating classical deficiency states. Although, for most vitamins the optimal levels are not yet established, for some vitamins such as folic acid, the health benefits have been clearly demonstrated for taking higher intakes to protect against spina bifida or other neural tube defects. A higher intake of vitamin B<sub>6</sub> has been linked with a lower risk of impaired mobility in older adults (Struijk et al., 2017). In general, water-soluble vitamins have a safety level of 100 times or more the RDA for which there is no clear indication of any adverse effect; this is mainly because water-soluble vitamins are

**Table 3** Population reference intakes (PRIs) for water-soluble vitamins in adult males and females

<i>Category</i>	<i>Age (years)</i>	<i>B<sub>1</sub> (mg/MJ)</i>	<i>B<sub>2</sub> (mg/day)</i>	<i>Niacin (mg NE/MJ)<sup>a</sup></i>	<i>B<sub>6</sub> (mg/day)</i>	<i>B<sub>12</sub> (μg/day)</i>	<i>Folate (μg DFE/day)<sup>b</sup></i>	<i>C (mg/day)</i>
<b>PRI</b>								
Males	≥18	0.1	1.6	1.6	1.7	4.0	330	110
Females	≥18	0.1	1.6	1.6	1.6	4.0	330	95

<sup>a</sup>NE: niacin equivalent (1 mg niacin = 1 niacin equivalent = 60 mg dietary tryptophan).

<sup>b</sup>DFE: dietary folate equivalents. For combined intakes of food folate and folic acid, DFEs can be computed as follows: μg DFE = μg food folate + (1.7 × μg folic acid).

Reproduced from EFSA, 2017. Technical Report: Dietary Reference Values for Nutrients, Summary Report. EFSA Supporting publication 2017, e15121.

excreted readily in the urine and are generally not stored within the body (the exception being vitamin B<sub>12</sub> which can accumulate in the liver) (Combs and Mcclung, 2017). Deficiencies can occur from inadequate intake, malabsorption or from an intake of drugs or food that interact with factors or compounds related to absorption. Water-soluble vitamin deficiencies in Western countries has been relatively uncommon, however with a rising consumption of nutrient lacking food, low micro-nutrient levels has been seen in overweight and obese adults (Kimmons et al., 2006). Deficiencies can also result from certain conditions, for example the thiamin deficiency disease, beriberi, was recently seen in a dialysis patient (Jankowska et al., 2017).

Nutrient reference values provide a good estimate of adequate intake for the majority of the population; however, some factors will influence the uptake in vivo.

### Availability and Bioavailability

The term bioavailability (biological availability) is used to describe the proportion of a nutrient in food that is utilised for normal body functions. The way in which vitamins are absorbed and utilised in the body can be affected by several factors that can be broadly divided into dietary-related factors and physiological factors related to the host, for example thiamin requirements are higher when the diet contains large amounts of carbohydrate or alcohol. In addition, the vitamin may not be in a form that can be readily absorbed by the body. For example, nicotinic acid occurs in cereals in a form that is not absorbed from the gut. Some synthetic vitamins have different absorbance rates compared to naturally occurring vitamins in foods due to matrix effects and inhibitors in some foods, for example, synthetic B<sub>12</sub> in fortified foods is readily available compared to natural, food-bound B<sub>12</sub> (Institute of Medicine, 1998). Other components in the diet may also interact with the vitamin, causing an enhancement, or depression, of absorption. Thus, the nutritive value of a diet for a given vitamin may be somewhat different from the amount analysed chemically. Absorption of water-soluble vitamins can be directly through the gut via passive diffusion, or via specific carriers. In some cases, the vitamin is absorbed via specific carriers when present in low doses and by passive diffusion when present in high doses (Combs and Mcclung, 2017). Not all the vitamins needed by a human are only acquired via the intake of food; vitamin B<sub>12</sub>, thiamin, riboflavin, vitamin B<sub>6</sub>, biotin and folate can be synthesized in small amounts by gut bacteria. In addition, biosynthesis of niacin from tryptophan and vitamin C from glucose does occur, however these amounts are not substantial for the requirements of a human being (Combs and Mcclung, 2017). Most vitamins need to be bound to a protein, enzyme or modified in some way to be biologically active; one of the exceptions to this rule is vitamin C.

### Adverse Effects

Along with the increasing evidence of health benefits from consumption of diets containing vitamins at levels much higher than the RDAs, there is concern over potential adverse effects and even toxicity. The European Food Safety Authority (EFSA) has produced guidelines on the upper levels of micronutrient intakes which are likely to result in adverse effects in the general population (European Food Safety Authority, 2006). For example, EFSA advise that the upper intake of vitamin B<sub>6</sub> should not exceed 25 mg/day for adults; intakes as low as 50–100 mg/day have even been associated with symptoms of nerve damage (Bender, 1997). Similarly, high doses of nicotinic acid have been linked to a decrease of LDL cholesterol and increase of HDL cholesterol (Carlson, 2005), however effects like this should be considered alongside the possible toxic effects, particularly for the liver. One of the main concerns of folic acid fortification is its potential to mask the diagnosis of pernicious anaemia, which is caused by vitamin B<sub>12</sub> malabsorption causing progressive and irreversible nerve damage.

### Effects of Processing on Vitamin Stability

Vitamin stability varies greatly and depends on several factors such as temperature, oxygen and acid or alkali strength. Table 4 provides general information on the stability of water-soluble vitamins under several conditions. Several processes have been developed to produce stabilised forms of the vitamins, including spray drying in a suitable matrix (e.g. gelatin) and encapsulation.

Conserving nutrients in processed products has been of growing interest to consumers. A major benefit of processing, for example canning and freezing, is that it generally preserves vitamin concentrations by inactivating food enzymes.

The amount of a vitamin in a processed product will be determined by the concentration in the unprocessed, raw product, of which there can be great variability. For example, carrots may vary in their vitamin C levels by as much as 35-fold. This can be due to several factors, including variety differences, climate and growing conditions, maturity at harvest, and postharvest storage conditions. Thus, the vitamin content of raw materials for processing can vary enormously, in many cases exceeding that found in normal processed food. Boiling rice can result in thiamin, riboflavin and niacin losses of around 45%, even washing milled rice can cause a dramatic loss of some B vitamins (Kyritsi et al., 2011).

The different chemical and physical properties of the vitamins mean that they vary widely in their degree of stability. In the pure crystalline form, they retain their activity for long periods of time. However, when foods are processed, vitamins are subjected to a range of conditions that can be detrimental to their stability such as light, moisture, oxygen, heat and pH, these being typical environmental factors to which the food will be subjected in the food chain (see Table 4). Vitamins that are unstable to oxidation are



**Table 4** Water-soluble vitamin stability under certain conditions

Vitamin	Vitamin	Stable (S)/Unstable (U) to						To enhance stability
		UV	Heat <sup>a</sup>	O <sub>2</sub>	Acid	Alkali	Metals <sup>b</sup>	
Thiamin	Disulfide form	S	U	U	U	U	U	Keep at neutral pH <sup>c</sup>
	Hydrochloride <sup>d</sup>	S	U	U	U	U	U	Exclude O <sub>2</sub> , at neutral pH <sup>c</sup>
Riboflavin	Riboflavin	U <sup>e</sup>	U	S	S	U	U	Keep in the dark, at pH 1.5–4 <sup>c</sup>
Niacin	Nicotinic acid	S	S	S	S	S	S	Good stability
	Nicotinamide	S	S	S	S	S	S	Good stability
Vitamin B <sub>6</sub>	Pyridoxal	U	U	S	S	S	S	Keep cool, work in subdued light
	Pyridoxol HCl	S	S	U	S	U	S	Good stability
Biotin	Biotin	S	S	U	S	U	S	Keep sealed, at neutral pH
Pantothenic acid	Free acid <sup>f</sup>	U	S	U	S	U	S	Cool, neutral pH
	Calcium salt <sup>d</sup>	S	U	S	S	S	S	Keep sealed, at pH 6–7
Folate	FH <sub>4</sub>	U	U	U	U <sup>g</sup>	S	U	Good stability <sup>c</sup>
Vitamin B <sub>12</sub>	Cyano-B <sub>12</sub>	U	S	S	U <sup>h</sup>	S	U <sup>i</sup>	Good stability <sup>c</sup> at pH 4–7
Vitamin C	Ascorbic acid	S	S	U <sup>b</sup>	S	U	U	Keep sealed, at neutral pH

<sup>a</sup>100 °C.<sup>b</sup>In solution with Fe<sup>3+</sup> and Cu<sup>2+</sup>.<sup>c</sup>Unstable to reducing agents.<sup>d</sup>Slightly hygroscopic.<sup>e</sup>Especially in alkaline solution.<sup>f</sup>Very hygroscopic.<sup>g</sup>pH < 5.<sup>h</sup>pH < 3.<sup>i</sup>pH > 9.

Adapted from Combs, G.F., McClung, J.P., 2008. The Vitamins, third ed. Fundamental Aspects in Nutrition and Health, pp. 33–58.

therefore degraded by heat, oxygen, metal ions, polyunsaturated lipids undergoing peroxidation and ultraviolet light (Combs and McClung, 2017). The rest of the water-soluble vitamins tend to be stable under most normal conditions. The most liable water-soluble vitamins are vitamin C, folate and riboflavin.

### Domestic Cooking

Typical percentage losses of vitamins on cooking for selected food groups are given in Table 5. Vitamin C is one of the most labile of all the vitamins and is readily leached into water on boiling, particularly when the surface of the food has been cut or damaged. Vegetables are an important source of vitamins in the diet and typical losses on boiling vegetables are quite variable. Steaming, microwaving and pressure cooking can reduce water-soluble vitamin losses for cooked vegetables (Bureau et al., 2015), however microwaving conditions, e.g. cooking time and water volume used can affect the vitamin retention.

Meat is the main source of B12 in the diet and is also a good source of other B vitamins. Dry cooking meat has been shown to result in a loss of B vitamins; the most pronounced being thiamin (Lombardi-Boccia et al., 2005). Losses are less when pot roasting

**Table 5** Typical percentage losses of vitamins on cooking for selected food groups

	Cereals		Milk		Meats	Fish	Vegetables	Fruits
Vitamin	Boiling	Baking	Boiling <sup>a</sup>	Baked dishes	Meat, grilled or fried	Baking	Boiling	Stewing
B <sub>1</sub>	40	25 <sup>b</sup>	10	25	20	30	35	25
B <sub>2</sub>	40	15	10	15	20	20	20	25
Niacin	40	5	0	5	20	20	30	25
B <sub>6</sub>	40	25	10	25	20	10	40	20
B <sub>12</sub>			5		20	10		
Folate	50	50	20	50		20	40	80
Pantothenate	40	25	10	25	20	20		25
Biotin	40	0			10	10		25
C			50				45	25

<sup>a</sup>In milk-based drinks and custards.<sup>b</sup>15% in bread-making and toasting.

Reproduced from Finglas, P., Roe, M., Pinchen, H., Berry, R., Church, S., Dodhia, S., Farron-Wilson, M., Swan, G., 2015. McCance and Widdowson's The Composition of Foods, seventh summary ed. Royal Society of Chemistry, Cambridge.

and braising are used. Some vitamins that leach into the meat juices during cooking will not be lost if the sauce or the gravy is eaten as part of the actual dish. This means that average losses in meat dishes are not higher than for grilled or fried meat, even though the cooking times are longer. Similarly, when using classic breadmaking techniques, 48% of the thiamin can be lost when making white bread. However, it was also shown that keeping the fermentation of yeast or sourdough separate during whole wheat bread making kept the thiamin levels close to the original level of the flour (Batifoulie et al., 2005).

### Other Processing Methods

Extrusion cooking is a common method of producing crisp, shaped cereal products such as breakfast cereals, snack foods and other textured foods. Various factors influence the retention of vitamins during extrusion, such as cereal type, sensitivity of the vitamin to heat, type of extruder. Heat sensitive vitamins are particularly susceptible to degradation during extrusion. Thiamin, riboflavin, niacin and vitamin B6 have been shown to have varying retention rates in different cereal types. For example, vitamin B6 was shown to be stable in maize but less so in oats (Athar et al., 2006). Moisture content, energy input, mass temperature, screw geometry, and rate of throughput are thought to influence retention. For example, short barrel (90 mm) extruders had a higher retention rate of B vitamins (44%–62%) compared to 20% for long barrel extruders (Brennan et al., 2011). It appears that retention can be improved by increasing both the rate of throughput and moisture content. Losses of vitamin C during extrusion of 25%–40% have been found.

Irradiation is an application that ensures the safety and quality of food by destroying microbes. Irradiation-induced changes in vitamin concentrations in foods during irradiation have been reviewed extensively in the literature. In practice, irradiation doses are usually lower than 10 kGy although even low doses can produce an effect on the level of vitamin in a product; losses of thiamin can be as much as 20% at an irradiation dose of 1 kGy. However, other vitamins can be relatively unaffected, for example a study showed that low irradiation of citrus fruits resulted in no loss of vitamin C. In others, an increase can occur due to potential that irradiation changes a precursor to the vitamin; this was seen in a study that showed a 25% increase of riboflavin in pork chops and chicken breasts when irradiated by up to 6.6 kGy. In addition, losses can vary depending on the structure of the food, for example whole grain products show small losses but these increase after milling (Kilcast, 1994).

It is well known that fermentation can increase the B-vitamin content (e.g. folates) of some foods, such as cheese, milks, yogurts and other dairy foods, and this is a potential area for production of foods with enhanced health benefits. *Streptococcus thermophilus* and *Propionibacteria* naturally produce folate and vitamin B<sub>12</sub> respectively, however microorganisms generally require vitamins for growth. This rule does not apply to *Lactobacilli* which grows without vitamins and produces vitamin B<sub>12</sub>. Gene mutation, incubation conditions and medium will also have an input on the amount of vitamin released by the microorganisms (Walther and Schmid, 2017).

Fortification of common foods can be an acceptable way of supplementing the population's diet with lacking nutrients. Recent studies have shown that even soaking rice in water-soluble vitamins can improve the nutrient composition, with up to 70% retention of the applied vitamin (Kyritsi et al., 2011). In addition, it has been shown that it is possible to encapsulate vitamins using different biopolymers via a spray-drying process (Estevinho et al., 2016).

### Conclusion

Water-soluble vitamins are an essential part of a human diet. As they cannot be stored in the body, a daily intake must be met to prevent any associated deficiencies from forming. Daily needs will vary from person to person due to different factors such as the form of the vitamin that is ingested and the way in which it is utilised in the body. They can be greatly affected by storage, cooking and processing techniques and it is therefore evident that the vitamin levels present will vary from food to food.

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 Public Health England: [www.gov.uk/government/organisations/public-health-england](http://www.gov.uk/government/organisations/public-health-england).

## Waxes

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### Introduction

A broad definition of “waxes” was dated back from 1975 when H. Bennett defined it as “an unctuous solid with varying degrees of gloss, slipperiness and plasticity, which melts readily” (Bennett, 1975). There are various definition of waxes. Waxes can be referred to fatty components obtained from plants, insects, animal skins, or mineral origin. They can also be described as hydrophobic organic substances of medium or long chain length (Post-Beittmiller, 1996). Generally, waxes contain very long chain ( $>C_{18}$ ) fatty acids, hydrocarbons, alcohols, aldehydes, ketones, esters, sterols, triterpenes, and flavonoids (Post-Beittmiller, 1996). Waxes are insoluble in water and soluble in specific organic solvents. Since waxes are complex mixtures, their physical properties vary dramatically depending on their sources, and are often used to describe specific products. Regardless if its broad definition and complex composition, there is no dispute that wax is broadly used in various industries. So far, there is not any review focused from the aspect of food science. Therefore, in this chapter, classic waxes from various sources as well as their food-related applications are discussed.

### Classification of Wax

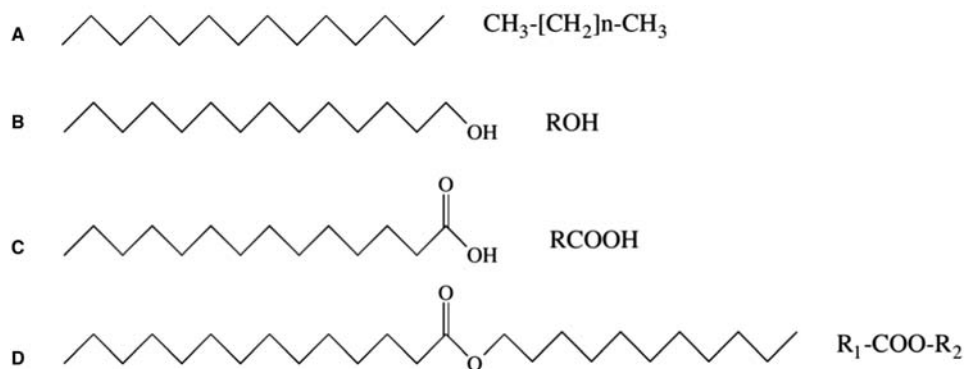
Waxes can be classified into synthetic and natural waxes. The synthetic waxes are generally low molecular weight polymers derived through polymerization of feed stocks, such as methylene, ethylene and  $\alpha$ -olefins. Natural waxes are from various origins, i.e., waxes from animals and plants, mineral waxes from petroleum, coal and peat. The composition of waxes largely depends on their sources. Most natural waxes from animals and plants consist of hydrocarbons, long-chain alcohols, long-chain carboxylic acids and long-chain esters (Fig. 1). While waxes of mineral origin contain almost exclusively long-chain hydrocarbons. The nature and classification of waxes, sometimes, may be misunderstood with fats and gums. For example, wool wax derived from lanolin is often considered as a fat because of its properties, although it is a wax in nature (Tinto et al., 2017). In this part, examples of some typical waxes from different sources are briefly introduced.

#### Animal Wax

Animal waxes are generally obtained from insects (bees, *Apis mellifera*), whale (shellac, *Physeter macrocephalus*), and sheep (*Ovis aries*), etc.

Beeswax is a naturally occurring wax secreted mainly by honeybees *A. mellifera*, for constructing honey combs (Tulloch, 1970a). Unhydrolyzed beeswax consists of approximately 71% esters, 15% hydrocarbons, 8% free fatty acids, and 6% other components (Tulloch, 1970b). Natural beeswax is brittle when cold, with melting temperature ranging from 62 to 65 °C. Beeswax is inert with high plasticity. It protects against corrosion, abrasion as well as moisture loss. Beeswax is one of the most useful commercially available waxes (Ogutcu et al., 2015; Martins et al., 2017). Commercial beeswax is used for metal castings, candle making, as well as in cosmetics, textiles, varnishes and food processings. Related food applications will be discussed in later part.

Other animal waxes include spermaceti (from whale) and wool wax (from sheep). Spermaceti is acquired from the frontal organ in the head of the sperm whale *P. microcephalus*, which is used as a sonar by the animal (Morri, 1973). Back in 15th century, it was used in medicine in England. However, it is no longer produced and is replaced by synthetic spermaceti that consists of pure acetal



**Figure 1** General structure of major constituents in waxes including (A) hydrocarbons (n-alkanes where  $n = 22-36$ ); (B) long chain fatty alcohols (ROH, where R is 12–34 carbon atoms in length); (C) long-chain carboxylic acids (where R is 12–34 carbon atoms in length); and (D) long-chain esters (where  $R_1$  and  $R_2$  are 10–20 carbon atoms in length).

palmitate or mixtures based on jojoba, because of the prohibition of whale capture (Crisp et al., 1984). Wool wax is secreted by the sebaceous glands in sheep skin, protecting sheep skin and fleece against exposure (Horn, 1958). Wool wax finds its intensive application in personal care products, such as facial cosmetics and lip products (Jover et al., 2006).

### Plant Wax

Plant waxes are generally the waterproofing components found in an amorphous layer on the outer surface of the plants. They are essential for plants as barrier protection against environmental stress. Many of them have gained GRAS (Generally Regarded as Safe) status approved by FDA (Marangoni, 2012), allowing for extensive food applications. Typical plant waxes include candelilla wax, carnauba wax, rice bran wax as well as sunflower wax, etc.

**Candelilla wax** is mainly obtained from the leaves of plant *E. antisiphilitica* Zuccarini native to northern Mexican and south-west Texax (Arato et al., 2014). Unpurified candelilla wax contains approximately 42% hydrocarbons, 39% wax, resin and sitosteroyl esters, 8% free wax and resin acids, 6% lactones, and 5% free wax and resin alcohols (Fraps and Rather, 1910). It is interesting to mention that majority of hydrocarbons are odd number in candelilla wax, and only trace quantities are found in even number. Candelilla wax is an FDA-approved food additive used for glazing in certain foods. Microemulsions of candelilla wax are also used as coatings for fruits (Hagenmaier and Baker, 1996).

The main source of **carnauba wax** is the Brazilian palm *Coernicia cerifera* Martius, also known as carnauba wax palm. It is found on the upper and lower surface of the palm leaves. One of the unique characteristic of carnauba wax is that it contains a high proportion of “unusual” chemical constituents such as un-esterified alcohols (12%), x-hydroxy esters (14%) and esters of hydroxylated cinnamic acid (30%), with the remainder being unidentified impurities (Vandeburg and Wilder, 1970). Carnauba wax is one the hardest plant waxes, with melting temperature ranging from 82.5 to 83 °C. Therefore, it is often used as a hardener to elevate the melting temperature of wax mixtures. Carnauba wax is the most commercially important plant wax. It has extensive applications and uses in foods, confectionery coatings, cosmetics, automobile and furniture wax, etc. (Tinto et al., 2017) Food related use of carnauba wax is outlined in the FDA regulation 21 CFR 184.1978.

**Rice bran wax** is another high melting vegetable wax found in husks of rice *O. sativa* (Feuge and Cousins, 1957). It is obtained as a by-product from the de-waxing of rice bran oil. The composition of rice bran wax is relatively homogeneous, with the major components being even-numbered aliphatic acids (wax acids) and higher alcohol esters, mainly C<sub>46</sub>–C<sub>62</sub> esters from C<sub>20</sub>–C<sub>36</sub> fatty alcohols and C<sub>20</sub>–C<sub>26</sub> fatty acids. Other constituents include free fatty acids (palmitic acid), phospholipids, phytosterols and squalene (Ishaka et al., 2014). The hydrocarbon content of rice bran wax is typically as low as 2%. Rice bran wax has been approved by FDA as a safe food additive (21 CFR, 172.890). It has extensive applications due to its relatively low cost and abundance in Asia. Food applications include fruit and vegetable coatings, confectionery and chewing gums. Other applications include candles, textiles, lubricants, cosmetics, etc. (Sabale et al., 2009)

**Sunflower wax** is found in the seed and seed hulls of *H. annuus* (sunflower) (Matyushenskii, 1960). It is obtained through the winterization of sunflower oil. Like rice bran wax, sunflower wax is also a hard, high melting wax mainly consisting of long chain saturated fatty esters (C<sub>40</sub>–C<sub>44</sub>). Sunflower wax has not been approved GRAS status by the FDA. Therefore, its applications are primarily in cosmetics, such as lipsticks, mascaras, lip balms, emulsions, etc. It functions as a consistency modifier and regulates the hardness, texture and mold release of cosmetic formulations. It can also be used as replacement of candelilla wax, carnauba wax and rice bran wax (Tinto et al., 2017).

### Applications of Waxes

Uses of waxes vary from consumer products, such as chewing gum, cosmetics, and candles, to industrial applications, such as hot-melt adhesives for carton sealing, flow modifier for plastic resins, and process additive for suspension polymerization. In regard of food applications, waxes are most widely used in coatings for food products to maintain quality and extend shelf life. They were less used in chewing gums nowadays. Novel applications include utilizing wax crystal networks for oleogelation as some waxes exhibit strong oil binding capacity. Other applications include delivery system such as serving as nanostructured lipid carriers, etc.

#### Traditionally Used as Edible Films or Coatings for Food Products

Mass transfer including water, oxygen and flavor, between food and the surrounding environment is a main cause for food quality deterioration. Edible films or coatings are developed to reduce such transfer and maintain the quality of food products. Lipid-based materials, especially waxes, are the most efficient substances to retard moisture loss and dehydration, due to their high hydrophobicity (Morillon et al., 2002). Waxes have been traditionally used as edible films or coatings for a variety of food products including fruits and vegetables, candies, cheese, egg shells, etc.

#### Advantages of Wax-Based Edible Coatings

**Retardation of moisture permeability:** The retardation of moisture loss and desiccation is one of the mostly notable benefits offered by waxes in coatings. Fresh produce such as fruits and vegetables may suffer from weight loss and loss of marketability because of dehydration. Processed food products may undergo undesirable changes due to water loss. Because of the apolar nature,

wax functions as a barrier against moisture migration and maintain the quality of food products. Waxes, wax emulsions as well as wax mixtures have been successfully applied to reduce moisture loss for a variety of products, including but not limited to blueberries (Chu et al., 2018), pineapples (Li et al., 2018), citrus (Hagenmaier and Baker, 1994; Kellerman et al., 2018), eggplants (Singh et al., 2016), walnut kernels (Kowalczyk et al., 2017) etc.,

**Regulation on internal atmosphere:** Waxing also creates modified internal atmospheres for fruits, which influences fruit metabolism, especially respiration, leading to change in fruit interior quality. Sometimes gentle atmosphere modification is used for fruits that are harvested un-mature and thus extend their shelf life (Baldwin et al., 1997). The efficacy of atmosphere modification depends on the type of waxes used, as waxes with different chemical composition exhibit different gas permeability. Reports showed that both grapefruits and oranges coated with polyethylene wax and carnauba wax had lower CO<sub>2</sub> and higher O<sub>2</sub> content than fruits coated with shellac (Hagenmaier and Baker, 1995). Wax treatment has also been reported to reduce internal browning of pineapple under chilling stress, but the mechanisms is not clear (Hu et al., 2012). In addition, waxing reduces spoilage and decay of fruits and vegetables. It has been reported that fresh cucumbers coated with carnauba wax microemulsion reduced decay by 17% compared to uncoated ones under simulated commercial conditions, with no reduction in weight loss (Baldwin et al., 1997).

**Improvement of appearance:** Waxing treatment improves the overall appearance of food products thus improves the marketability. Some waxes provide a glossy appearance when coated on fresh fruits, vegetables and confectionery products. Use of wax coatings also maintains the strength of peel tissue, avoids surface abrasion during handling of fresh produce, thus reducing spoilage from infection at injury sites. It has been reported that avocado coated with candelilla-based films alleviated the sharp change in color by browning (Saucedo-Pompa et al., 2009). Another study reported that cherry tomatoes coated with rice bran wax delayed the decrease in firmness during 10 days of cold storage (Zhang et al., 2017).

## Novel Application in Oleogelation

### Gelation of Oil by Waxes

In recent years, waxes have been used as effective structuring agents to immobilize a high amount of liquid oil (>95%wt) within a three-dimensional network and creates a semisolid, thermo-reversible soft materials, called oleogels. Compared to traditional hardstock fats, where higher melting, solid TAGs (>0.2) form a three-dimensional crystal networks, oleogelation can be employed as an alternative strategy to formulate lipid-based food products with zero trans fats and significantly reduced saturated fats. In order to form a wax-based oleogel, specific amount of wax is first dispersed in a selected liquid oil, normally at elevated temperature, until the wax crystals completely dissolve. Upon cooling, the high melting wax gelators form crystals of characteristic size and shape. These crystals further assemble into a three-dimensional colloidal network via noncovalent interactions, and entrap the liquid oil within the network through surface tension and capillary force (Co and Marangoni, 2012; Singh et al., 2017).

### Natural Wax Gelators

Among various oleogelators that have so far been explored, natural waxes are of particular interest for their excellent oil-binding properties, capability of gelling oil at low concentration (as low as 0.5%wt), the wide range of properties that can be tailored, economical values (abundance in resources and relatively low in cost), and their natural origin as well as availability of some waxes with GRAS status. So far, most natural waxes that have been found with oleogelation capability are plant origin, such as rice bran wax, carnauba wax, candelilla wax, sunflower wax, sugar cane wax, and berry wax (Mattice et al., 2018). There are also some wax-based gelators from animal sources, the most notable one being beeswax (Ogutcu et al., 2015). The gelation of edible oil by rice bran wax was first reported by the group of Ueno at Hiroshima University, Japan (Dassanayake et al., 2009). It is a highly efficient gelator with a critical gelator concentration (CGC) of 1% (w/w) in canola oil (Dassanayake et al., 2009), and 0.5% wt/wt in soybean oil by controlling cooling rate (Hwang et al., 2012). Sunflower wax is another highly efficient gelator with a CGC of 1% (w/w) in canola oil, and 0.5% (w/w) in soybean oil at specific cooling rate (Hwang et al., 2012). Candelilla wax has a moderate gelation efficiency of 2% (w/w) in canola oil (Blake and Marangoni, 2015). Carnauba wax is considered as one of the least efficient gelators due to the formation of spherical, grain-like crystals with low aspect ratio that are less capable of entrapping oil. Even though, the CGC is 4% (w/w) in canola oil (Blake and Marangoni, 2015). Considering the economical values and the small amount (0.5%–4%, wt/wt) of waxes required to structure liquid oil, these natural wax gelators are very promising in industrial applications on a large-scale basis.

## Physicochemical Properties of Wax Oleogels

Given the complex composition of each wax, it varies greatly in physicochemical properties. No two wax-based oleogels are identical. Therefore, it is of great importance to identify the precise composition of each wax, in order to truly understand how the subsequent gels behave in specific manners. Doan et. al. have conducted a systematic investigation on the chemical profile of some natural waxes and established correlation between their chemical composition and gelling properties. The general chemical composition of some common wax gelators have been shown in Table 1. The order of importance for oil gelation was reported as hydrocarbons > fatty acids > wax esters > fatty alcohols.

The microstructure of wax-based oleogels is also unique to the type of wax used. It has been demonstrated that waxes with more homogeneous composition, such as rice bran wax and sunflower wax, tend to have thinner and needle like fibers or platelets (Lim-pimwong et al., 2017). A more complex system, such as candelilla wax and carnauba wax, allows for co-crystallization of multiple



**Table 1** General chemical composition of some natural waxes investigated through HPLC-ELSD on the Alliance system<sup>a</sup>

(%)	RBW	SFW	BSW	CLW	CRW	BRW
HC	0.29 ± 0.29	0.17 ± 0.15	26.84 ± 1.04	72.92 ± 2.23	0.41 ± 0.30	0.03 ± 0.01
FAL	0.22 ± 0.22	0.32 ± 0.38	6.42 ± 0.90	2.20 ± 1.02	30.74 ± 2.48	4.24 ± 1.10
FFA	6.00 ± 2.12	3.29 ± 0.16	8.75 ± 0.75	9.45 ± 1.14	6.80 ± 0.76	95.70 ± 1.11
WE	93.49 ± 2.63	96.23 ± 0.19	58.00 ± 0.68	15.76 ± 0.35	62.05 ± 3.03	0.02 ± 0.02

<sup>a</sup>HC: hydrocarbon; FAL: free fatty alcohol; FFA: free fatty acid; WE: wax ester; RBX: rice bran wax; SFW: sunflower wax; BSW: beeswax; CLW: candelilla wax; CRW: carnauba wax; BRW: berry wax.

Adapted with permission from Doan, C.D., To, C.M., De Vrieze, M., Lynen, F., Danthine, S., Brown, A., Dewettinck, K., Patel, A.R., 2017. Chemical profiling of the major components in natural waxes to elucidate their role in liquid oil structuring. *Food Chem.* 214, 717–725, © Elsevier.

components, hindering the tight molecular packing and prevent the ordered crystal structure (Morales-Rueda et al., 2009). Such systems usually result in the structural features of a larger scale. Wax-based oleogels exhibit very interesting and complex rheological characteristics of both flocculated suspensions and semi-dilute polymer solutions. The rheological profile is also affected by the chemical composition of various types of waxes. It was found that waxes consisted of saturated fatty acids and high melting fatty acids produce viscous and hard oleogels (Dassanayake et al., 2012).

### Other Applications

Other food-related applications of waxes include plasticizing in chewing gums and fabricating microencapsulation systems for bakery additives (Al-Widyan and Small, 2004; Lakkis, 2007). Microencapsulation of bakery additives allows for better manipulation of dough characteristics during bread-making process. When it comes to medicine drugs or poisonous baits, some natural waxes have been used to preserve the bioactive compounds and achieve controlled release (Tinto et al., 2017; Soleimanian et al., 2018).

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## Introduction to the Volume: Food Adulteration & Contamination

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Protecting consumers from foodborne contaminants that may lead to a possible health risk is vitally important to avoid harm, but also to retain consumers' trust and confidence in our food supply chains. This requires a thorough understanding of the potential contaminant hazards that may be introduced at any of the many steps along the food chain, i.e. from "farm to fork".

This volume of the Encyclopedia entails more than 50 articles, addressing those foodborne hazards identified as most significant in terms of (i) health impact (acute or chronic), (ii) major recent developments in the scientific knowledge and/or regulations in the past years, and (iii) that have a significant degree of public/media attention. The authors have focused largely on "what is new", providing a concise expert assessment that highlights progress as well as current knowledge gaps.

The readers will notice that the topics are spread rather broadly, encompassing food fraud, food defense, natural toxins, process contaminants (and processing technologies to mitigate risks), packaging migrants, genetically modified organisms (GMO), endocrine disruptors, nanotechnology, allergens, pesticides, veterinary drugs, and biocides. Due to the rapid advancement in detection techniques (including untargeted approaches) and data management, selected chapters deal with next generation sequencing (NGS), chemical profiling, "omics", and big data applications. Although most of the chapters are related to chemical hazards, a few have been included that emphasize microbiological risks and associated detection methods.

To avoid food safety issues, mechanisms need to be in place to conduct reliable risk assessments and identify emerging risks timely, and when necessary act on these appropriately. Thus, separate chapters are dedicated to emerging food safety risks and advanced concepts of risk assessment. The management of chemical hazards through HACCP at operational level, as well as proportionate risk management measures, are essential elements to reliably manage and control foodborne exposure to undesired contaminants, with the end goal to protect consumers and build trust in our food systems and supply chains. The legislative landscape particularly for chemical contaminants has evolved nearly exponentially in the past decades, especially within the EU, with unfortunately little alignment visible at a global level and apparent disconnect to the way food materials are traded. In some cases, manufacturers are faced with major challenges in terms of compliance, exemplified by traces of "dual use" substances. These may enter the food chain inadvertently, leading to serious economic consequences if not managed early in the process with the involvement of all stakeholders.

A key topic covered by several chapters in this volume is food fraud, which encompasses a wide range of malpractices, including adulteration, mislabelling, and counterfeiting. Our food supply chains can be highly complex and global in scope, with the possibility of multiple intermediate steps from "farm to fork". Several incidences in the past years have contributed to eroding the trust of consumers in the quality & safety of our foods, such as the "horsegate" scandal that surfaced in 2013. This scandal involved the fraudulent use of horse meat added to beef and not declared as such across several EU countries. The occurrence of traces of the anti-inflammatory drug phenylbutazone in some horse meat samples exacerbated the issue, due to possible health concerns which after testing luckily revealed a low risk to public health. The authorities reacted quickly, introducing EU-wide measures to better control the European meat supply ([EC Recommendation, 2013](#)). In this context, major advancements were also made related to the development of meat speciation testing such as for example NGS technologies as well as mass spectrometry based methods. These methods achieve detection limits down to 0.1% or lower on a weight basis and surpass the traditional Polymerase Chain Reaction (PCR) and ELISA meat speciation tests, in both performance and scope.

To effectively combat food fraud, processes that reach beyond traditional chemical risk-assessment need to be implemented, i.e. a food fraud prevention management system, ideally incorporated within a food manufacturers food safety management system. In economically motivated food adulteration, a plethora of possible fraudulent acts can be considered, mainly at the food raw material/ingredient preparation step. The inherent vulnerability of a material will depend on factors such as its physical state (e.g. liquid, powder), its level of processing, and its composition. In general, the higher the degree of processing, the greater the vulnerability of the material. For example, the composition of a fish powder is far more difficult to verify (particularly by rapid methods) than the whole fish or a single filet ([Cavin et al., 2016](#)). Other drivers that determine vulnerability are the material cost – which incidentally may fluctuate significantly over time – and the degree of supplier engagement/transparency. The latter includes any verification measures that may be in place at the suppliers end to ensure traceability and detect adulteration (mislabelling, substitution, dilution, concealment, unapproved enhancement etc.).

A segment of the foodborne chemical contaminants dealt with in this volume are dedicated to the mycotoxins, representing six major compounds/classes of relevance (aflatoxins, ochratoxin A, zearalenone, fumonisins, patulin, and the trichothecenes). In the past decade, mycotoxins have accounted for 30%–60% of all food and feed rejections at EU borders, signifying challenges in managing these contaminants at a global level. In fact, one additional concern today are the effects of climate change and its impact on fungal growth at the crop stage and toxin profiles, which may shift fungal occurrence patterns more toward southern and central Europe (example, aflatoxins in maize). Besides a more general overview chapter on mycotoxins in food and feed, specific contributions on aflatoxins and ochratoxin highlight latest information on occurrence and associated risks. Research today on mycotoxins continues to focus on the development of both screening and rapid techniques, but also continues to reveal new risks, such as the

“modified forms” that may contribute to the overall human exposure (EFSA, 2014). These modified forms (e.g. the *Fusarium* toxins zearalenone, nivalenol, T-2 & HT-2 toxins, and fumonisins) - that are poorly understood from a toxicologically perspective - are either bound, conjugated or “hidden” (complexed or physically entrapped by macromolecules). Today, there is a clear lack of analytical standards and reference materials, which hinders routine analysis and the gathering of reliable data for risk assessment purposes.

Another intensive area of research is the occurrence of toxic plant alkaloids in foods and their significance to human health, notably the pyrrolizidine alkaloids (PAs) and tropane alkaloids. More than 600 PAs have been identified in roughly 5% of all flowering plants, mainly of the families Boraginaceae, Asteraceae and Fabaceae. PAs have been associated with food poisoning in humans, with hepatotoxic and carcinogenic effects (1,2-unsaturated PAs). Similar to mycotoxins, PAs produce typical « spot » contamination, thereby they are unequally distributed in the raw material and require tedious sampling procedures. In addition, there is a lack of harmonized analytical methods, and no consensus on exactly which compounds to analyse. Similar to PAs, tropane alkaloids are natural secondary plant metabolites (mainly present in the seeds of *Datura* spp.) that may lead to human poisoning. More than 200 compounds (e.g. atropine/hyoscyamine, scopolamine) have been identified, and limits within the EU set for cereal-based foods for infants and young children, at 1 ng/g for each scopolamine and atropine (Commission Regulation (EU) No 2016/239, 2016). The *Datura* seeds are similar to millet/sorghum/quinoa seeds in terms of weight and color, making separation highly challenging.

Of the foodborne chemical contaminants, probably the most studied over the past decade are those formed during processing, particularly when food is subjected to thermal treatment. Consequently, several chapters are dedicated to these substances, i.e. acrylamide, furan/methyl furans, polyaromatic hydrocarbons, biogenic amines, MCPD esters and glycidyl esters, N-nitrosamines, furfuryl alcohol, heterocyclic aromatic amines, advanced glycation end products. These undesired substances may occur at different amounts in a wide range of processed foodstuffs that we ingest on a daily basis, as well as those foods prepared in a domestic (kitchen) environment. Of the substances listed, acrylamide-encompassing several articles - is the most intensively investigated over the past 15 years and deserves this heightened attention. These contributions on acrylamide help to (i) better understand the human health risks; (ii) describe chemical pathways of formation in food; (iii) find ways to mitigate its formation, also at the agricultural stage; (iv) elaborate on measures that can be introduced to reduce levels in the concerned foodstuffs. Importantly, a USA FDA approach in the form of guidance to the food industry is provided. In this context, the EU recently introduced mandatory risk management measures for acrylamide in foodstuffs (Commission Regulation (EU) 2017/2158 of 20, 2017), which on a positive note will undoubtedly accelerate the usage of the FoodDrinkEurope (FDE) acrylamide “Codes of Practice” across the food industry. Regulations have also been drafted for glycidyl esters, that are formed in all refined vegetable oils during the thermally driven deodorization process. The food industry is encouraged to continue research into mitigation options for those contaminants/foods of concern, ideally sharing this information openly in a non-competitive manner, as exemplified over the past 10 years by the “Acrylamide Toolbox” (FDE, 2013), and more recently the MCPD esters/glycidyl esters Toolbox (BLL, 2016).

Reviews on endocrine disruptors, biocides and nanoparticles summarize the status of research in these areas but also highlight the challenges and constraints. In the case of biocides, the use of cleaning and disinfection agents can be considered a part of the production cycle, avoiding the excessive growth of microorganisms. Recently, issues have arisen related to the use of chlorinated sanitizers such as hypochlorite and the presence of the undesired byproduct chlorate. Produce such as vegetables washed with chlorinated water will retain up to 20% of the chlorate present in the wash water (or potable water in case chlorinated), leading in most cases to amounts above the current EU legislative limit for chlorate in foods (10 ng/g). The risk of use (byproduct formation and exposure) versus benefit (microbial reduction or elimination) requires careful evaluation prior to introducing any maximum levels, as the avoidance of highly effective sanitizers may lead to more serious, acute microbial risks with possibly fatal consequences.

We have chosen to allocate several chapters to allergens, that also warrant careful consideration in economically motivated fraud. For example, a material/ingredient may be partially substituted with an allergenic food, risking serious injury to health or even fatalities. Better food labelling to address inconsistencies among countries, identifying thresholds of elicitation of allergy symptoms, and regulatory gaps in how to deal with unintended allergen contamination of foods are topics discussed in these contributions. A dedicated chapter has been included on rapid and confirmatory testing methods for allergens, including current deficiencies in the analytical methodology and how these could possibly be overcome.

Other “hot topics” in this volume are the pesticides, with emphasis on the evaluation process and impact of MRLs on global trade, a much disputed issue. Excellent regulatory overviews and/or recent analytical developments are presented in chapters related to acrylamide, veterinary drugs, allergens, GMOs, and the dioxins/polychlorinated biphenyls.

Modern analytical techniques continue to evolve in terms of sensitivity, coverage of analytes and speed of analysis. Analytical equipment is becoming more “affordable”, easier to use and the data can be interpreted faster. Researchers will undoubtedly continue to detect undesired chemicals or residues of contaminants in foodstuffs at low to trace levels, but need to understand that not all chemicals or hazards need to be managed, i.e. may not represent a health concern. What is lacking today is a clear prioritization of the risks and agreement on management measures, taking into account the feasibility to reliably measure the contaminants, as well as mitigate and effectively lower the levels of exposure.

The food industry has an important role to play in being transparent with new scientific findings, so that food regulators are better equipped to control undesired contaminants, while not inadvertently placing undue restrictions on their citizens’ food supplies.

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## New Breeding Techniques: Detection and Identification of the Techniques and Derived Products

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### Glossary

**Agroinfiltration** an *Agrobacterium* based method used to induce transient expression of genes in a plant, generally to express a protein

**Cis-genesis** a transgenesis, with DNA sequences transferred between individuals from the same species, the same pangenome

**CRISPR-endonuclease** a technique using initially Double strand DNA endonuclease and RNA guide to modify genome or epigenomes

**Graft** the result of the insertion of a shoot or twig into a slit of a living plant, named the rootstock, from which it receives sap for further growth.

**Epigenome** originally a set of chemical modifications of DNA and chromatin's proteins that modify the genes' expression. Can also be used to also encompass epitranscriptomic modifications.

**Epitranscriptome** a set of biochemical modifications of the RNA (the transcriptome)

**Floral dip** a genetic modification, without plant tissue culture or regeneration, by modified *Agrobacteria* infiltrated through vacuum

**Intragenesis** a transgenesis, with DNA sequences transferred between closely related organisms.

**ODM** Oligonucleotide directed Mutagenesis. A generic terme covering several techniques of chromosomal or episomal genetic modification by introducing chemically synthesized oligonucleotide to modify targeted sequences

**Pangenome** very complex genomic context due to large variations in gene and regulatory elements contents among closely related varieties or ecotypes

**RdDM** RNA-directed DNA methylation. An epigenetic modification of DNA (methylation) due to the action of natural or introduced small interfering homologous RNA

**SDN** Site Directed Mutagenesis. A targeted genetic or epigenetic modification due to endonuclease such as "native" or modified ZFN, TALEN or Crispr-endonuclease

**TALEN** Transcription activator-like effector nucleases. Initially an artificial complex made with a double strand endonuclease domain fused to a DNA binding domain for targeted genetic and epigenetic modifications

**Transgenesis** a set of methods to introduce exogenous nucleic acids, either from distantly or closely related organisms, for genetic and epigenetic modifications by random or targeted insertion

**ZFN** Zinc-Finger Nuclease. Initially an artificial complex made with a double strand endonuclease domain fused to a DNA binding domain for targeted genetic and epigenetic modifications

### Introduction

The world of detection, analytical traceability still faces many challenges, whether to specify the geographical origin of a product, prevent adulteration of food by non-compliant products or pathogens, or the multiplexing of isothermal amplification methods.

In this chapter we focus on another challenge of detection and identification, the one what the Advocate General of the European Court of Justice recently advocated to consider as GMOs, but maybe exempting it of risk assessment and traceability/labeling, if no recombinant DNA was used in the modification processes of the species considered (Goldstein, 2014). These are 'new techniques of breeding' (NBT), i.e. of genome, epigenome and epitranscriptome modifications.

The challenge, beyond the easy and cheap documentary traceability which can easily be set up in the supply chains once big retailers wish it, is to both detect the final product of these NBT techniques and especially, in front of a non-traced element, to identify the NBT technique initially used.

The NBT techniques as grouped by the European Commission in the 2007 first report of the Dutch COGEM include several techniques that may lead to epigenome (RdDM, interfering RNA) modifications of mutations with intentional or not epimutations (epimutation of DNA and chromatin, or of RNA = epitranscriptome), sequences insertions ... ex: ODM, meganucleases, ZFN, TALEN and CRISPR systems for genome modification and epigenome (Claeys and Vialatte, 2016), transient or stable expression systems of transforming nucleic acids (agroinfiltration, floral dip ...) new formats of traditional processes for which the nature of the components varies (such as the graft of a non-GM scion on a GM rootstock), or new denomination of techniques according



to the origin of genetic sequences (cis-, intra- and trans-genesis), and finally a set of techniques grouped under the generic term of synthetic biology which was later on omitted.

Many of the techniques and molecules considered can be combined with each other or modified (e.g.: double-stranded endonuclease transformed into nickase) and involve the expression, penetration with or without the stable or provisional insertion of nucleic acid molecules, in the form of DNA (e.g. plasmids) and/or RNA (e.g. mRNA) and/or ribonucleoproteins complexes. In this later case, most authors did not ensure that RNP were free of contaminating DNA, a defect common to many, even commercial, preparations of purified proteins.

This short document does not allow to detail each of the specific characteristics to identify the implemented technique. We will also not discuss non-intended changes of organelle genomes or extrachromosomal DNAs despite their retrotransposition and persistence capabilities as episomes (Koo et al., 2018; Lanciano et al., 2017).

Finally, this chapter will not focus on the assessment of inherent risks, while several areas such as epigenetics remain largely unknown (EFSA et al., 2016).

## Overview

It is often argued that organisms contain many mutations, making them both 'Natural GMOs' and 'unable' to distinguish NBT induced mutations induced from so-called 'natural' mutations.

## Genomes Stability

Due to the evolution, genomes are essentially stable.

Evolution has indeed implemented a large number of genome protection mechanisms against transposable elements, invasive DNA ... and more generally non-self recognition systems, such as immunity in animals or PAMP in plants (Boller and He, 2009; Boyd et al., 2013; Iriti and Faoro, 2007; Liu et al., 2017b; Muthamilarasan and Prasad, 2013). As for prokaryotes, the Crispr-endonuclease defense system and the NHEJ, MMEJ ... are only some of the numerous elements of genomes' repair and protection against invasive DNA (Doron et al., 2018).

Plants like animals protect themselves from mutations not only by isolating germ cells with low reproductive rates during the early development, but also somatic cells through "stem cells" such as apical and axillary meristems. The mechanisms in place explain the very low rate of mutations transmitted to offspring even by old plants and old parts of plants as it can be observed for several years with old trees (Burian et al., 2016; Grant and Mitton, 2010; Lanner, 2002; Ledford, 2017; Sarkar et al., 2017; Watson et al., 2016). Millennia during which it is intuitively difficult to think that they could accommodate a growing number of genetic mutations ...

The work on the Napoleon's oak on the campus of the University of Lausanne is a very good example of this resistance of plant genomes to mutations and thus of their elimination during cells' cycles (Kuhlemeier, 2017; Schmid-Siegert et al., 2017).

Cellular systems repair DNA damages even the neutral mutations that are generally the most numerous to 'survive'. This corresponds well to the very modest rates of the residual spontaneous mutations found in both prokaryotes and eukaryotes (Badran and Liu, 2015; Lynch, 2010).

Given the very low known values of the spontaneously appeared mutations which are kept (Oksman-Caldentey and Barz, 2002), it is therefore wrong to say that apart from stressful situations (ranging from meiosis to the physical stress of mutagenic radiation, high temperature or drought, to those induced by cell cultures and mutagenic chemical products) many mutations persist in plant tissues. Moreover, it is well known that, in addition to the lack of reproduced experiments, the rates of spontaneous mutations differ according to the study methods used, the species and parts of the genome considered.

Apart from a selection pressure and a better "fitness" granted by some rare mutations or epimutations, conserved naturally born mutations therefore appear empirically very low and mostly neutral before disposal, as it can be seen in plants with vegetative reproduction.

## The Circulation of Informative and Detectable Molecules

A recurrent question in varietal selection, revived by the NBT, concerns the remote influence of a gene or of an epiallele, or of its expression products, thus beyond the cells in direct contact via the plasmodesma?

The outstanding long-distance information between the various parts of the plant, but also of animals via e.g. the placenta, between a parasite plant and its host and finally of the ingested nucleic acids on the animal's genes is now well documented (Avramidou et al., 2015; Citovsky and Zambryski, 2000; Fuentes et al., 2014; Goldschmidt, 2014; Hannapel et al., 2013; Lewsey et al., 2016; Shahid et al., 2018; Springer and Schmitz, 2017; Stegemann and Bock, 2009; Stephenson et al., 2018; Thieme et al., 2015; Zhang and Hu, 2017). Information is conveyed via hormones, proteins, electrical signals, but also nucleic acids (mRNA, miRNA, siRNA, large DNA fragments ...). More than 3000 genes are involved in grapevine in such transmissions at different frequency s according to the environment (Yang et al., 2015).

Finally, on the contrary of pompous titles (Kyndt et al., 2015), horizontal gene transfers that participate in evolution remain rare in eukaryotes. They cannot suggest that “GMO by horizontal transfer” are the norm, especially when the responsible living organisms differ (Chen and Otten, 2017).

It is therefore possible to find inheritable genetic, epigenetic and epitranscriptomic modifications in the distal parts of the rootstocks, and therefore in the detached fruit.

## Detection and Identification Techniques

Detection methods, aimed at ensuring a difference, can be used to identify an organism or techniques at its origin.

### Target Detection Methods

The methods of detection, qualitative and/or quantitative cover a very broad spectrum of techniques allowing

- (i) the observation of phenotypes (seed germination in the presence of a herbicide),
- (ii) the study of gene expressions (immunology, transcriptomics, exome sequencing),
- (iii) the detection of coding or non-coding genetic targets by signal or target amplifications (DNA or RNA), followed or not by hybridization ... (PCR, LCR, LAMP, NASBA, HIP, <sup>1</sup> Q  $\beta$  Replicase, SDA, <sup>2</sup> RCA, <sup>3</sup> RPA, <sup>4</sup> HDA, <sup>5</sup> NEAR, <sup>6</sup> SHERLOCK<sup>7</sup> ...), isotherms or not, <sup>8</sup> with or without a combination of techniques and targets (singleplex to multiplex, SNPLex, DNA chips after amplification ...), from single nucleotide variant (SNV) to large size with edge fragments with high fidelity DNA polymerases, helicase, nickase or recombinase (Barabaschi et al., 2016; Chang et al., 2012; Daher et al., 2016; Thomas et al., 2014; Zhang et al., 2017).
- (iv) the sequencing of modified or not DNA and RNA (Sanger method, high throughput sequencing according to various technologies and platforms [multiparallel, single molecule] thus genomes (whole or reduced to the exome), epigenome, transcriptome and epitranscriptome, per tissue or single cell) (Holst-Jensen et al., 2016; Pauwels et al., 2015).

Finally, chromosome walking, asymmetric PCR, *in silico* or effective genomic subtraction between genomes or transcriptomes are available as needed (Darrasse et al., 1994; Fu et al., 2010; Gong et al., 2018; Pawelkiewicz et al., 2017; Tengs et al., 2009).

Many of the necessary devices are available in miniaturized forms thus allowing amplification or sequencing to be used from the laboratory to the field.

These techniques allow unambiguous detection and identification over a very wide range of sequences, from the point mutation of an SNV to the insertion of sequences of any size (as for GMOs resulting from transgenesis), from chromosomal rearrangements to multiple copies of sequences. It is thus possible to unambiguously detect, on the contrary of what was asserted by the some people, by SNPLex, LCR or OLA, alone, with or without hybridization of the amplicons, any point modification in a specific 20 nt sequence of a genome (Beerli et al., 1998; Gaj et al., 2013).

These tools for studying polymorphism are relatively easy because they are often accompanied by other tools, for example data bases, statistics and decision tools (Schmid and Bennewitz, 2017; Willems et al., 2016).

Various combinations of techniques make it also possible to increase the sensitivity of the detection (enrichment by prior scavenging of target sequences, DNA chips, prior chromatographies, etc.), biosensors (DNA chips, etc.) with for example Raman spectrometry (Arulandhu et al., 2016; Kadam et al., 2017). All the detection techniques mentioned in this article and several other ones not listed here can be used in detection/identification of the products and techniques used, as they are in marker-assisted selection.

Results are generally gathered in genetic maps for varietal improvement, barcoding or meta-barcoding, and genotype/phenotype relationship identification. The choice of techniques (simplex vs. multiplex, junction of large junction fragments or SNV) to implement, targets to combine (genetic, epigenetic, etc.) according to the uses and costs will be largely facilitated by the use of databases, artificial intelligence, statistical tools and decision support systems (Dobnik et al., 2018; Marschall et al., 2018).

### Strategies for Identifying NBT Techniques and Traceability Targets for Derived Products

The majority of the reports and articles dealing with the subject of the detection of NBT products or the identification of the techniques at their origin do not establish, voluntarily or otherwise, a clear distinction between (i) the needs of the operators of the sectors, (ii) those of the owners of patents and producers of NBT products and (iii) those of the competent authorities in charge of the labeling of GMOs or the withdrawal from the market of unauthorized GMOs in the EU. Finally, a constant confusion has been maintained between (i) traceability, in the ISO documentary meaning, an easy and inexpensive procedure to implement when a large retailer requires it, (ii) detection that is limited to ensure the presence/absence of a detectable element, and (iii) the determination of the origin (NBT or “natural”) or the owner (producer of this GMO).

Confusion has thus been rapidly settled between the routine techniques used for enforcement, such as detecting the edge fragment PCR of GMOs derived from transgenesis, and detection and identification strategies prelude to routine analyzes.

It is therefore necessary to precisely distinguish the objectives sought as well as the targets (proteins, nucleic acids of the DNA, exome, epimutations, metabolites, etc.) and the techniques used (detection of phenotypic or genetic modifications, by amplification or sequencing or spectrometry ...) in combination or not.

The deployment of the variety of detection and identification methods used is always part of technical strategies that vary according to the purpose. Depending on the importance (significant proven risk, unauthorized product entering the EU area, high cost of products possibly to be withdrawn from the market, etc.), various methods performed by personnel of different qualification levels can be mobilized. Feedback from laboratories is important, hence the importance of the guidelines and meetings of the European ENGL network<sup>9</sup> with its national network heads.

Considerations of habit, cost, ease of use, available materials and staff training are not absent in the choice of techniques and the willingness to detect and identify targets, especially in the period of budget restriction of these last two decades.

Lastly, the States have favored self-checks in private companies in recent decades, with the enforcement laboratories of the Member States intervening, with ever-decreasing resources, only on a case-by-case basis (crises, doubts, legal procedure, etc.).

## The Matrix Approach

Detection and identification techniques of an organism or technique can rely as much on unambiguous signatures as on convergent clusters of evidence. A strategy more and more considered by risk assessment agencies under the name of "weight of evidence". Such a multi-target approach has been developed under the name of matrix approach in the case of the detection of known GMOs, for reasons of cost and speed of analysis, and unknown, supported when necessary by decision support systems (Dobnik et al., 2018; Holst-Jensen et al., 2009, 2013).

These convergent clusters of evidence can then be developed, combining technical and target, according to the needs and requirements of analysts and ordering party (complete control, routine traceability, legal framework with reinforced evidence, certainty *versus* speed, cost optimization. with documentary traceability ...).

More generally, the matrix approach is analogous in its principle to the anthropometric methods developed by Bertillon (Farebrother and Champkin, 2014). This principle is also the basis of biometric methods for digital fingerprint identification, facial and iris recognition, or DNA fingerprinting as used by the police (Jeffreys, 2013).

The data gathered for the matrix approach of GMOs identification, issued or not from NBT techniques, are nothing more than indices like those collected by the technical and scientific police and whose evidence can be statistically evaluated (Aitken, 2018; Kirk, 1953).

The matrix approach is therefore nothing more than a particular use of polymorphism, similar to that breeders use in markers assisted selection.

## Detection and Identification Targets

### Stresses Impacts and Consecutive Scars

The implementation of NBT techniques involves the use of the same set of old related techniques used for transgenic GMOs. Namely, the preparation of plant protoplasts or tissues, cells cultures, the vectorization of molecules so that they reach their nuclear target, generally a selection of the transformed cells followed by the elimination of the selection factors, new cells cultures for callus and seedling regeneration, all for the few non recalcitrant plant species (Marx, 2016b; Yau and Stewart, 2013). All these stressful techniques, sources of somaclonal variation, leave detectable scars in the genomes and epigenomes (Altpeter et al., 2016; Ledford, 2016; Miguel and Marum, 2011; Neelakandan and Wang, 2012).

These remaining scars have long been valued by seed producers in varietal selection (Karp, 1995). Conversely, these breeders attempt to reduce the effect of these unwanted mutations and epimutations in nuclei and organelles, generators of off-type during *in vitro*-propagation (Miguel and Marum, 2011; Rout et al., 2006).

Again, the identification of these scars results in genetic maps and marker-assisted selection irrespective of the target cell compartment (Bonev and Cavalli, 2016; Ciabrelli and Cavalli, 2015; Dekker, 2016; Dekker et al., 2017; Elsner, 2017).

These characteristic scars of cell lineages are obviously not specific to plants. Any change even a point mutation, like SNV,<sup>10</sup> usually affects the 3D structure of genomes, genes regulation, conformation of proteins, epistasis ... inducing various genomic disorders, factors of diseases as cancer for animals (Bianco et al., 2018; Editorial, 2018; Hehir-Kwa et al., 2016; Li et al., 2015a; Marx, 2017; Mendizabal et al., 2014; Niel et al., 2015).

In animals, these scars, which can be induced even by the immersion oil of the microscopes and may affect the development of the embryo, are known to be caused by various diagnosis and monitoring effects of preimplantation diagnoses and *in vitro* fertilization (Cyranoski, 2017; Dirix et al., 2005; Guil and Esteller, 2009; Hayward et al., 2017; Martinez et al., 2017; Marx, 2016a; Mulero-Navarro and Esteller, 2008; Scarpa et al., 2017; Zhao et al., 2014). More generally, all *in vitro* manipulations, including those used in assisted reproduction induces, by their epigenetic effects, Large Offspring Syndromes (Chen et al., 2017d; Ventura-Juncá et al., 2015).

There is therefore no domain or organism for which stresses, especially *in vitro* ones, do not induce inheritable scars which are identifiable, traceable and can be organized in patterns.

### The Persistence of Scars, Signatures and Unintended Effects During Backcrossing

Most of the changes intentionally or unintentionally induced by the related techniques, or NBT are transmitted to their cells offspring thus allowing the identification of products and techniques to their origin.

Indeed, the number of backcrosses effectively performed with Elite varieties for the marketed modified products is generally below the 6 backcross (Ferreira et al., 2016) necessary for a ca 95% of genomic “purification”. Moreover, far from these theoretical statistical values, the number of backcross needed to ‘clear’ a genome varies enormously with the species and the varieties considered, the number of loci to be separated, the relationships between loci that can be located in large non-Mendelian segregated blocks or co-segregate with unwanted loci, and the genomes’ size which may be allopolyploids (Wang et al., 2017c, 2018a, 2018b). These cleaning issues of genome cleaning are particularly true for the Elite varieties originating from selection process which may have led to fitness decreases (Bertheau, 2016; Kremling et al., 2018).

In the genomes can thus persist millions of ‘uncleanable’ base pairs (over 500 million in the case of *Triticum aestivum*), all detectable in subsequent generations (Batista et al., 2017; Holland, 2007; Hollick, 2017).

Finally, vegetatively propagated plants or those resulting from micropropagation, due to either phytosanitary or mass production reasons cannot ‘benefit’ of such ‘cleaning’, even partial, of their generally polyploid genomes and epigenomes (Bado et al., 2017).

As a result, much, if not the majority, in several instances, of unintentional mutations and epimutations, caused in a variety modified by any of the related techniques or NBT have a very good probability of ending up in its offspring.

### Limitations of the Identification Techniques of Unintended Changes

Numerous authors using NBT are claiming that they either drastically reduced the number of unintended changes (up to a 15000 FACTOR), i.e. generally off-targets, or they were unable to detect one.

In view of these claims it should be reminded that the majority of them are restricting their searches for cost reasons to partial sequencing of areas predicted by still imperfect off-target software (Cyranoski and Reardon, 2015; Haeussler et al., 2016; Kodama and Komamine, 2011; Tycko et al., 2016). Because of the currently important associated costs, most authors do not perform whole genome sequencing, especially in the case of species without a reference genome. Finally they do not search for epigenetic and epitranscriptomic modifications.

With the unexhaustive searches the authors are thus missing numerous mutations, including structural ones such as chromosome inversions, and exon skipping (Anderson et al., 2017; Kapahnke et al., 2016; Xiao et al., 2013; Zhao and Wolt, 2017).

The search for genetic, epigenetic and epitranscriptomic modifications due to related techniques and NBT therefore requires complete and reliable sequencing of genomes and epigenomes. Which is currently far from being the case.

### Sequencing

Genome sequencing is not just a simple alignment of nucleotides for which many errors already exist due to systematic biases of platforms for example (Alic et al., 2016; Goodwin et al., 2016). The multiple readings needed from deep-sequencing and now from ultra-deep-sequencing, not to mention ‘coverages’ generate large amounts of data whose analysis requires trained personnel, time, high-performance computers and thus important funding (Dahlö et al., 2018).

The work continues with the annotation step, again to be drastically improved, in order to detect point mutations and indels, variations in the number of copies and structural variants, not to mention the relation with functional changes (Hwang et al., 2015; Poplin et al., 2018; Xu, 2018). The proliferation of sequencing programs of thousands of plant varieties or animals shows how far we are from having reliable reference genomes, even for humans or important food species (Hehir-Kwa et al., 2016; Kehr et al., 2017; Wang et al., 2018c; Weller et al., 2017).

The same goes for epigenomics and transcriptomic variations related to alternative splicing and epitranscriptomic variations. If a number of sequencing errors could be overcome by avoiding tissue, i.e. multicellular, sequencing in favor of isolated cells (Ranasinghe et al., 2018), there is also much progress to be made (Yang et al., 2012).

Apart from the still costly whole genome sequencing, chromosome walking, asymmetric PCR, *in silico* or effective genomic subtraction between genomes or transcriptomes can be mobilized to discern more quickly and at a lower cost the differences between modified and conventional genomes (Darrasse et al., 1994; Fu et al., 2010; Gong et al., 2018; Pawelkowicz et al., 2017; Tengs et al., 2009).

The detection of unintentional changes therefore requires more sequencing than the one presented in the majority of published articles (Hardwick et al., 2017; Lam et al., 2012; O’Rawe et al., 2013).

### Sequence Analysis

Assembly, alignment and sequence comparison give rise to many errors in the absence of good reference genomes, errors also enhanced by the presence of repeated sequences.

Only a few rare species better studied, for which large funding has been spent have already been committed, and allow fairly reliable analysis (Shendure et al., 2017; Sims et al., 2014). Guidelines have yet to be enacted as to what to know and do (Domínguez Del Angel et al., 2018) and not only in the field of medical diagnostics (Santani et al., 2017). This need is found even for the

easiest sequencing such as exomes with 'gold standard' because of the current software flaws (Chowdhury and Garai, 2017; Diroma et al., 2017; Eisenstein, 2017; Goldfeder et al., 2016; Hwang et al., 2015).

Computers and analysis software as well as the training of staff are sources of many approximations that cannot ensure the absence of off-targets and other mutations in most, if not all, cases of NBT induced changes.

### Sequence Databases

Finally, the sequences databases still contain numerous errors of both sequences and annotations. Many improvements, including a drastic curation of databases, are still needed, even with 'gold standard' before asserting the absence of unintended effects (Jones et al., 2007; Zischewski et al., 2017).

More generally, the deficiencies detected in the quality of sequencing and the need for quality control and reproducibility enhancement led to the Massive Analysis and Quality Control (MAQC) society (Shi et al., 2017). It is also envisaged to develop aptitude tests and to set up a laboratories' accreditation system as in many other areas of detection (Davies et al., 2016; Hardwick et al., 2017).

While it is therefore necessary to remain skeptical about claims of no unintentional changes following the use of random or targeted mutagenesis techniques, we can expect when taking into account these limitations to discern many of these modifications in order to identify NBT techniques and analytically trace their products.

### Identification of Detection Targets

Historically, random mutagenesis techniques, applied to cell cultures or not, preceded the more or less targeted mutagenesis which evolved for plants from random transgenesis to more targeted NBT techniques.

### Effects of the Old Related Techniques

Apart from the graft, the NBT techniques employ the same intermediate *in vitro* steps, and their variants, as those used in transgenesis, techniques that we will call later *related techniques* (Ghosh et al., 2017; Heidebrecht, 2017; Lowder et al., 2016; Ran et al., 2017; Smith, 2017; Weeks and Yang, 2018).

These steps include protoplast production or tissue isolation for cell cultures, transformation with NBT products (ex: DNA, RNA or RNP system Crispr-endonuclease) generally vectorized (see below) which must pass the plasma and nuclear membranes while damaging the organelles' genomes (Liu et al., 2017a; Marx, 2016b; Mout et al., 2017; Thakore et al., 2018; Wang et al., 2016).

Then the few modified cells have to be selected before the selection system is more or less easily removed, by site-specific recombinase systems like Cre-Lox accompanied with the usual removal scars. Finally, after a new phase of cell culture, the whole process of cell transformation finishes with a callus then seedling regeneration step of the few non-recalcitrant plant species.

This set of *in vitro* techniques leaves sets of genetic and epigenetic scars, whose use, under the name of somaclonal variation of this somatic embryogenesis, has been a source of genetic diversity for plant breeders for decades.

### Scars of Cell Cultures and Seedlings Regeneration

This somaclonal variation stemmed from DNA mutations and epimutations of DNA, chromatin and RNA associated with the mobilization of transposable elements generating thus unstable genomes and epigenomes with hypermutations (Altpeter et al., 2016; Aydin et al., 2016; Kaeppler et al., 2000; Krishna et al., 2016; Marx, 2016b; Miguel and Marum, 2011; Neelakandan and Wang, 2012; Stroud et al., 2013; Yau and Stewart, 2013).

This somaclonal variation, making these plants GMOs according to the European 2001/18 directive, has never been considered in the risk assessments, nor labeling and post-market monitoring obligations even for GMOs issued from transgenesis (EFSA et al., 2016; Schouten et al., 2017).

These old related techniques have very little evolved since the beginnings of transgenesis in the 80–90s. They always require a laboratory specific know-how that can be lost due to lack of specific training, as it was recently noted in a recent conference (Ledford, 2016).

As a stress memory, the epigenomes influence the 'choice' of proliferation or quiescence of the daughter cells (Kedziora and Purvis, 2017; Yang et al., 2017). The estimation of the duration required to erase this 'memory' of stress depends on the techniques used to evaluate it.

The effect of such *in vitro* stress on isolated cells or multicellular clusters on their further development as bodies and reprogramming are well documented for animals (Ventura-Juncá et al., 2015). The intracellular injections are similar, not only by the sizes of the involved molecules, to the delivered "packages" of nucleic acids, proteins and/or ribonucleoprotein complexes of NBT.

It is only recently that the richness and unpredictability of unintentional changes has been better documented.

The *in vitro* resulting genetic and epigenetic modifications differ from the natural ones. For instance inheritable CHH<sup>11</sup> hyper-methylations are observed in *Arabidopsis* and rice (Springer and Schmitz, 2017) in which the mutation frequency (SNP, indels, base substitutions) is multiplied by a factor 248 compared to that, spontaneous, of *Arabidopsis thaliana* (Miyao et al., 2012). *Arabidopsis* cell cultures also induce more cytosine methylation, particularly in the heterochromatin regions, with in addition to class modifications of the small interfering RNAs (Tanurdzic et al., 2008).



Compared to wild rice, cell cultures enrich the epigenome by inheritable hypomethylated CG (Stroud et al., 2013), as observed in several other cases (Berdasco et al., 2008; Kumar and Van Staden, 2017; Tanurdzic et al., 2008). Other unintentional changes such as duplication genome result in tetraploid with methylation patterns differing from diploids (Springer and Schmitz, 2017).

We could multiply the number of examples of inheritable genetic and epigenetic modifications induced by related techniques but not addressed in the current risk assessments procedures (Arpaia et al., 2017; Filipecki and Malepszy, 2006; Neelakandan and Wang, 2012; Wolt, 2017). Yet epigenetics is increasingly seen as providing a significant part of progress in varietal selection, when it is not considered as a cause of heterosis (Gallusci et al., 2016; Jaligot and Rival, 2016; Springer and Schmitz, 2017).

Markers of *in vitro* cultures are therefore numerous, detectable (Bairu et al., 2011) and easily organized as the markers of assisted selection.

### Reagents' Delivery Scars

As for transgenesis issued GMOs most NBT use vectorization systems which damage cells, as for instance the very large molecule Cas9 DNA, RNA or RNP.

We thus find residues of these delivery systems (viruses, *Agrobacterium* ... but also biolistic, PEG, electroporation, lipofection with or without nanoparticles ...) targeting the site of action (nucleus and organelles) as DNA of plasmids, encoding or not RNA, or the recent RNP. These RNP use partially purified protein with contaminating recombinant DNA, which can be inserted in the genomes, as it was previously observed even for the commercial highly purified DNA polymerases used in PCR (Altpeter et al., 2016; Cameron et al., 2017; Chen et al., 2015; Engelke et al., 1990; Gelvin, 2003, 2008; Iulia et al., 2013; Kim et al., 2017; Songstad et al., 2017; Ulker et al., 2008; Witt et al., 2009).

*Agrobacterium* remains still the most effective vector (Pitzschke, 2013) with always many genetic and epigenetic traces. Those traces often small and difficult to detect originate not only from the plasmids, but also from the bacterial genome or from extrachromosomal circular elements never taken into account in the risk assessments of transgenesis issued GMOs (Gelvin, 2008; Ito and Machida, 2015; Schouten et al., 2017; Singer et al., 2012). The mechanism of integration of T-DNA is still unknown even if it seems to integrate preferentially into active genes with additional epigenetic modifications, which can facilitate the discovery of the scars of this reagents' delivery system (Bourras et al., 2015; Gelvin, 2017; Shilo et al., 2017).

Aborted natural infections of *agrobacteria* can be distinguished from those resulting from *in vitro* techniques by the scars' sequences and the bacterial origin (Chen and Otten, 2017).

These reagents' delivery scars are all accountable for determining the use of *in vitro* techniques.

### Scars of the Transformed Cells Selection Systems and Their Removal

Despite an improvement in transformation efficiencies, the selection of modified cells and the subsequent excision systems such as Cre-Lox or R/RS is still relevant (Germini et al., 2018; Manimaran et al., 2011). We thus find the usual scars of these selection systems, possibly with 'episomes' over several generations (Cheng et al., 2014; Scabill et al., 2008; Srivastava and Ow, 2003; Zhang et al., 2016b).

In conclusion, depending on the size, the type and location of inserted fragments, patterns, genetic and epigenetic maps can therefore be established for related techniques in use for NBT (Alkan et al., 2011). Moreover, the improvement of the algorithms, which for instance make it possible to differentiate microorganisms within complex species mixtures in metagenomic studies, should still benefit the establishment of these genomics and epigenomics patterns of scars left by related techniques (Albanese and Donati, 2017).

All these reviewed scars and other ones are constitutive of the identification tool of *in vitro* techniques.

### Random Mutagenesis Markers

Induced mutagenesis results either from the random action of mutagenic physical or chemical agents, or *in vitro* mutagenesis by random insertion, or finally by targeted modifications of the genomes and epigenomes (Oladosu et al., 2016). Among these methods, *in vivo* methods always result in chimeras (Bado et al., 2017). On the contrary the *in vitro* cultures can benefit from the totipotency of plant cells resulting in homogenous modified organism.

The *in vitro* application of chemical agents such as EMS,<sup>12</sup> ENU<sup>13</sup> or physical, be there *in vivo* or *in vitro*, such as UV or neutron flux,<sup>14</sup> induce mutations types depending on the agents used as well as the doses and duration of application. Various signatures, such as the types of mutation and epimutation caused, such as homo- or hetero-zygotes point mutations and transversions, their frequency of occurrence and location, at relatively constant or random intervals, can characterize the constitutive elements of the random mutagenesis, basis of Tilling and its variants (Blumenstiel et al., 2009; Doitsidou et al., 2016; Flibotte et al., 2010; Henry et al., 2014; Lehrbach et al., 2017; Li et al., 2001, 2015b; Roberts and Gordenin, 2014; Smith and Yun, 2017; Songstad et al., 2017; Till et al., 2007).

The induced mutation and epimutations rates exceed by several orders of magnitude those of spontaneous mutations, which can lead to hypermutation situations (Botstein and Shortle, 1985; Roberts and Gordenin, 2014). Again, the types of the provoked mutations and hypermutations can in themselves be signatures of past events (Fleta-Soriano and Munné-Bosch, 2016; Jaligot and Rival, 2016; Probst and Mittelsten Scheid, 2015; Wibowo et al., 2016). They also provide a higher level of knowledge through density differences e.g. in the form of hotspots, by the possible vicinity of changes, their positioning in areas frequently transcribed or not, the participation or not to structural rearrangements ...



This information constitutes a huge information source from the past of organisms, and thus of the mutagenesis techniques and culture conditions previously applied. However, this information is very rarely exploited, in addition to the organelles' scars due to stress are never taken into account (Chen et al., 2017; Sablok et al., 2017).

Genome and epigenome sequencing and omics can now complement or replace the polymorphism patterns (Batista et al., 2017; Endo et al., 2015; Fonseca et al., 2015; Henry et al. 2014, 2015; Li et al., 2016).

As with somaclonal variation, breeders collect the information in genetic and epigenetic maps (Doitsidou et al., 2016; Flibotte et al., 2010; Lehrbach et al., 2017; Li et al., 2001; Revollo et al., 2018). Due to the cost and information management difficulties these polymorphism profiles are often reduced in practice to some SNPs and QTL phenotypes more easily manageable. They are nonetheless identifiers of the technique initially and thus can be used to monitor the product in selection schemes and then in production (Daher et al., 2016; Godbout et al., 2017).

The increasing use of isogenic and single cells and of their sequencing will uncover more and more genomic and epigenomic scars issued from *in vitro* manipulation. The inheritable scars could help establishing cell lineages, histotypes, cell lines stratifications, and other tissue subtyping usable for identifying all kind of cell manipulations (Alexandrov et al., 2016; Chen et al., 2017b; Hayward et al., 2017; Kuipers et al., 2017; Lal et al., 2017; Letouzé et al., 2017; Nawy, 2017; Pennisi, 2018; Pfeifer, 2016; Villani et al., 2017; Wang et al., 2017a, 2017b; Zou et al., 2018).

There are thus clearly no technical barriers to differentiating between organisms with spontaneous natural mutations from random *in vivo* and *in vitro* induced mutations.

### Signatures of the NBT Techniques Used

In this short document we will only consider a few general aspects of these many NBT techniques, with multiple combinations and numerous modifications resulting in very numerous variants (eg: double-stranded cleavage activity of a nuclease transformed into a nickase or a methylase activity added to he nuclease activity).

Many NBT techniques initially focused on DNA modification before targeting the epigenome. However, all nucleic acid sequence change, whether of endogenous or exogenous origin, seeks to be accompanied by epigenetic signature (Sullivan et al., 2015). Epigenetics seems again to perform a cellular memory of all stress, including genetic manipulation, usable even after cisgenesis and intragenesis (Henikoff and Greally, 2016).

NBT techniques using mainly (except Floral dip and graft and some applications of RNAi resulting in chimeras (Shim and Kwon, 2010)) *in vitro* techniques unless producing chimaeras, their products are discernible *ab initio* from those resulting from spontaneously arising mutations. The challenge to differentiate NBT techniques themselves from derived products appears attainable...

It is therefore only a question of bringing together in some relevant subsets some of the scars and signatures left by the different techniques used to produce the NBT derived produce. Thus, essentially to perform the very classic work of any scientist involved in genetic diversity analyses, such as a breeder.

The detection/identification targets will in part vary with the type of NBT involved. The scientific and technological watch as conducted for ca. 2 decades by the ENGL laboratories will therefore remain relevant.

For example, in the case of grafting a non-GM scion onto a GM rootstock, it will be wise to first look for the DNA, mRNA and small RNA circulating from the GM part to the non-GM part or in both directions (Avramidou et al., 2015; Citovsky and Zambryski, 2000; Cuerda-Gil and Slotkin, 2016; Gallusci et al., 2016; Hannapel et al., 2013; Hovel et al., 2015; Koepke and Dhingra, 2013; Lewsey et al., 2016; Panda et al., 2016; Sharma, 2013; Zhang and Hu, 2017). But any other circulating signal influencing the distal parts of plants and animals can also be used in the initial phase of identifying the technical origin of the product (Savadi, 2017; Servick, 2017; Springer and Schmitz, 2017; Thieme et al., 2015). These epigenetic changes induced in the scions by the circulating RNAs allow the induction of canonical or not RdDM mechanism in addition to the activation of transposable elements.

Some quantification is foreseeable in a near future (Eisenstein, 2017; Li et al., 2017). Of course, for traceability purposes the signals originating from the GM parts will be more actively sought in the detached non-GM parts, such as fruit.

Generally speaking, NBT such as interfering RNA and oligonucleotides cause numerous false positives and false negatives (Heigwer et al., 2018; Jackson and Linsley, 2004; Kodama and Komamine, 2011; Mockenhaupt et al., 2015; Smith et al., 2017; Xu et al., 2006). All these unintended mutations and epimutations, as well as off-target effects are difficult both to predict and detect (Casacuberta et al., 2015; Riba et al., 2017).

We also frequently find vectorization residues (Sarita et al., 2017). It is likely that the tender<sup>15</sup> for a systematic review launched in 2016 by EFSA will provide a better understanding of the scars, signatures and other unintended effects left by non-coding RNAs and by extension on all types of interfering RNA and OdM. In the case of these interfering RNA the uncover of the 'seed regions' of the off-targets will facilitate the identification of the use of interfering RNA (Buehler et al., 2012; Das et al., 2013; Marine et al., 2012; Sigoillot and King, 2011).

Generally speaking, distal off-target effects (mutations and epimutations) therefore contribute to the set of signatures in addition to the targeted changes (Khan et al., 2016. Ramesh, 2013; Sullivan et al., 2015; Thakore et al., 2016).

On another issue, the use of nucleases, such as CRISPR-Case9 generates traceable indels inherited by the daughter cells, with even some specific 'patterns' such as the occurrence frequency and rather specific sizes (Junker et al., 2016. Songstad et al., 2017). These results are similar to those highlighting cells genealogies bearing genomic signature of the origin and history of the cells as imprinted alleles (Callaway, 2017; Frumkin et al., 2005; Junker et al., 2016; Kalhor et al., 2017; Rodrigues and Zilberman, 2015;

Sulston et al., 1983; Waters et al., 2013; Woodworth et al., 2017). Unintentional changes that can lead to a single barcode (Nawy, 2018; Williams, 2016).

If the transcriptome sequencing of isolated cells is still problematic for establishing cell lineages, the current technical improvements offer hope that tracking all the cells and their offspring will become accessible (Smaglik, 2017).

For SDN techniques based on Crispr-endonuclease targeting either DNA or RNA the signature of the technique can be constituted by the proximity (approximately 3–4 nucleotides) of a nuclease specific PAM sequence and of the desired mutation (within a target sequence of about 20 nucleotides). Recurrent detection of differences (on target and off-targets) and close PAM<sup>16,17</sup> sequence [s] would form a strong predictor, not to say an unambiguous signature of the employed technique. As PAM sequence vary among Cas nuclease, the analyst could even predict the kind(s) of the used nuclease (s). The suspicion of the artificial origin of the on-target mutation(s) will be reinforced by the type and frequency of off-targets still present, detection of exon skipping and structural mutations, the indel profiles already mentioned, and finally a large set of scars due to the *in vitro* related techniques. Some variants, such as modifications to change the double strand nuclease into a nickase, which require the proximity of 2 PAM, will increase the univocal side of the modifications signatures (Kleinstiver et al., 2015; Zhang et al., 2016a). The proxy-Crispr tool developed by Merck requires a succession of PAM sequences also providing an unambiguous signature of the NBT used (Chen et al., 2017a).

These SDN changes limited to the proximity of PAM sequences explains the race for discovering new endonucleases to expand the accessible part of genomes as well as the sequences' size to be integrated. Without going into details, such recognition sites also are discernible for the meganucleases, and TALEN as well as ZFN systems, be they canonical or modified.<sup>18</sup> The efficiency and specificity of ZFN and TALEN are indeed dependent on the sequence context as for the other SDN technologies, sequences usable as signature (Gaj et al., 2013; Gao et al., 2018; Luo et al., 2016; Sander and Joung, 2014).

Finally, as part of a matrix approach, we shall remember that stable modifications, particularly knocking and gene stacking, do not occur anywhere in the genome. Breeders are therefore looking for these 'safe harbor' that we can use in our matrix approach (Cantos et al., 2014; Rudgers and Sastry-Dent, 2015; Songstad et al., 2017).

If we remember that the bacterial adaptive immunity system introduce foreign sequences in genomes (ca 85% for archaea and ca 50% for eubacteria genomes), we cannot be surprised that genome changes induced by CRISPR-Cas9 be also 'saved' in the genomes.<sup>19</sup> These stress recordings would lead to a kind of barcode through a succession of mutations induced in Crispr sequences (Duarte, 2016; Kupferschmidt, 2016; McKenna et al., 2016; Nawy, 2016). The induced changes, and probably any biological event including environmental signals (Borkowski et al., 2016; Roquet et al., 2016), could therefore be tracked as it seems to be the case for humans (Perli et al., 2016) and bacteria (Barrangou and Doudna, 2016; Shipman et al., 2016; Wang and Qi, 2016).

Spontaneously occurring and induced univocal signatures therefore seem to be available for identifying NT and tracing their products. These biological recording systems of modifications might be imposed on laboratories and companies. To protect their patented products, there is nothing to prevent seed companies from developing an official registration system for SDN changes and other technical stresses (Ledford, 2018; Sheth et al., 2017; Tang and Liu, 2018). But the polymorphism patterns we presented above for related techniques and NBT could be sufficient in a more cost-effective way.

A large set of signatures and scars specific to the use of NBT techniques are therefore accessible by conventional polymorphism studies for the identification of NBT at the origin of a product. Signatures and scars which can complete with the previously considered ones.

### The 'Matrix Approach' for Differentiating *In Vivo* and *In Vitro* Modification Techniques and for the Identification of NBT

We previously saw that several elements, scars and signatures, could be linked to *in vitro* and NBT techniques. The next step is now to rationally organize them into patterns for unequivocally identifying the NBT and their products. It is essentially the day to day job of scientists in charge of studying genetic diversity and breeders in charge of exploiting it.

The availability of prior knowledge of what to target makes detection and identification easier and cost-effective. However, this knowledge does not constitute yet a prerequisite for initial identification. Here we meet strategies developed for identifying unknown objects or persons from clues as done by forensics and biometrics using e.g. facial and iris recognition, or fingerprinting.

Detection and identification can indeed much rest on unequivocal relationships with an element, like the fragments of the edge of the current transgenesis issued GMOs, that result from a body of convergent evidence, indirect presumptions and knowledge as it was done for detecting unknown GMOs by the matrix approach (Bohanec et al., 2013; Holst-Jensen et al., 2013).

Data bases, such as CRAVAT, decision support systems, data mining and artificial intelligence helps the analyst to gather the numerous data of polymorphism, select the relevant combinations of markers and typing, put into place the appropriate detection tools according to the purpose (Bohanec et al., 2016; Dobnik et al., 2018; Golestan Hashemi et al., 2018; Guinney et al., 2015; Holst-Jensen et al., 2016; Mallah et al., 2017; Paape et al., 2018; Roberts and Gordenin, 2014).

Agricultural production already uses such 'big data' approaches, whether for field diagnosis of deficiencies or diseases, or improvement of marker plants. Marker-assisted selection with or without high-throughput phenotyping uses strategies, statistical analysis software and other tools similar to those used to identify NBT techniques and products (Bhat et al., 2016; Crossa et al., 2017; Jahufer and Luo, 2018; Martinelli et al., 2015; Poland and Rutkoski, 2016; Valente et al., 2013; Varshney et al., 2016).

It is therefore surprising that the European Commission has not followed up on the proposal made in 2013 by its ENGL network to develop tools for the identification of NBT and derivatives. The proof of concept would have been available at the time of deciding the status of NBT derived products, while currently markets could be overloaded by consumers not desired products.

Socio-economic and political considerations are probably the cause of this failure to meet the labeling and information needs of consumers.

### Distinction Between Natural and Induced Mutations

It is often argued that targeted mutations due to NBT techniques are 'natural' and therefore indistinguishable from those spontaneously arising. Assumption under which the identification of techniques would be impossible and the traceability of products questionable.

As we have seen before, it is more of a political assertion than a scientific truth.

There are several ways of considering a mutation and thus of determining whether its origin is natural or induced, according to the philosophical definition that one wants to take from nature. Claiming the 'naturalness' of a mutation is of the same line of reasoning that asserting the current climate change is natural and not due to anthropic changes.

More prosaically, such an assertion puts forward only the tree that hides the forest.

The probability of occurrence of mutations and their maintenance in the absence of selection pressures are so low (the estimated probability of a mutation in the order of a substitution at a given moment by sampling natural populations would be from 0.01 to 0.0001 [Wakeley, 2008](#), [Watterson, 1975](#)), that human intervention is necessary to accelerate the occurrence of genetic diversity, as recognized by all plant breeders. In view of such probability values, the simple increase of mutations frequency is a constituent element of the artificial character of the mutations and therefore participates in the convergent body of evidence.

Finally, more generally, considering a mutation *per se*, isolated from its context, cannot constitute by itself a sufficient factor of proof of naturalness. As usual in logic, we must indeed consider all available information such as genomes and epigenomes modifications, their frequencies, their types ... associated to this element. In short, we need to consider a situation analogous to that of biometric identification systems such as fingerprints, iris recognition or facial recognition for which several indices converge toward the identification of an individual, and consequently of his past, as in the matrix approach developed above.

Logically, and according to the very similar approach of forensic medicine, any assertion, and more generally the formulation of a proposition, is admissible only according to the context ([Hicks et al., 2015](#)). Taking into account this context, there is therefore no difficulty in distinguishing a mutation spontaneously appeared, in non-particularly stressful conditions, from an induced mutation such as those that we have been preoccupied with so far.

### Detection in Routine

As usual, routine detection use only a relevant subset if not a single element of the initial identification matrix.

#### Quantification

The quantification required for labeling in the EU can be ensured for the majority of technical methods to modify genomes and epigenomes listed above, such as for Crispr-endonuclease induced mutations ([Harayama and Riezman, 2017](#)).

Qualitative methods can determine contents against a predetermined threshold through multiple control plans such as those used in seed quality control ([Kobilinsky and Bertheau, 2005](#); [Laffont et al., 2005](#); [Remund et al., 2001](#)).

#### Costs and Feasibility in Routine

Documentary traceability is a tried- and- tested method that can only be put in motion by the will of a few large operators such as supermarket chains who face the transparency demands of consumers ([Bertheau, 2013](#)). The market will do the rest ...

Traceability remains the selected method of supply chains for its ease of implementation, with or without blockchain as recently experimented in China by Carrefour, for its very low cost and control by operators, but it cannot protect consumers from frauds. Analytical controls and therefore the methods for detection and identification remain indispensable.

We have previously seen that methods for identifying NBT techniques exist and are already used in a similar form by breeders and seeds' companies. Routine detection methods are only simplified derivatives of this set of polymorphism's analyzes. These routine techniques, the majority of which use methods such as PCR or NASBA, which are well known to GMO control laboratories, can therefore be quickly taken over for validation by the EURL-GMFF, with the support of ENGL laboratories, and then directly applicable by all operators at all stages of the supply chains, thanks to the miniaturization of devices since 2001 ([Marx, 2015](#)).

To give an idea of the evolution of costs, it may be recalled that sequencing companies have recently announced they would provide in a short time full genome sequencing costs equivalent to the current PCR detection tests, i.e. 100 to 250 euros, list price, depending on the variant requested. Miniaturized amplification or sequencing devices such as the Oxford Nanopore MinIon allow field studies in the laboratory.

Legal and technical frameworks are all in place to ensure that the reference material is provided and analytical traceability of NBT products is quickly implemented as requested by consumers.

## Conclusion

Proof of concept of the ability to identify NBT techniques at the origin of certain products should be readily available as soon as the European Commission decides to provide the means as in the late 1990s with research programs on transgenesis issued GMOs.

As 30 years ago for transgenic GMOs, the analytical traceability and labeling of NBT products is technically accessible; it is part of a political choice and therefore partakes in the balance of power between stakeholders.

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<sup>1</sup>Hinge initiated primer dependent amplification (HIP).

<sup>2</sup>Strand Displacement Amplification (SDA).

<sup>3</sup>Rolling Circle Amplification (RCA).

<sup>4</sup>Recombinase Polymerase Amplification (RPA).

<sup>5</sup>Helicase-dependent amplification (HDA).

<sup>6</sup>Nicking Enzyme Amplification Reaction (NEAR).

<sup>7</sup>Specific High-sensitivity Enzymatic Reporter unLOCKing (SHERLOCK).

<sup>8</sup>Thus needing cheaper amplification apparatus, usable on field

<sup>9</sup>European Network of GMO Laboratories.

<sup>10</sup>Single Nucleotide Variant, point mutation often used for characterizing an organism by its SNP as for instance locating a trait such as QTL (Quantitative Trait Locus).

<sup>11</sup>H étant A, C, ou T.

<sup>12</sup>Ethyl-methane sulfonate: transitions C-to-T and G-to-A (GC-to-AT), point mutations.

<sup>13</sup>N-ethyl-N-nitrosourea: A-T base transversions and AT-GC transitions (Nolan et al., 2002). And GC-AT transitions.

<sup>14</sup>Small deletions.

<sup>15</sup><https://www.efsa.europa.eu/en/consultations/call/160412>.

<sup>16</sup>Protospacer Adjacent Motif: DNA sequences necessary for anchoring the Cas9 (or Cpf1...) double strand endonuclease or nickase (single strand endonuclease after an enzyme modification)

<sup>17</sup>Depending on the Crispr-Cas variant used (or another modified or not enzyme) techniques, one or more specific PAM sequences of the enzyme(s) used may be required and therefore found in a close vicinity of the (intended or unintended) mutated sequence(s).

<sup>18</sup>For producing ZFN or TALEN derived nickases...

<sup>19</sup>Not surprisingly, the Crispr systems are originally a system of bacterial "adaptive immunity" inserting new sequences of aggressive phages in the bacterial genome. ....

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# Biogenic Amines in Food: A Review of Factors Affecting Their Formation

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## Glossary

**Decarboxylases** enzymes which remove a carboxyl group from organic compounds, i.e. catalyzing the decarboxylation of amino acids leading to the production of the correspondent amines.

**Secondary metabolic pathways** Specialized metabolisms, which produce small molecules that are not required for microbial survival

**Non-thermal treatments** Food processing methods that are alternative to heat processing and which do not use heat to enhance microbial safety. These methods can reduce microbial contamination, while retaining the sensory attributes and nutrient content similar to raw or fresh products.

## Overview

Biogenic amines (BAs) are organic bases, which can be present in foods and cause several adverse reaction in the consumers. They are produced by microorganisms (mainly bacteria) through the action of decarboxylases, which are enzymes selectively acting on specific amino acids by removing the carboxyl group with the formation of the correspondent amine and CO<sub>2</sub>. The most important BA in foods and the relative precursors (shown in Fig. 1) are histamine, tyramine, 2-phenylethylamine, tryptamine, cadaverine and putrescine, being this latter a polyamine obtained through a direct decarboxylation of ornithine or through the agmatine deiminase pathway, which follow the decarboxylation of arginine to agmatine (Landete et al., 2008a; Marcobal et al., 2012; Wunderlichová et al., 2014). In addition, other polyamines (spermine and spermidine) can be produced with a more complex pathway that starts from putrescine (Bardócz, 2005).

The decarboxylative pathways are activated as cell response to acid stress, due to their contribution to maintain intracellular pH. In addition, BA production can lead to the energization of the membrane proton-motive force, with the transfer of a net positive charge outside the cell, bringing supplementary energy. In this framework, such decarboxylations belongs to the secondary transport systems responsible for electrogenic exchanges which can support the primary metabolism in environmental critical conditions (Konings, 2006; Pereira et al., 2009; Perez et al., 2015).

The presence of BA in food can constitute a risk for the consumer since they may cause severe health effects. Ingestion of food containing high amounts of BA is implicated in various pharmacological and toxicological reactions, i.e. headache, heart palpitations, vomiting and diarrhoea. Due to their relatively low threshold toxic levels and the severity of symptoms they may cause, the most dangerous BA are histamine and tyramine, which are responsible for symptomatology known as "scombroid fish poisoning" and "cheese reaction", respectively (Hungerford, 2010; McCabe-Sellers et al., 2006).

Several bacteria are able to produce BA. Among Gram negative, enterobacteria and pseudomonads have the greatest potential in the accumulation of histamine, putrescine and cadaverine (Landete et al., 2008a; Morii and Kasama, 2004; Wunderlichová et al., 2014). Anyway, BA are accumulated also by Gram positive bacteria. In fact, the decarboxylase activity has been described in members of several microbial groups, such as staphylococci, *Bacillus* spp. and, especially, Lactic Acid Bacteria (LAB). In particular, this latter group includes the most important tyramine producers (enterococci and some lactobacilli) (Marcobal et al., 2012). Moreover, the production of other BA (namely histamine and putrescine) has been observed in LAB strains (Landete et al., 2008a; Wunderlichová et al., 2014). It is also important to underline that, in many cases, the activities of decarboxylases are expressed independently of cell viability and that these enzymes are particularly resistant to harsh environmental conditions, even after the lysis of the cells responsible for their production (La Gioia et al., 2011; Rossi et al., 2011; Gardini et al., 2012).

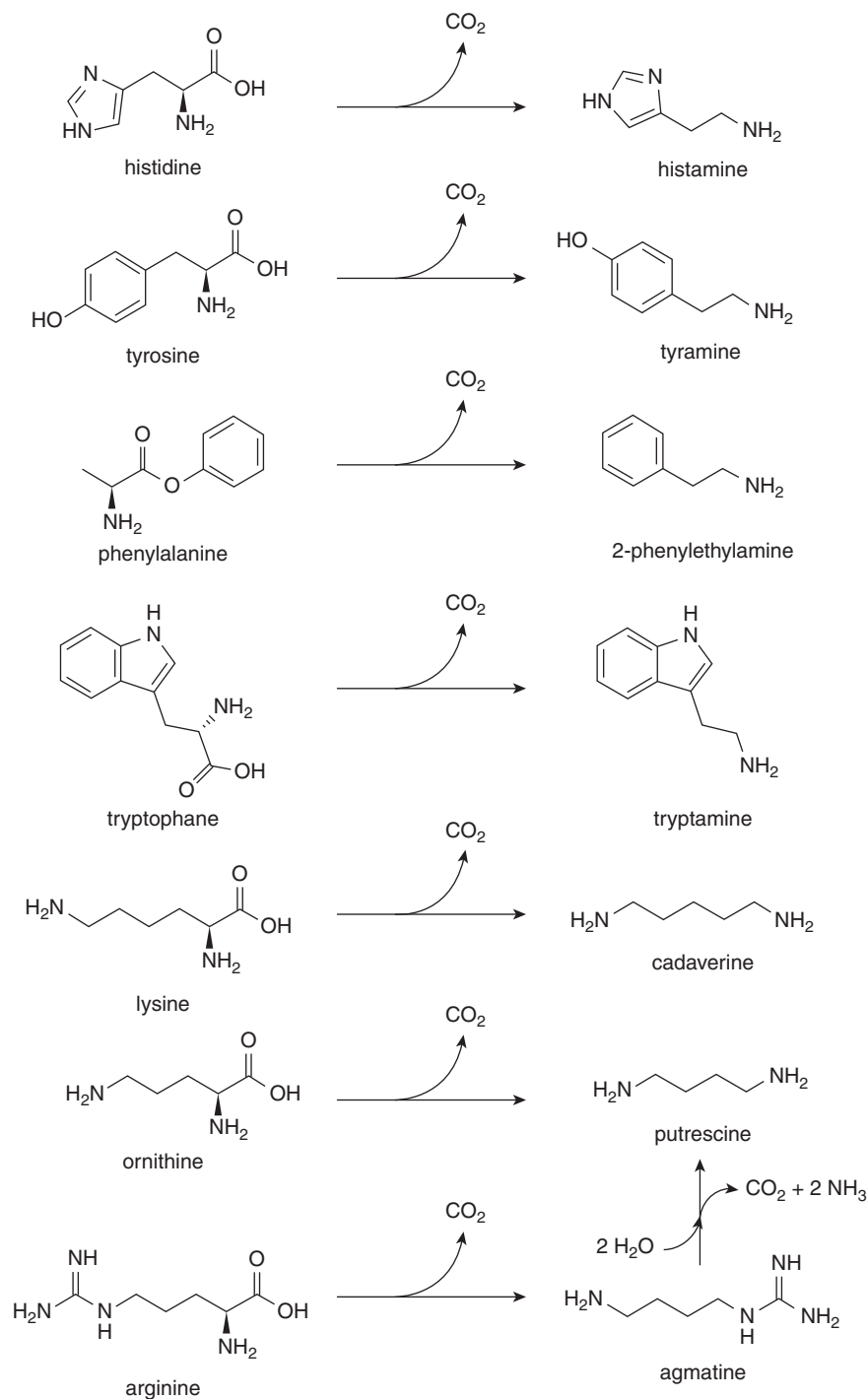
The genetic organization of decarboxylase clusters has been reviewed for tyramine (Marcobal et al., 2012), histamine (Landete et al., 2008a) and putrescine (Wunderlichová et al., 2014). Generally, enzymes responsible for specific amino acid decarboxylation are organized in operons in which some genes are present, such as those responsible for the production of the decarboxylase, the antiporter permease (amino acid/BA) and a correspondent amino acid-tRNA synthetase.

## Occurrence in Foods

### Non-fermented Foods

The presence of BA is dependent on the precursor availability in food (proteins and/or free amino acids) and their contamination by decarboxylating microorganisms. In non-fermented foods, these compounds have been related to poor food hygienic quality because are associated with a massive growth of spoilage microorganisms (>7 log cfu g<sup>-1</sup>) with decarboxylating activity. For





**Figure 1** Most important biogenic amines in foods and their relative precursors.

this reason, several Authors proposed BA content as a microbial quality index, as indirect indicators of excessive microbial proliferation (Özogul and Özogul, 2006; Ruiz-Capillas and Jiménez-Colmenero, 2004).

In particular, scombroid fish (such as tuna, sardines, anchovies, mackerel, etc.) have been associated with cases of histamine intoxication because of their high histidine content and the proliferation of histidine decarboxylating Gram-negative bacteria (i.e. *Photobacterium* spp. *Enterobacteriaceae* such as *Morganella morganii*, *Enterobacter aerogenes*, *Raoultella planticola* and *Klebsiella oxytoca* and *Aeromonas hydrophila*) (Knöpe et al., 2014; Prester, 2011). Regarding fresh meat, when properly stored, it contains only low levels of spermine, spermidine and putrescine, which directly derive from the animal, but an improper storage can



promote the growth of an environmental Gram negative microflora that can produce high concentrations of cadaverine and histamine (Jairath et al., 2015).

### Fermented Foods

On the other hand, fermented foods are often characterized by the presence of different BA. The accumulation of these compounds is mainly caused by decarboxylase positive non-starter microflora, which can be present in the raw materials and in the environment and can massively grows when spontaneous fermentations occur or during the ripening period (when the selected starter cultures can be replaced by wild strains) (EFSA, 2011). BA, and in particular histamine and tyramine, can be accumulated in relevant amounts in dairy products, mainly in ripened cheese and in those obtained from raw milk. These products represent a suitable matrix for BA accumulation especially for the precursor availability (deriving from casein proteolysis) and the presence of wild microflora, which can prevail during ripening phases. BA concentrations up to 2000 mg kg<sup>-1</sup> has been detected (Linares et al., 2011).

Fermented sausages are another suitable substrate for BA accumulation. The presence of high precursor concentrations and, usually, the impossibility to eliminate the original spontaneous microflora are the reasons for high BA content, mainly tyramine, found in some cases in these products, especially in those obtained without the addition of starter cultures (Suzzi and Gardini, 2003; Latorre-Moratalla et al., 2010).

### Alcoholic Beverages

Differently from cheeses and sausages, the presence of precursors in wine and beer is relatively scarce. Nevertheless, the high amount of ethanol, which inhibits the action of the natural liver detoxifying enzymes for BA (amino oxidases), enhances the toxicity of BA. Then, also small amounts of these substances may have negative effects on people consuming alcoholic beverages (Ancín-Azpilicueta et al., 2008). The accumulation of BA, mainly histamine, cadaverine and putrescine, in wine is attributed to the metabolism of LAB and, for this reason, the activity of the bacteria responsible for malolactic fermentation has to be strictly controlled. Also fermented vegetables can be a favorable environment for the accumulation of BA. These aspects have been studied particularly in sauerkraut (Rabie et al., 2011) and in table olives (Medina-Pradas and Arroyo-López, 2015).

### Regulation aspects

Concerning the regulatory aspects regarding the presence of BA in foods, there is no legal regulation with the exception of fishery products, for which the maximum acceptable level of histamine is defined by several national and international Authorities. The European Commission Regulation (EC) 2073/2005 and successive amendments indicated a maximum histamine amount between 100 mg kg<sup>-1</sup> (m) and 200 mg kg<sup>-1</sup> (M) (n = 9, c = 2) in fishery products from scombroid species. The level is increased to 200 mg kg<sup>-1</sup> (m) and 400 mg kg<sup>-1</sup> (M) for fishery products subjected to enzyme maturation in brine (EC, 2005).

EFSA (2011) conducted a qualitative risk assessment concerning BA in fermented foods in the European Union. According to the conclusion of this investigation, no adverse health effects have been observed for the consumption of 25–50 mg of histamine per person per meal, even if smaller amount of this BA (few mg) can be dangerous for patients with histamine intolerance. The level of attention for tyramine is consistently higher (600 mg per person per meal). This dose is drastically lowered in individuals using third generation monoamino-oxidase inhibitor drugs, which should not be exposed to levels higher than 50 mg per person per meal.

### Factors Influencing the BA Formation in Foods

Several intrinsic and extrinsic factors can affect the formation of BA in foods (Gardini et al., 2016; Naila et al., 2010). Anyway, the first requirement to reduce the risks due to BA is related to the microbiological quality of the raw materials, and, in particular, to the absence or low presence of wild microorganisms with specific decarboxylases activities. In addition, amino acid decarboxylases responsible for BA production are generally stable and active under a wide range of environmental conditions, even when they are released in the medium after death and lysis of the cells that produced the enzymes. The increase of BA due to the presence of cell free extracts of culture able to produce decarboxylases has been demonstrated *in vitro* and in food such as cheeses (Gardini et al., 2016).

### Environmental Factors

The main environmental factors affecting microbial activities in foods are temperature, *a<sub>w</sub>* (and then salt concentration) and pH. These same factors affect BA formation by influencing both the metabolisms of microbial cells and/or the activity of decarboxylase enzymes independently on the cell viability.

Each microbial species presents its own optimum temperature and can grow in a more or less wide temperature range. This is obviously true also for decarboxylating microorganisms. Anyway, the better conditions for cell growth do not necessarily coincide with the optimum condition for BA production. In addition, the optimum conditions for BA accumulation by pure enzymes or cell free extracts do not always coincide with those characterizing living cells. The interaction between these two aspects is responsible

for the final BA amount. For example, in a tyraminogenic strain of *Enterococcus faecalis* inoculated in model system, after 24 h of incubation the maximum activity of the decarboxylating enzyme was detected at 37 °C, while the maximum tyramine content due to cell metabolism was found at 20 °C (Bargossi et al., 2015). This can be explained by the fact that the tyrosine decarboxylase is more active under harsh conditions for the cells, which require supplementary energy to counteracting stress conditions. In any case, the ability to produce BA is generally limited by the decrease of temperature and thus, the control of the cold chain during food storage and commercialization remains one of the main tool to avoid the accumulation of these undesired products after manufacturing. In the case of fermented foods, the temperature adopted in the first days of fermentation is crucial for the potential BA accumulation. In fact, in this first step of process, the members of the microbiota characterizing the ripening are selected (Gardini et al., 2008).

Increasing salt concentration significantly contributes to the limitation of the decarboxylating activities. However, the amounts of salt needed to control the BA production are often too high for food applications (Tabanelli et al., 2012). This is particularly true for LAB responsible for tyramine production (such as enterococci) and their decarboxylases, which can maintain their activity even at concentration higher than 20% (Bargossi et al., 2015). Gram negative aminobiogenetic bacteria are more sensitive to NaCl. Species belonging to *Photobacterium* spp. enterobacteria or pseudomonads are limited in BA production by salt concentration of 4%–5%, even if their decarboxylases are still active in these conditions (Morii and Kasama, 2004). Nevertheless, it has been demonstrated that initial salt concentrations of about 5%, which are added in some traditional meat fermented products, can significantly contribute to the limitation of BA production. However, such concentrations are not compatible with the trend of the industry to reduce NaCl in foods.

Since decarboxylation is a cellular activity to counteract acidic stress, several studies were addressed to exploit the relationship between pH and BA accumulation. Also in this case, the effect of pH is different if the decarboxylase activity of living cells, pure enzymes or cell free extracts is considered. From a genetic point of view, the transcription of many decarboxylase cluster genes is induced by low pH and favors the cell reaction to acidic stress (Marcobal et al., 2012; Perez et al., 2015). Tyrosine decarboxylases showed their maximum activities generally at pH comprised between 5 and 6 (Bargossi et al., 2015; Moreno-Arribas and Lonvaud-Funel, 2001). By contrast, the maximum tyramine accumulation of living LAB cells is always observed at pH lower than these values (between 4 and 5), as an active stress response. Optimum pH for histidine decarboxylase in Gram negative bacteria (*Photobacterium* spp and *M. morganii*) are close to the neutrality (between 6 and 7) (Kanki et al., 2007) while histidine decarboxylase of *Streptococcus thermophilus* has an optimum at pH 4.5 (Tabanelli et al., 2012).

As reported above, in general, the literature data indicate a great variability in the cell decarboxylase responses to the environmental factors. This is due not only to the intrinsic differences between bacterial metabolic pathways but also to the matrix considered and to great heterogeneity characterizing decarboxylase activity, even within strains of the same species.

Due also to this heterogeneity, in fermented foods the possibility to act on environmental factors in order to modulate BA production is often limited by the fermentation and ripening conditions and by health trends, as in the case of the reduction of NaCl content. For this reason, in this kind of product, the main tool to counteract BA accumulation is the choice of starter cultures without decarboxylating activity, able to rapidly and persistently colonize the environment and showing the capacity to inhibit or reduce the growth of aminobiogenetic wild microorganisms through a rapid acidification. Moreover, some starter cultures can produce antimicrobial compounds such as bacteriocins or other substances active against pathogens but also against aminobiogenetic bacteria. Recently, it has been demonstrated that the use of nisine producer strains of *Lactococcus lactis* can limit BA production by *S. thermophilus* and *E. faecalis* (Tabanelli et al., 2014).

### Technological Factors

Given that the ability to produce BA is often a strain rather than a species feature, in the last year a particular attention has been posed in the selection criteria of starter cultures, which are genetically and phenotypically screened to be sure of the absence of amino acid decarboxylase potential.

Linares et al. (2012) and Latorre-Moratalla et al. (2012) reviewed the use of selected starter cultures aimed to limit BA accumulation in dairy products and in fermented sausage production, respectively. In general, the use of the so-called autochthonous starter cultures, i.e. strains isolated from the same type of fermented product, reduced BA concentrations in these products. Also in wine, the choice of selected LAB strains unable to produce BA is essential for malolactic fermentation process in winemaking (Moreno-Arribas et al., 2003).

Beside to their role as a starter, some bacteria can be exploited for their potential in BA degradation. In fact, many microorganisms, among which LAB, can produce amino oxidases, which are the enzymes responsible for BA detoxification. Alvarez and Moreno-Arribas (2014) reviewed these aspects and reported this ability for some *Lactobacillus*, *Pediococcus* and *Oenococcus* strains, while Herrero-Fresno et al. (2012) showed that the use of two strains of *Lactobacillus casei* able to degrade histamine and tyramine could reduce the accumulation of these BA in Cabrales-like mini-cheeses. In matrix poor in BA precursors (such as wine) some Authors reached better results with the use of *Lactobacillus plantarum* able to deaminate BA (Capozzi et al., 2012).

However, it is well known that the use of selected starter cultures alone cannot be sufficient to assure the reduction or inhibition of BA production in fermented foods. For this reason, other technological factors can be considered for controlling amino acid decarboxylation, i.e. the use of antimicrobial substances and technological additives.

It is reported that BA accumulation is higher in nutritionally poor environments, being decarboxylative activities secondary metabolic pathways (Konings, 2006). Several authors tested *in vitro* BA production in relation to sugar supply. *S. thermophilus*

produced increasing amount of tyramine in the presence of limiting amounts of lactose (0.1%) (La Gioia et al., 2011) while increasing fructose and glucose concentration inhibited histamine accumulation of *Lactobacillus hilgardii*, *Pediococcus parvulus* and *Oenococcus oeni* (Landete et al., 2008b). In many fermented foods, the sugar amount added can modify the equilibrium among the microbial populations including decarboxylating bacteria. Bover-Cid et al. (2001) found that sausages produced without the addition of sugar in minced meat mixtures had significantly higher tyramine and cadaverine concentrations, concluding that sugar omission is detrimental for product quality and safety.

Preservatives can reduce the BA formation through the inhibition of biogenic bacteria growth and interfering with the equilibrium among microbial populations (Gardini et al., 2016; Naila et al., 2010). In fermented sausages, the presence of potassium sorbate, ascorbic acid and nitrites affects BA formation (Naila et al., 2010). Also sulphur compounds were used to control BA accumulation and, in particular, the addition of sulphur dioxide (SO<sub>2</sub>) in wine after malolactic fermentation prevented amine formation by aminobiogenetic LAB, due to the inhibiting effect on cell metabolism rather than to the repression of the decarboxylases activity (Landete et al., 2008b).

Natural antimicrobial compounds, such as essential oils and spices, could replace traditional additives in food since can exert antibacterial activities against many species, including decarboxylating bacteria. For this reason, these substances can be useful for the reduction of BA in foods (Komprda et al., 2004). In particular, essential oils containing eugenol, thymol and carvacrol reduced BA formation depending on their concentrations and bacterial species (Cai et al., 2015; Özogul et al., 2015). Also spices can influence BA accumulation in food, due to their content of several molecules among which capsaicin (in pepper), cinnamic aldehyde (in cinnamon) and allicin (in garlic) that exert antimicrobial and antioxidant activity (Naila et al., 2010).

Among technological factors, food packaging can influence both qualitatively and quantitatively BA production. In fact, the use of modified atmosphere and, specifically, the absence of oxygen inside packaging (obtained also through the use of oxygen scavengers) may delay or reduce the production of BA, selecting spoilage microorganism pattern and inhibiting microbial population endowed with decarboxylating properties (i.e. enterobacteria producing putrescine, cadaverine and agmatine). The presence of carbon dioxide (CO<sub>2</sub>) can act as bacteriostatic agent against many BA forming bacteria in refrigerated foods. In particular, high CO<sub>2</sub> concentrations have been tested in fish and meat and results showed a lower production of histamine, putrescine and cadaverine (Naila et al., 2010; Gardini et al., 2016).

Besides packaging, several non-thermal treatments could determine a lower BA content, due to the reduction or delay in microbial growth along the shelf life. Among these treatments food irradiation, pulsed electric fields (i.e. short electricity pulses that cause microbial inactivation with minimal detrimental effect on food quality attributes) and pressure treatments can be mentioned. Among these latter techniques, High Hydrostatic Pressure (HHP) up to 600 MPa can limit BA content by controlling decarboxylating microflora both in dairy and meat products (Novella-Rodríguez et al., 2002; Ruiz-Capillas et al., 2007). Also High Pressure Homogenization (HPH) can be an important tool to control the BA accumulation in dairy foods (Lanciotti et al., 2007).

## Conclusions

The negative effects of BA are strictly dependent on the conditions of the consumers. In other words, it is difficult to define limits associated with negative symptoms. Healthy consumers can ingest BA even in relative high amount without serious diseases because of the capacity of their organisms to detoxify them through the natural enzymes assigned to this function (amine oxidases). However, food industry has to face the request for a BA control of increasing segments of consumers less protected against BA (younger and elder people, immunocompromised, patients taking specific drugs). In addition, the presence of BA is undoubtedly a marker of improper procedures in the production and storage of foods. These considerations, such as the search for an always higher quality of foods, have brought several national and international food authorities to focus their attention on the content of BA in foods. If they should be absent in non-fermented foods, the question may be different for fermented foods, in which their presence could be, at some extent, unavoidable. Nevertheless, any effort to reduce BA concentration also in these products has to be done. As described here, there is not a unique or specific procedure to lower this risk, but a variety of actions can be taken to limit amine accumulation. An acceptable final result could be reached only modulating the factors considered above with the aim to inhibit the growth of decarboxylating microorganisms and the activity of their decarboxylases.

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## Plant Alkaloids

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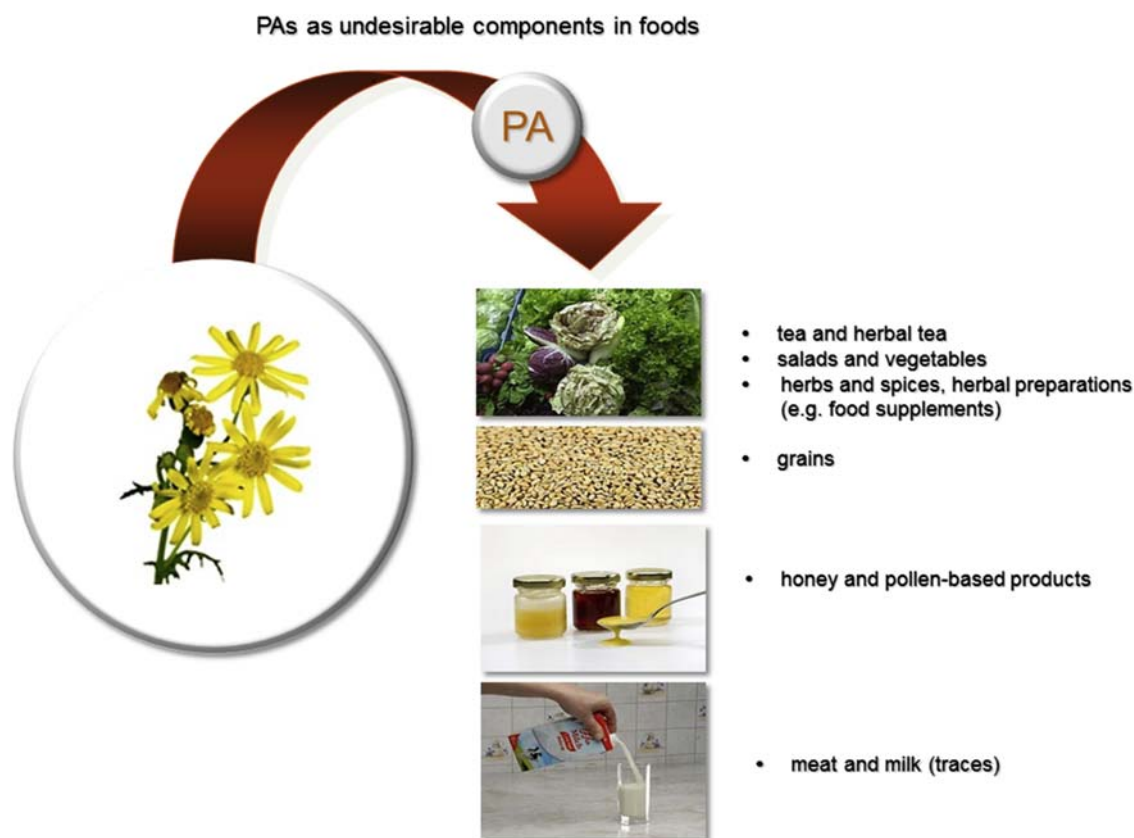
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### Overview

Natural sources of food are very well accepted by the consumer because of the positive image in regard to their secondary plant phytochemicals which may have for example anti-oxidative effects. However, plants (e.g. tea) or plant-based products such as food supplements may also contain natural contaminants. Since recently, therefore phytochemicals such as 1,2-unsaturated pyrrolizidine alkaloids (PAs), tropane alkaloids (TAs), and ergot alkaloids (EAs) are in the focus of Risk Assessment as examples for natural contaminations of food and feed. After rapid absorption upon oral intake these natural phytochemicals can induce very different tissue toxicity with acute and after long term ingestion chronic effects (e.g. PAs). Whereas EAs and TAs are believed to be non-genotoxic phytochemicals contain the PAs examples of derivatives that are proven to be genotoxic and carcinogenic in animal experiments. Therefore, PA exposure via common foods, contaminated with PA-producing plants or parts thereof raised concern, especially regarding the potential of genotoxicity and carcinogenicity of PAs. In summary, besides well-known classical contamination the contamination of food and food products with natural phytochemicals is a challenging food safety issue.

### Pyrrolizidine Alkaloids (PAs)

Over 500 different pyrrolizidine alkaloids (PAs) and their N-oxides are known and they are reported to occur in more than 6000 plant species (e.g. [Mattocks, 1986](#); [IPCS/INCHEM, 1988](#); [BfR, 2016](#); [EFSA, 2011](#)). PAs are secondary plant compounds and are undesirable substances in food and feed ([Fig. 1](#)). Plants containing pyrrolizidine alkaloids primarily belong to the Asteraceae, Boraginaceae and Fabaceae. PAs are esters of 1-hydroxymethylpyrrolizidine (necine base), which may bear a hydroxyl group at position 7, and aliphatic monocarbon or dicarbon acids (necine acids). PAs can occur as monoesters or diesters depending on the esterification of one or both hydroxyl groups. Cyclical diesters result from esterification with two carboxyl groups of a dicarbon acid.



**Figure 1** PAs as undesirable components in Foods.



Depending on the necine base, a distinction is primarily made between PAs of the retronecine, heliotridine, otonecine or platyne-cine type. PAs with 1,2-unsaturated necine structure esterified with at least one branched C5-carboxylic acid are associated with hepatotoxic, carcinogenic and genotoxic effects. This includes the unsaturated PAs of the retronecine, heliotridine and otonecine type but not the saturated pyrrolizidine alkaloids of the platyne-cine type (e.g. [Mattocks, 1986](#); [IPCS/INCHEM, 1988](#); [BfR, 2016](#); [EFSA, 2011](#)).

Food items that may contain unsaturated PAs include (herbal) teas, vegetables, honey, grain-derived products, eggs, milk and meat. The occurrence of PAs in food is mainly the consequence of contamination of food via PA-producing plants (e.g. [BfR, 2013a, b, 2016](#); [Mulder et al., 2015](#); [EFSA, 2011, 2016, 2017](#)).

PAs are generally rapidly absorbed upon oral intake. Following partial metabolism, excretion occurs primarily via the kidney and to a lesser extent in the faeces. In the organism unsaturated PAs are metabolised to pyrrole derivatives through hydrolysis, N-oxidation and dehydrogenation. N-oxide metabolites that are formed are highly soluble in water and are excreted extremely quickly in the urine, and this metabolic pathway is therefore seen as a detoxification pathway. However, the transformation of the uncleaved esters to toxic pyrrolic esters in the liver due to mixed-function oxidases, constitutes a toxification reaction. These pyrroles are highly reactive alkylating agents reacting with nucleic acids and proteins to form adducts and are considered active metabolites that are causing the hepatotoxic, hepatocarcinogenic and genotoxic effects (e.g. [Mattocks, 1986](#); [IPCS/INCHEM, 1988](#); [EFSA, 2011, BfR, 2016](#)).

The acute and chronic liver toxicity of unsaturated PAs is well known due to case reports in humans and animals and outbreaks of human poisonings by grain crops contaminated with seeds of unsaturated PA containing plants. Symptoms of acute PA poisoning are abdominal pain, ascites, vomiting, nausea, diarrhoea, oedema, jaundice and fever. The poisoning is typically associated with hepatic veno-occlusive disease (HVOD) involving obstruction of the small veins with sudden onset of hepatomegaly and ascites, and which may be lethal. A low long-term exposure to unsaturated PAs through the intake of food is known to yield chronic HVOD leading to cirrhosis of the liver. Full recovery from HVOD is possible. A chronic disease may develop among survivors of an acute HVOD or with longterm intake of small quantities of unsaturated PAs and result in liver cirrhosis. Other organs like the lungs (pulmonary hypertension) and the cardiovascular system (cardiac right ventricular hypertrophy) may be affected too. Unsaturated PAs exert foetotoxic and teratogenic effects in higher doses (e.g. [Mattocks, 1986](#); [IPCS/INCHEM, 1988](#); [EFSA, 2011, BfR, 2016](#)).

Some unsaturated PAs and corresponding plant materials have been evaluated by the International Agency for Research on Cancer (IARC). IARC classified lasiocarpine, riddelliine and monocrotaline to be possibly carcinogenic to humans (group 2B) ([IARC, 1976,1983,1987,2002](#)).

Several national and international bodies have carried out risk assessments of the intake of unsaturated PAs via food considering the toxic effects of PAs as cumulative ([COT, 2008](#); [BfR, 2013a, b](#); [EFSA, 2011, 2017](#)).

Regarding neoplastic lesions the British Committee on Toxicity of Chemicals in Food, [Consumer Products and the Environment \(COT\) in 2008](#) and the German Federal Institute for Risk Assessment (BfR) in 2013 and 2016 recommended to use a BMDL<sub>10</sub> (the lower confidence limit on the benchmark dose associated with a 10% response) of 0.07 mg/kg body weight (bw) per day derived from a 2 year carcinogenicity study of lasiocarpine in male rats as reference point to assess the exposure for any PA ([NCI, 1978](#); [COT, 2008](#); [BfR, 2013a, b](#); [EFSA, 2011, 2017](#)). A margin of exposure (MOE) of at least 10,000 was considered to be unlikely to be of high priority for risk management. PAs have also been evaluated as undesirable substances in food and animal feed by the [European Food Safety Authority \(EFSA\) in 2011 and 2017](#) ([EFSA, 2011, 2017](#)). In its latest evaluation EFSA established a new reference point of 0.237 mg/kg bw per day as BMDL<sub>10</sub> to assess the carcinogenic risks of PAs, based on a 2 year carcinogenicity study of riddelliine in female rats ([NTP, 2003](#); [EFSA, 2017](#)).

According to recent assessments dietary exposure to PAs may occur at levels that could be of concern with respect to carcinogenicity, especially for subpopulations which consume relatively large amounts of specific PAs-containing foods such as herbal teas. However, intake of PAs from the regular diet is not associated with risks of acute toxicity ([Mulder et al., 2015](#); [EFSA, 2011, 2016, 2017](#)).

## Tropane Alkaloids (TAs)

Tropane alkaloids (TAs) are natural components contained in certain plants such as henbane, thornapple and deadly nightshade. Tropane alkaloids-containing plants are found in numerous families, such as Solanaceae, Erythroxylaceae, Convolvulaceae, Brassicaceae, and Euphorbiaceae. A total of more than 200 different TAs have been identified in plants (e.g. [BfR, 2013c](#); [EFSA, 2013](#); [Mulder et al., 2016](#)). Some of these alkaloids are also used in medicinal products, for example atropine (a mixture containing equal amounts of the isomers (–)-hyoscyamine and (+)-hyoscyamine), (–)-hyoscyamine (–)-scopolamine and cocaine. In the modern food chain TAs, such as (–)-hyoscyamine (–)-scopolamine occur in food and feed, due to contamination with seeds of especially *Datura* spp. plants containing high concentrations of hyoscyamine and scopolamine (e.g. [BfR, 2013c](#); [EFSA, 2013](#)). In 2003, cases of domestic food poisoning with a typical syndrome of TA toxicity has been reported from Slovenia due to ingestion of a traditional dish made of buckwheat flour contaminated with *Datura stramonium* seeds. Outbreaks of acute toxicity due to contamination of crops with *Datura* seeds are also reported from Ethiopia and Botswana. Intoxications of children, teenagers and adults by TAs are reported mainly from abuses because of the hallucinogenic effects or from accidental exposure ([Perharič, 2005](#); [EFSA, 2013](#)).

Several tropane alkaloids are muscarinic receptor antagonists and used as powerful anticholinergic drugs. Some of them are hallucinogenic. Atropine, hyoscyamine, and scopolamine are used therapeutically for different medical indications, e.g. for treating spasms in the gastrointestinal tract, the biliary ducts or the urinary tract or for the prevention of kinetosis. The lowest single therapeutic dose for oral use is 1.4 µg/kg b.w. for (–)-hyoscyamine, 6 µg/kg b.w. for atropine (corresponding to approx. 3 µg/kg b.w. (–)-hyoscyamine), and 2.5 µg/kg b.w. for (–)-scopolamine (e.g. BfR, 2013c; EFSA, 2013). Known adverse effects of these alkaloids at the lowest oral therapeutic doses are slight cardiac slowing, dryness of the mucosa of the upper digestive and respiratory tract and reduced perspiration. At higher therapeutic oral doses an increased heart rate and mydriasis have been observed. Unlike (–)-hyoscyamine and atropine, (–)-scopolamine at levels of oral therapeutic doses causes a depression of the central nervous system (CNS) (e.g. BfR, 2013c; EFSA, 2013).

For risk assessment purposes, EFSA derived an acute reference dose (ARfD) as health-based guidance value of 0.016 µg/kg body weight (b.w.) for the sum of (–)-hyoscyamine and (–)-scopolamine (group ARfD). The derivation is based on observations in a study in healthy young male adults regarding the deceleration in the heart rate and CNS effects, such as drowsiness, headaches and nausea (Perharič et al., 2013a, b; EFSA, 2013).

Cocaine is a natural constituent of the coca leaf and it is a drug of abuse in many countries. It has local anesthetic properties. However, its therapeutic use nowadays is very limited. Cocaine affects in particular the central nervous and the cardiovascular systems and has the potential to induce mentally dependence. The BfR has undertaken a health risk assessment of the cocaine content of a coca leaf extract-containing soft drink and concluded that no health risk is to be expected from consumption of this product because of its low cocaine content. According to information in the scientific literature, the lowest dose that may lead to an adverse effect is a daily intake of 4800 µg cocaine per person. Assuming a high daily consumption of 1.7 L, the margin of safety between the consumed amount of cocaine and the amount upwards of which adverse effects may occur, is a factor of approximately 7000 (BfR, 2009).

## Ergot Alkaloids (EAs)

Ergot alkaloids (EAs) are found in the Western diet due to contamination with ergot. Ergot is the dried sclerotium of the fungus *Claviceps purpurea* developing mainly on rye, *Secale cereale*. More than 50 different EAs have been characterised. EAs are derivatives of lysergic acid (e.g. EFSA, 2012, 2017; BfR, 2013d).

Ergots contaminate flour or animal feed when harvested together with the grain (e.g. BfR, 2013d; EFSA, 2012, 2017).

Pharmaceutically ergotamine tartrate is used for the treatment of migraine, the usual oral dose being 1 to 2 mg. Not more than 6 mg should be given per day and the total weekly dose is limited to a maximum of 12 mg. Salts of ergometrine are used in the active management of the third stage of labour in oral dosages of 0.2 to 0.4 mg, 2 to 4 times daily. Ergometrine maleate causes intense uterine contractions in the range of the therapeutic dose (e.g. EFSA, 2012).

Ergotism is the disease caused by chronic consumption of rye infected by EAs, which is known since the Middle Ages in Europe, also as St. Anthony's fire. Ergotism exists in a convulsive (neurologic) and a gangrenous (vasoconstrictor) form. Convulsive symptoms are weariness, giddiness, dimness of sight, convulsions, muscle contractions, painful flexions of joints, tingling of the skin, headaches, fever, hallucinations, mania, and delirium followed by death. Gangrenous ergotism starts with tingling effects in the toes and fingers, is followed by dry gangrene of toes and limbs and may lead to loss of limbs. Typical adverse effects known from the medical use of ergotamine and ergometrine salts in high dosages include symptoms of ergotism (e.g. EFSA, 2012; BfR, 2013d).

EFSA (2012) derived a BMDL10 of 0.33 mg/kg bw per day for the incidence of tail muscular atrophy in a 13-week rat feeding study of ergotamine. Based on this reference point, which was considered representative of the vasoconstrictive effects of EAs, a group acute reference dose of 1 µg/kg bw and a group tolerable daily intake of 0.6 µg/kg bw per day were established. With respect to the uterus contracting properties of the ergot alkaloids, BfR considers pregnant women as a high risk group for high intake of cereals with high contamination of ergot alkaloids (EFSA, 2012; BfR, 2013d).

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# Pyrrolizidine Alkaloids: Analytical Challenges

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## Introduction

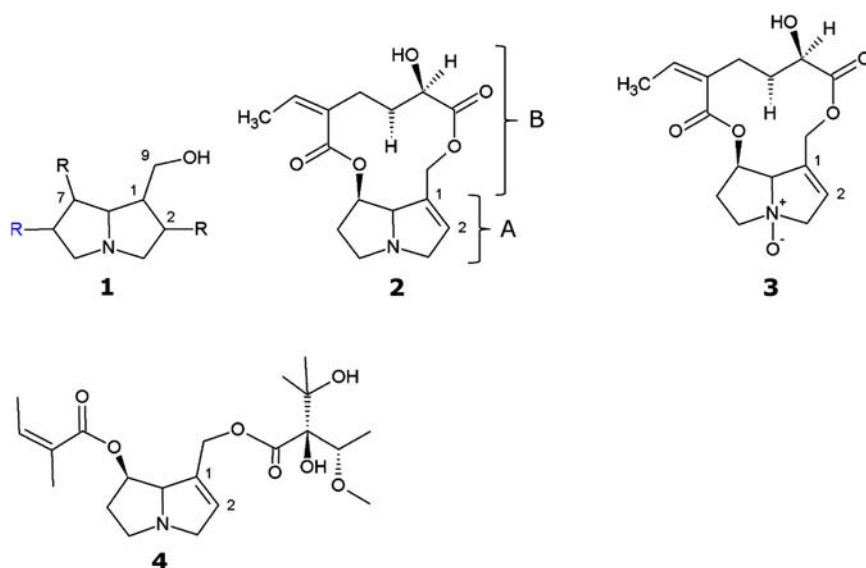
Pyrrolizidine alkaloids (PAs) are toxic secondary plant metabolites active against herbivores. It has been estimated that about 6000 plant species worldwide, representing 3% to 5% of all flowering plants, may contain PAs (EFSA, 2011; Smith and Culvenor, 1981). PAs are mainly found in the plant families of the *Boraginaceae*, *Asteraceae* (tribes Senecioneae and Eupatorieae) and *Fabaceae* (genus *Crotalaria*). The toxicity of a few PAs has been studied and they possess either acute or chronic toxic effects. PAs with a double bond in the 1,2-position are the most toxic PAs and are thus of special interest. The acute toxicological effects to livestock are known and described in the literature (Boppre, 2011; Fletcher et al., 2011; Molyneux et al., 2011; Molyneux, 2010; Wang et al., 2011; Wiedenfeld and Edgar, 2011). They lead to cirrhosis of the liver, and in case of acute intoxication it is well described that PAs cause the veno-occlusive disease (VOD) (Kakar et al., 2010; Prakash et al., 1999; Zhe and Ji-Rong, 2010). In addition, in experimental animals some PAs such as ridelliine and lasiocarpine have been tested and genotoxic, hepatotoxic and carcinogenic effects have been shown.

PAs are considered as contaminants or rather as plant toxins which can contaminate the food chain. From their behavior of contamination, e.g. their inhomogeneous distribution in the food or their concentration range, PAs are comparable to mycotoxins. The source of PAs is quite different. PAs enter the food chain at a very early state of the production chain due to harvesting of PA-containing plants. Examples are herbal infusions which can be contaminated with plant parts of PA containing weeds or Rucola which can be contaminated with ragwort (*Senecio jacobea*) during harvest. After harvesting it is almost impossible to sort out the PA containing plant parts which lead to a contamination of food and feed. Thus, the only possibility is to avoid foreign plants in the crop or to avoid harvesting the PA-containing plants. This is possible e.g. by applying a good and very strict agricultural practice.

## Chemical Structure of PAs

PAs have a common, so-called necine base (1, Fig. 1) of two fused nitrogen containing 5-membered rings as a backbone structure and this necine part can be esterified with miscellaneous acids (EFSA, 2011; Mattocks, 1986; Röder, 1995). This acidic part is called necic acid (B in Fig. 1). The necine base can either be saturated or can show one unsaturation in the 1,2-position.

The basic nitrogen of the condensed ring system can occur as a tertiary base as well as the corresponding N-oxide (PANO) (Fig. 1 (3)). In plants, the PANOs are the preferred form of the PAs. Furthermore, on C-2, C-6 or C-7 they can bear one or two hydroxy moieties which can lead to stereoisomers (Röder, 1995). Depending on the kind of the necic acid which is bound on the necine



**Figure 1** Chemical structures of pyrrolizidine alkaloids: (1) main necine base backbone of PAs including the typical numbering (R = H or –OH) (2) macrocyclic PA type senecionine ((A) necine base part (B) necic acid part) (3) senecionine-N-oxide (4) open chain diester type PA lasiocarpine.

base, the PA can occur as monoester, open chain diester and macrocyclic PA type forms. For this reason, a wide variety of different structures can arise in nature and as contaminants in food.

Moreover, these facts and the possibility of different stereocenters in the chemical structure of PAs lead to more than 600 known structures (EFSA, 2011; Kempf et al., 2010b; Kempf et al., 2008; Mädege et al., 2015). Also, for the toxicity of PAs, the structure is an important prerequisite. The PA toxicity is due to the existence of a carbon–carbon double bond in 1,2-position of the necine base. Furthermore, an ester functionality in position C-7 or C-9 or both positions is required. If these requirements are met, the PAs can undergo biotransformation, mainly by liver cytochrome P-450 monooxygenases, into the corresponding dehydropyrrolizidine alkaloids (pyrroles) (Jago et al., 1970; Kempf et al., 2010b, p. 159).

## The Pathway of PAs Into the Food Chain and Levels

The pathway how PA end up in the food chain was already described above. The main commodities in Western countries which are affected by PA contamination regarding chronic toxic effects are teas, infusions, honey, herbal drugs and medicines, phyto food supplements, herbs and spices and with lower prominence cereals and cereal products (Beuerle and Kempf, 2010; Boppré et al., 2008; Huxtable, 1980; Huybrechts and Callebaut, 2015; Kempf et al., 2009, 2010a, 2011a, 2011b; Oberlies et al., 2004).

PAs have regularly been determined in several commodities. Following a convention samples were analyzed on a spectrum either of 17, 21 or 28 individual PAs.

The levels are diverse and reach in herbal infusions from lower than  $10 \mu\text{g kg}^{-1}$  for the sum of 17 PAs up to more than  $3000 \mu\text{g kg}^{-1}$ . The occurrence in black and green tea is of lower importance because only a few highly contaminated samples have been reported (Bundesinstitut für Risikobewertung, 2013, 2016). For honey, especially for raw honey, high levels (more than  $600 \mu\text{g kg}^{-1}$  retronecine equivalents) of PAs are reported in the literature. For special *Jacobaea vulgaris* honeys levels up to  $12\,000 \mu\text{g kg}^{-1}$  have been reported (Kempf et al., 2011a,b). However, also for food supplements, PAs have been reported as a contaminant (Bundesinstitut für Risikobewertung, 2016).

When no grain cleaning processes are in place during food production, it is known that also acute intoxication of humans and animals can occur by consuming these products. As a consequence, liver diseases like VOD can occur (Kakar et al., 2010; Tandon et al., 1976; Wang et al., 2011). In general, the chronic toxic effects are the major concern regarding human health.

## Analytical Approaches for PAs

Analytical methods for PAs show a broad range of different techniques that were and still are used. In the literature, methods are described using chromatographic techniques like thin-layer-chromatography (TLC) methods (Bredenkamp and Wiechers, 1987; Mattocks and Jukes, 1987; MATTOCKS, 1967; Parvais, 1994), capillary electrophoresis, classical high-performance liquid chromatography (HPLC, LC) with ultraviolet (UV) or diode array detection (DAD) (Brown et al., 1994; Hösch et al., 1996; Xiong et al., 2009b; Yang et al., 2001; Yu et al., 2005), gas chromatographic approaches with nitrogen phosphorous detector (PND) or mass spectrometry (MS) (Joosten et al., 2010; Witte et al., 1993). During the past 10 to 15 years HPLC tandem mass spectrometric (MS/MS) techniques in the low concentration range ( $<10 \mu\text{g kg}^{-1}$ ) (Beales et al., 2004; Betteridge et al., 2005; Crews et al., 2009; Fu et al., 2010; Hoogenboom et al., 2011; Mulder et al., 2015; Zhou et al., 2010) are more frequently applied. These chromatographic techniques are used for structure elucidation but mostly for the quantification of PAs. Numerous methods are described in the literature to elucidate the level and the distribution of the different PAs. Likewise, some of them describe the levels of PAs in different commodities like tea, honey, pollen or flour.

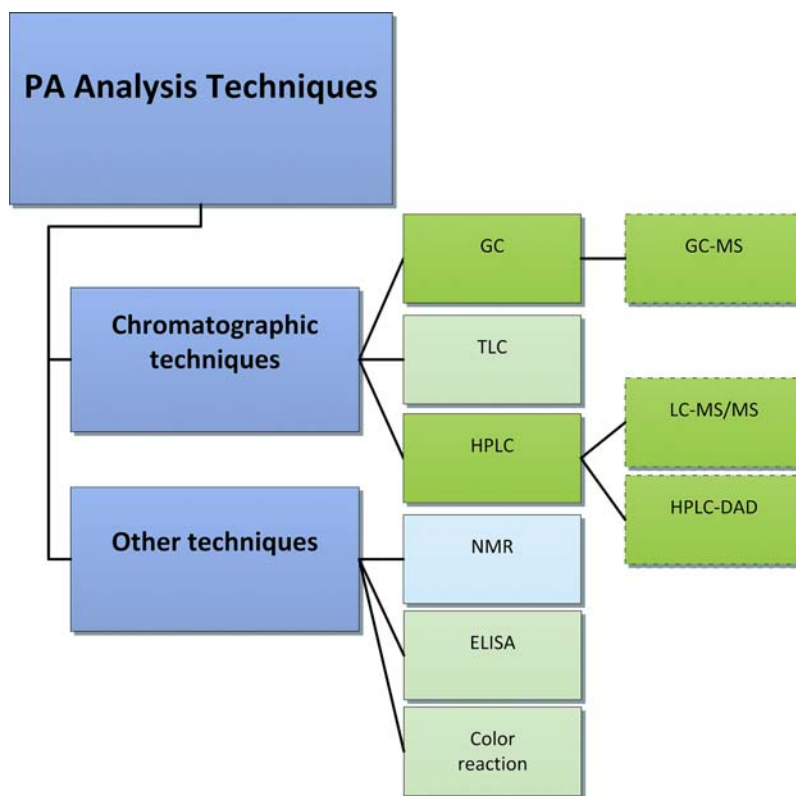
Besides chromatographic methods, techniques, such as enzyme-linked immunosorbent assays (ELISA) are also used for the analysis of PAs (Crews et al., 2010). The ELISA methods are more generic to detect PA structures, but it is known that these applications suffer from limited cross reactivities which prevent a broader use (EFSA, 2011). Additionally, none of them are commercially available.

Modern analytical methods for quantification use chromatographic techniques coupled with mass spectrometry. In the last ten years more and more sensitive and selective methods with mass spectrometric detection, tandem MS as well as high resolution MS, were established and described. Furthermore, the mass concentration range becomes nowadays much lower than before. Current analytical methods usually have a working range down to low  $\mu\text{g kg}^{-1}$ . LC-MS/MS is now the method of choice for detection and quantification of PAs followed by GC-MS methods.

## Analyzing PAs Using LC and GC Techniques

As described above, various analytical techniques can be used for analyzing PAs in different applications. In food and feed analysis, the quantitative chromatographic techniques are the most relevant applications, although with ELISA quantitative results can be obtained but only for the whole group of PAs, not for individual substances (Fig. 2).

A general problem for analyzing PAs is the limited number of commercially available standards. To date, around 50 substances out of more than 600 possible compounds are obtainable as reference standards.



**Figure 2** Overview of analytical techniques for PAs (dark green = quantitative, sensitive; light green = semi quantitative, not for individual PA; light blue = not quantitative).

The most relevant chromatographic techniques which allow the determination of individual PAs down to 0.5 to 2  $\mu\text{g kg}^{-1}$  and their challenges will be described in more detail below.

### GC-MS

For the analysis of PAs using GC-MS, first methods were described in the late 1980s and frequently applied in the following years. Different setups were described in the literature and the GC columns used differ from polar to unpolar stationary phases, such as OV-1, DB-1, DB-5, DB-17 and DB-1701. Modern capillary GC give satisfactory separation of several PA-isomers and existing retention indices relieve the identification of different PAs (Crews et al., 2010; EFSA, 2011; Witte et al., 1993).

A general limitation of GC methods for PAs is the fact that the PANO cannot be volatilized without destruction in the injection system of the GC. Thus, the tertiary bases of the PANOs need to be chemically converted. This is mainly done upstream by a reduction step using  $\text{Zn}/\text{H}^+$  (Witte et al., 1993, p. 188) (Kempf et al., 2008, p. 1194). This step is time consuming (3 hours (Kempf et al., 2011a, p. 335)) depart from the other steps of the sample preparation. Also, for some of the PAs, thermal decomposition and the formation of diesters from monoesters are described (Crews et al., 2010, p. 331).

By using GC methods, it is not possible to measure the analytes as they are, the PAs must be derivatized. Often used reagents are boronate derivatives, tetramethylsilane or a combination of both (Crews et al., 2010; Wretensjö and Karlberg, 2003).

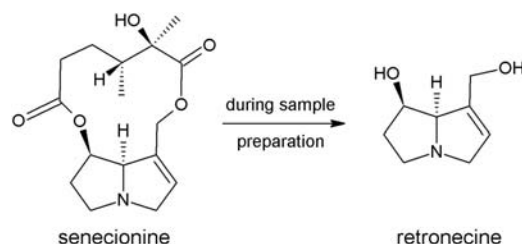
For the detection, nitrogen-phosphorous-detectors (NPD) or flame-ionization-detectors (FID) in parallel are common (Joosten et al., 2010). But the most common detector for PAs is the mass spectrometric detector with electron-impact ionization (EI) mode. The mass spectrum is typical for PAs and shows unique signals for the necine base part of the structure. Characteristic fragments are e.g.  $m/z$  93, 120, 136 and 137 for retronecine type PAs, and macrocyclic PAs with a retronecine base typically show intense ions at  $m/z$  93, 119, 120 and 136 (Crews et al., 2010, p. 331). PAs do not show intensive molecular ion peaks (Schulzki, 2010).

In many cases GC-MS is used to compile a PA profile of the plant or e.g. of the phytopharmaceutical which shall be free of PA. In these cases, it is not the aim to quantify (LOQ 0.06 mg/kg) all the individual substances. It is rather the aim to identify the PA signal using typical  $m/z$  fragments and to quantify these signals by using one or two PAs for the calibration. For example, senecionine is used for retronecine type PAs and senkirine for otonecine type PA according to Schulzki (2010).

A completely different approach is to convert PAs into their basic structure moiety retronecine and quantify this compound (Fig. 3). This method is described by Kempf and Beuerle (Kempf et al., 2008).

This approach allows to detect and quantify most of the 1,2-unsaturated PAs as retronecine-equivalents. This represents a measurement of PAs as a single sum parameter. The PAs are extracted by means of acidic liquid extraction, a  $\text{Zn}/\text{H}^+$  reduction



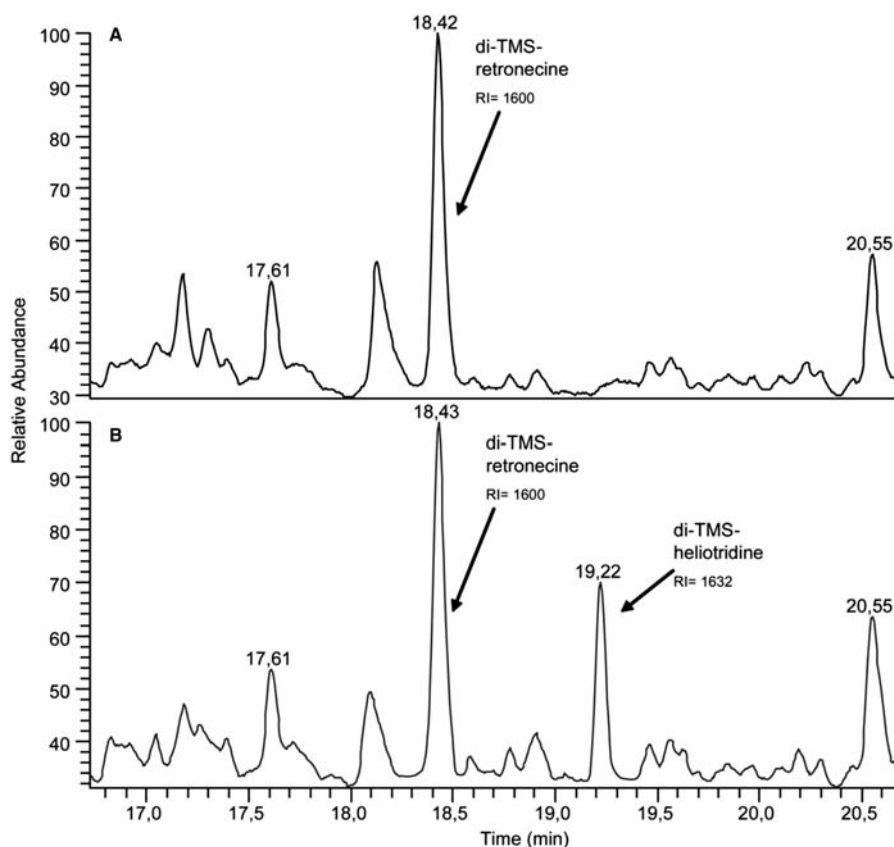


**Figure 3** Conversion of PAs into their basic structure moiety retronecine during sample preparation (Kempf et al., 2008).

step and solid phase extraction (SPE) cleanup with a strong cation exchange material. Afterwards the essential reduction with lithium aluminum hydride to yield the necine base structure is performed. N-Methyl-N-(trimethylsilyl)trifluoroacetamid (MSTFA) is used as derivatization reagent. The di-TMS derivatives are quantified using an internal standard (ISTD). As ISTD heliotridine, a naturally occurring PA is used. Hence, all samples have to be analyzed a priori in duplicate to proof that no heliotridine is naturally occurring in the sample. A general lack is that all PAs of the otonecine type are not covered by this method. The method is very time consuming because the two reduction steps take 6 hours per sample, and the total analysis time is approx. 10 hours without chromatographic determination ([Kempf et al., 2011a](#)) ([Fig. 4](#)).

The typical concentration range for the GC-MS methods depends on the setup but it is usually in the range of  $10 \mu\text{g kg}^{-1}$  (retro-necine equivalents) for the sum parameter method described by Kempf et al. or for the PA profiles in the range of  $60 \mu\text{g kg}^{-1}$  (EFSA, 2011; Schulzki, 2010).

GC-MS/MS has not been used for PAs very often. Schoch et al. described a GC-MS/MS method already in the year 2000 but to date, where sensitive GC-MS/MS equipment is available in a number of laboratories, this technique has not gained increased importance for the determination of PAs (Schoch et al., 2000). A possible reason for this could be the limitations (no PANOS etc.) and the relatively complex cleanup procedure as described above.



**Figure 4** SIM mode chromatogram ( $m/z$  93, 183, and 299) of a PA positive honey sample (A) Without internal standard heliotridine and (B) after the addition of heliotrine (shown as di-TMS- heliotridine; RI [DB1] = 1632) (Kempf et al., 2008, p. 1197).

## LC-MS/MS

Liquid chromatography coupled with tandem mass spectrometry is a very widely used technique, especially for the target analysis of contaminants in food and feed. Generally, LC-MS/MS allows a very sensitive quantitative detection of contaminants, such as the PAs. It has a number of advantages compared to GC-MS based methods despite it is more cost-intensive than GC-MS equipment, both for purchasing and maintenance. Nevertheless, LC-MS/MS became the method of choice for separation and detection of PAs over the last approx. 15 years.

One of the main advantages of LC-MS/MS is the possibility of simultaneous detection of the tertiary forms of PAs and the PANOs. This is possible within one analytical run, allowing a substantially decreased time and effort for sample clean-up. The chemical reduction steps mentioned for GC based methods are not necessary by using an LC-MS/MS approach. Additionally, modern column materials allow to achieve a suitable separation of individual PAs and PANOs. Very often C<sub>18</sub> based separation columns are used. Chromatographic conditions both with acids (e.g. formic acid) or bases (e.g. ammonium hydroxide) as modifier for the aqueous eluent part and either methanol or acetonitrile for the organic eluent part can be set-up. Electrospray ionization (ESI) is the most frequently used method as ionization technique (Betteridge et al., 2005; Crews et al., 2009; Hoogenboom et al., 2011; Lucchetti et al., 2016; Xiong et al., 2009a,b). Atmospheric-pressure chemical ionization (APCI) is a good choice for PAs in their tertiary forms but it shows lower sensitivity for the PANOs (Beales et al., 2004). For ESI, most frequently H<sup>+</sup>-adducts are formed in the ion source.

For the specific and sensitive detection of PAs in food and feed matrices targeted MS methods are described by using single reaction monitoring (SRM) and multiple reaction monitoring (MRM). It lowers the effort for sample cleanup steps. For these purposes, ion trap or triple quadrupole (QqQ) instruments are used. For the sensitive and selective detection of the PAs, collision induced dissociation (CID) fragments of the adduct ions formed in the ion source is the method of choice. These CID fragments are typical for PAs. Characteristic CID fragment ions are m/z 94, 118, 120, 122, 138, 136, 150 and 168 for 1,2-unsaturated PAs/PANOs (Dubecke et al., 2011; Hoogenboom, 2010; Kempf et al., 2011a; Xiong et al., 2009a; Zhou et al., 2010).

Concerning sample preparation, it is very often the same approach of acidic conditions for the extraction of the sample material. To clean up the raw extracts, SPE is used either with strong cation exchange (SCX) or with a reversed phase material. This step usually leads to a concentration of the extract for a more sensitive detection. Hence matrix effects can be decreased by an appropriate dilution of the extracts, a better signal to noise ratio is the result.

Although LC-MS/MS has several advantages, also some challenges need to be faced. Due to the fact that LC-MS/MS is usually (due to sensitivity reasons) used as target approach one challenge is that only approx. 50 substances can be purchased commercially, but standards are required for quantitation. By using the typical CID transitions described above it is also possible to identify "unknown" PAs (where no standards are available), however with high uncertainty as the structure cannot be proven without an authentic reference compound. Furthermore, the typical CID transitions are in the lower m/z range and even below m/z 100. Measuring small molecules with MS/MS approaches is a general problem as the MS-transitions have a relatively high uncertainty and can be affected by e.g. matrix interferences. To overcome this problem, isotope labeled internal standards (ISTD) should be used, however to date no labeled standards are commercially available. Cramer et al. (2013) described a sum parameter method with the use of deuterated 7-O-9-O-dibutyryl-[9,9-<sup>2</sup>H<sub>2</sub>]-retronecine as ISTD. However, this standard is not commercially available. Owing to matrix effects in LC-MS/MS, which may lead to signal suppression or enhancement, these effects must be considered to get correct quantitative results. Alternative methods which can be used instead of an ISTD, are standard addition or matrix matched calibration. Both methods are not easy to handle in routine analysis. For matrix matched calibration, there is the need of suitable blank material, which is sometimes not possible. Standard addition is also complicated to perform because of the high price of reference standards and the high effort which is needed for the sample preparation.

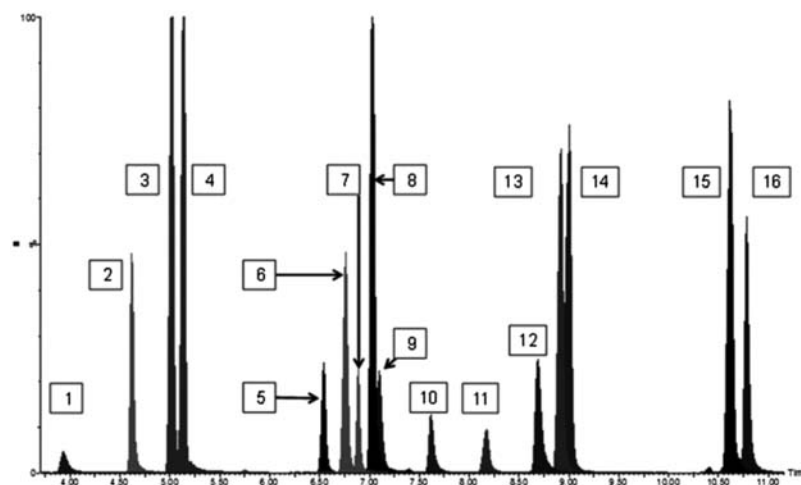
The next important issue where attention must be paid are the mass transition itself, the ion ratio, and the chromatographic separation. Since there are a lot of stereoisomers within the group of PAs, this leads to isobaric compounds (same m/z values) which generate the same mass transition. To distinguish between the different isomers (e.g. intermedine/lycopsamine) a suitable chromatographic separation is needed. This can be managed by using modern UHPLC separation columns with 1.7 µm particle size. The advantage of this material is not so much a faster chromatography, which is often not possible with complex matrices like food and feed, but the high chromatographic resolution. However, not in all cases a sufficient separation can be obtained. In these cases, the result can only be calculated as a sum, as it is not possible to distinguish between the individual PAs.

Compared to GC-MS analysis, LC-MS/MS is more sensitive with reported limits of detection between 1 µg kg<sup>-1</sup> and 10 µg kg<sup>-1</sup> depending on the compound and the procedure of sample preparation (Bundesinstitut für Risikobewertung, 2014; de Nijs et al., 2014).

## LC-High Resolution Mass Spectrometry (HRMS)

As LC-HRMS is much more expensive, both in purchase and maintenance compared to GC-MS and LC-MS/MS, this analytical equipment is not available in many laboratories. The advantages of LC-HRMS as screening methods are enormous. But for quantification purposes, LC-MS/MS is still the method of choice.

Analyses searching for so called "known unknowns", which means PAs where no reference standard is available but the structure is known, can be performed by use of LC-HRMS based methods. Nevertheless, the challenges with the stereoisomers and the isobaric compounds remain. The identification using CID with PA-typical fragments as described above is possible with high



**Figure 5** Chromatogram of a honey sample spiked at 1 ng g<sup>-1</sup>: (1) monocrotaline; (2) monocrotaline-N-oxide; (3) intermedine; (4) lycopsamine; (5) retrorsine; (6) heliotrine; (7) seneciophylline; (8) retrorsine-N-oxide; (9) heliotrine-N-oxide; (10) seneciophylline N-oxide; (11) senecionine; (12) senecionine-N-oxide; (13) echimidine; (14) senkerkin; (15) lasiocarpine; (16) lasiocarpine-N-oxide. (Huybrechts and Callebaut, 2015)

resolution. But without any respective reference standard this is difficult to confirm, and quantitative results can only be obtained with a considerable uncertainty.

Structural screening for PA relevant substances ("non-target" screening) can be performed by using LC-HRMS as well as with LC-MS/MS approaches applying CID fragmentation on PA-typical *m/z* values. The major advantage here is to use the exact mass for the unequivocal identification of PA CID fragments.

## Conclusion

PAs are a broad group of natural plant alkaloids which can contaminate the food and feed chain. They exhibit a number of toxicological effects, of which chronic toxicity is most relevant. As PAs occur at low levels, analytical methods must be sensitive and specific allowing low LODs. There is still a lack of data regarding the exposure of humans with individual PAs though various food commodities. Therefore, a target approach for individual substances is of interest. Otherwise, a toxicological assessment cannot be performed because the PAs have a different toxicological potential depending mainly on their structure.

By applying chromatographic techniques for analyzing food and feed, a number of challenges must be considered. Since the past 15 years, LC-MS/MS became the method of choice. While there is a lack of isotopic labeled standards, only matrix matched calibration or standard addition is a possibility to take potential matrix interferences into account. Chromatographic procedures with a high resolution (e.g. UHPLC) are needed to achieve an optimized separation between the individual substances.

To date, it is uncertain whether all relevant PAs are covered as only around 50 reference standards are commercially available. Sum parameter methods cover a lot of relevant structures, but it must be proven that they produce reproducible results and even these methods do not cover all relevant structures. In addition, structural relevant information gets lost e.g. by hydrolysis and a toxicological conclusion cannot be drawn. The toxicological potential of a sample can even be overestimated by this approach.

Analyzing PAs needs experienced laboratories which know how to deal with natural substances. There can be numerous gaps and pitfalls which must be considered. Therefore, the analysis of PAs is to date not a routine method which can be implemented easily as a standard analytical procedure.

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# Big Data Applications in Food Safety and Quality

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## Glossary

**Big data** Data sets whose size or type is beyond the ability of traditional relational databases to capture, manage, and process with low-latency.

**Blockchain** A digital ledger in which transactions are recorded chronologically.

**Food fraud** Deliberate and intentional substitution, addition, tampering, or misrepresentation of food, food ingredients or food packaging.

**Machine learning** A method of data analysis that automates analytical model building.

**Microbiome** The microbial composition of an environment.

**Predictive microbiology** Field of study which uses mathematical models to predict microbial response to various abiotic and biotic factors.

## Introduction

Big data and data analytics has evolved to become an indispensable facet across all industries, enabling new advances in management strategies, product development, and data insight that has never before been possible. A widely accepted definition of big data is inspired by a Meta (now Gartner) report (Douglas, 2001) from 2001 which never mentions Big Data and predates the popularity of the term and its widespread use. IBM analytics has defined big data as data sets whose size or type is beyond the ability of traditional relational databases to capture, manage, and process the data with low-latency (IBM: What is big data? - Bringing big data to the enterprise. <http://www-01.ibm.com/software/data/bigdata/>). And it has one or more of the following characteristics – high volume, high velocity, or high variety (Beyer and Laney, 2012).

However, the term big data encapsulates more than increased magnitude of data and includes statistical ideas that have revolutionized data analysis. This revolution in statistical analyses of vast amounts of various forms of data is how big data has permitted disruptive innovation across seemingly unrelated fields and has generated revolutionary ideas that has changed the course of entire industries. Traditionally, scientists have approached scientific questions by formulating a hypothetical model and carefully designing experiments to accept or reject the proposed model. The more modern statistical method, fueled by the revolution of big data, is to refrain from relying on hypothetical models and allow the data itself to identify pertinent variables and patterns that shape the observed outcome. Closely related is the development of artificial intelligence (AI) and Machine learning in which machines, via algorithms, learn from input data, predict for future data, and perform tasks guided by the data (Bishop, 2006; Hastie et al., 2009; and Mohri et al., 2012).

A simplistic view of the process for creating models from big data is represented in Fig. 1. A more realistic view, however, looks more like the scheme in Fig. 2. Big data problems are often categorized into supervised learnings and unsupervised learnings. Supervised learnings deal with data that is accompanied by a label, also referred to as response, target variable, or dependent variable. Labels are the variables that need to be predicted. A label can be quantitative, a regression problem, or categorical, a classification problem. In contrast, features, also referred to as inputs, independent variables, or predictors, are individual measurable properties or characteristics observed in every instance. Unsupervised learnings, on the other hand, deals with unlabeled data, where the goal is to find hidden patterns and structure in the data from the observed features.

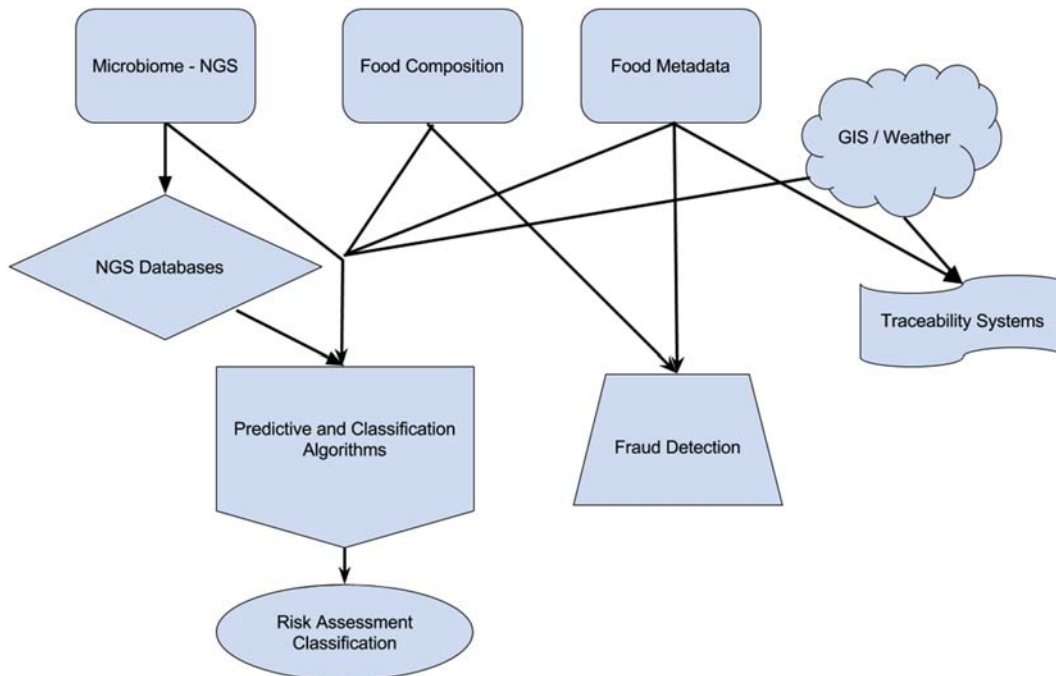
A significant feature of modern statistical platforms and what brings intelligence to these algorithms, is the ability to update the model and continue learning as newer data is collected. In fact, many applications include a feedback line that keeps training and testing the model with the updated set of data and monitors the performance of the model continuously.

The following sections describe the workflow of general predictive algorithms with a focus more in supervised learnings, as unsupervised learnings are generally more challenging to formulate, train, and evaluate.



Figure 1 Analysis of data, general schema.





**Figure 2** General schema for a supervised learning problem.

### Data Collection, Cleaning and Storage

Collection of the data is the first step for any big data application. Even though in big data the emphasis seems to be on quantity, very often quantity cannot cover the lack of quality. In particular maintaining diversity while avoiding bias is an important aspect of good data collection. The unbiased choice of data does not mean equal numbers of data points from different sectors, but numbers that are close to proportional to the population of each sector. Cleaning and preprocessing is another essential step when dealing with large quantities of data. This is more the case since larger and larger quantities of new data are unstructured.

Data storage and security of data create further challenges of collecting and analyzing data. The issue of data security is a hot topic. The data related to food safety, on one hand ties to liabilities of the public and private sector, and on the other hand is connected to medical and health data which are among the most sensitive categories of data.

### Splitting Data, Feature Reduction, and Normalization

In supervised learning problems, the data has to be divided into training and test data. The test set is generally a smaller subset which needs to be kept separated from the training process and all aspects of model selection.

Often the data used in a problem is highly dimensional and contains too many features. Keeping all the features not only creates performance deficiencies but may lead to overfitting, which is a major concern in all prediction problems. To help, a step for feature selection and feature extraction is added where some features are eliminated and some others are merged into smaller number of features (Blum and Langley, 1997). The feature extraction is often accompanied by dimensionality reduction algorithms. Finally in the normalization step, features are brought to the same scale, which often improves the performance of the final model significantly.

### Training and Model Selection

Training of the model involves first choosing the type of model and then training the model using the training dataset (Claeskens and Hjort, 2008). Once a model is chosen the test set is used to evaluate the predicted performance of the model on future data. One common pitfall when choosing a model is heavily reliance on the training data set (James, 2003). In this case, the trained model may perform very well on the training set and fail miserably on the test set or the future data due to what is called overfitting (Hawkins, 2004 also Burnham and Anderson, 2002).

A common remedy to the overfitting problem is to seek models with more consistent performance on the training data (Brodely and Friedl, 1999). Another approach, called cross-validation, is a further splitting of the data outside the test set to training set and the cross-validation set (James et al., 2013). Then the cross validation set is used to minimize overfitting and select the main architecture of the model.

## Evaluation and Metrics

When training of the final model is complete, it is evaluated against the test set which has not been used in any of the steps for development of the model. Mean squared error and the coefficient of determination, i.e., R-squared-error, are important metrics for regression problems. For classification problems, accuracy score is the most common metric, and precision, specificity, and recall, sometimes referred to as sensitivity, scores are other notable metrics.

## Big Data and Food

The use of big data in the food industry is still new. There are four general categories of such applications which are briefly explained below and expanded with more specific examples in the remainder of the chapter.

- Predictive models: Applications that can be used to provide food safety and quality risk assessments, including pathogen risk prediction, shelf life prediction, and precision agriculture.
- Classification algorithms: Applications determining presence or absence of pathogens, pesticides, or existence of any other major modification that affects the food
- Anomaly detections: Detection of fraud or any isolated defect among samples of the same product or samples of products in the same category.
- Accessible large datasets: Having organized, comprehensive data sources by itself is an asset and can be implemented in many applications. Traceability technologies rely on such data collections. Other examples of ever expanding major data sources related to food are the GenomeTrakr, NCBI genomic database, and NARMS.

## Agriculture

Big data-enabled technology has an increasing adoption rate in agriculture and is expected to continue to become more widespread in the coming years. In the agricultural sector, big data is used in numerous aspects of farming and crop production such as precision agriculture (soil conditions, yield monitoring, field mapping, crop scouting, weather forecasting, etc.), decision making tools, and in emerging areas of on-farm food safety. Pre-harvest food safety hazards can be assessed through the use of geographical information systems technology (GIS) and geo-referenced data systems to identify associations between the environment and a pathogen (Strawn et al., 2013).

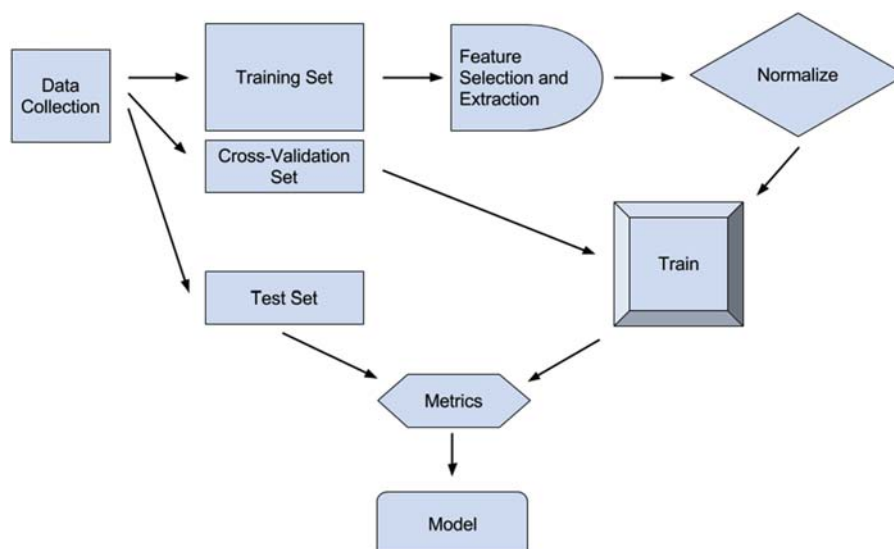
Similarly, by monitoring the conditions of crops in the field, areas with an increased incidence of aflatoxins can be identified before entering the food chain (Armbruster and MacDonell, 2014). Quantitative models have also been developed to predict the contamination of the mycotoxin deoxynivalenol (DON) on wheat in northwestern Europe using a variety of data, including weather data (van der Fels-Klerx et al., 2012). Phytobiome and soil microbiome analysis can be used to identify microbial communities that are vital to plant health by imparting increased abiotic (e.g. drought tolerance) and biotic (e.g. disease resistance) stress tolerance. By gathering a vast amount of phytobiome and soil microbiome data, machine learning tools can be used to identify those microbial communities that may play a role in crop disease resistance/suppression, yield increase, and drought tolerance, and harness that information to develop new agricultural products (Marasco et al., 2012). There are several pioneer companies breaking new ground in this field including Indigo and AgBiome located in Boston, MA and Research Triangle Park, NC, respectively.

As big data analytics continues to disrupt and revolutionize a multitude of industries, it is possible that the future of big data applications in the agricultural sector will bridge data associated with crop production, including weather, soil, environmental conditions, pest management strategies, phytobiome composition, and nutrient availability into one all-encompassing system, allowing the agricultural sector to make more informed decisions that will lead to higher yields, greater nutritional value, and decreased production costs (Fig. 3).

## Food Authenticity

With the rise of consumer demand for food transparency, consumers are more concerned than ever with where their food comes from and expect companies to provide clear, accurate and useful food-related information for the products they purchase. The Center for Food Integrity conducted a study in 2015 that revealed that consumers not only want transparent food product labeling and ingredients, they want companies to be transparent about their business practices as well (CFI, 2015). Big data has played a major role in allowing companies to ensure authenticity and detection of food fraud in the products they sell. Advances in sequencing technologies and the availability of public databases has enabled the development of genomics-based authenticity and food fraud detection tests with greater accuracy than previously available.

Since the 2013 horse meat scandal in which foods advertised to contain beef was found to contain undeclared horse meat, consumers have a heightened level of concern about food authenticity and adulteration (Lawrence, 2013). Sequencing-based tests enable high-throughput analyses using curated, proprietary, or publicly available databases which allow companies to verify suppliers, ensure product integrity, and reduce the risk of food adulteration and contamination. Such tests include, but are not



**Figure 3** The use of big data applications in food safety and quality to create a FSQA system.

limited to, GMO detection, pesticide residue, allergen contamination, missing ingredients, halal validation, antibiotic detection, heavy metals, animal species identification, and hormones (Ellis and Goodacre, 2016).

### Composition Trends to Enhance Quality Control

One way of monitoring food authenticity is by tracking product composition trends. Monitoring the relative abundance of each component within a product is an effective way of identifying unexpected changes that may be problematic or cause the product to become out of compliance with standard specifications. This approach provides monitoring which is flexible and can be different for different products, and even different part of the product's content, and also it can evolve and update when there are gradual changes in the production. Additionally, if monitored over time, composite trends can also be used to show long-term trends and even potentially identify seasonal trends.

An example of this type of monitoring system is implemented by Clear Labs Inc., where NGS technology is used to quantify and identify the DNA content of food samples. Using data generated from numerous samples of the same product, composition trends are monitored and appropriate alerts are automatically generated when something unusual is observed. However, while it is useful to be able to quantify sample components to facilitate a detailed analysis of products, monitoring DNA composition trends do not require any DNA/Mass model conversion and are effective to monitor for product specification compliance and enhance quality control systems.

### Fraud Detection

Food fraud can be a major economic and public health threat. Some of the earliest cases of food fraud, dating back thousands of years, involved olive oil, tea, wine, and spices. Although the vast majority of fraud incidences do not pose a public health threat, there have been some cases that have harmed or had the potential to harm the public. One of the most well-known cases of economically-motivated adulteration (EMA) involved the addition of melamine to high-protein feed and milk-based products to artificially inflate protein values in products (Johnson, 2014). Similar to methodologies previously described to investigate composition trends, monitoring similar products across brands and suppliers can be used to identify these fraudulent products at any point throughout the supply chain process. These compliance specifications may include many parameters such as ingredient substitution, GMO percentage, presence and quantities of allergens, toxic, and toxigenic organisms, and matching with label claims. This big data approach to fraud detection can enable high-throughput, reliable analysis for fraud and adulteration detection. Such analysis will have additional advantages of providing specifications that depend on the product, help to identify sources of adulteration, and establish industry standards for manufacturer or suppliers.

### Traceability

Tracking down contaminated food has historically been a very time-consuming process, sometimes requiring weeks for food companies and retailers to identify what products they need to pull from the store shelves. The advent of blockchain-based systems, however, has the ability to reduce that time to seconds. A blockchain is a continuously growing list of records protected from revision and tampering. In the food safety world, blockchain technology can be used to digitally record the distribution of products

throughout the farm to fork continuum (Ahmed and Broek, 2017). Walmart, Unilever, Dole, Nestlé, and several other food giants have already partnered with IBM to explore how to use blockchain technology as a data management system across a complex network that includes farmers, broker, distributors, processors, retailers, regulators, and consumers (Yiannas, 2015).

### **Predictive Microbiology via Microbiome Analysis**

Predictive microbiology is an established field which studies the responses of microorganisms to various factors that may impact growth (e.g. temperature, water activity, pH, humidity, matrix, etc.) using mathematical models to make statistically sensible predictions (Perez-Rodriguez and Valero, 2013). Big data, however, has expanded these applications far beyond their historical boundaries. Classical predictive models relied heavily on imperfect prior assumptions of the distribution of data. However, in the new world of AI and machine learning, those assumptions are unnecessary and the distribution is pushed among the unknowns which are discovered and updated by the machine and the data to create models with greater accuracy than ever before.

#### **Shelf Life**

Traditionally, shelf life estimation begins with the hypothesis that the shelf life of a product follows a standard mathematical model, often an exponential model. Subsequently, through regression analysis and statistical goodness of fit tests, the model and its parameters are generated based on the limited number of available observations. Such predictions are often limited to known models of spoilage and a completely new model is required if newer data suggests a different model, or if the product varies over time.

Additionally, another way to predict shelf life for a wide range of food products is through analysis of the microbial composition of the food as measured by the food microbiome.

There are many intrinsic and extrinsic factors that influence food quality and shelf life including, but not limited to, food storage conditions, packaging, pH, temperature, water activity, and nutrient availability. Traditionally, microbiologists have relied on culturing techniques to measure the microbial composition of foods and make deductions about spoilage potential and shelf life. In contrast, utilizing a metagenomics approach to measure the microbial composition of foods via sequencing analysis will help recover some of those missing pieces and provide a more complete picture.

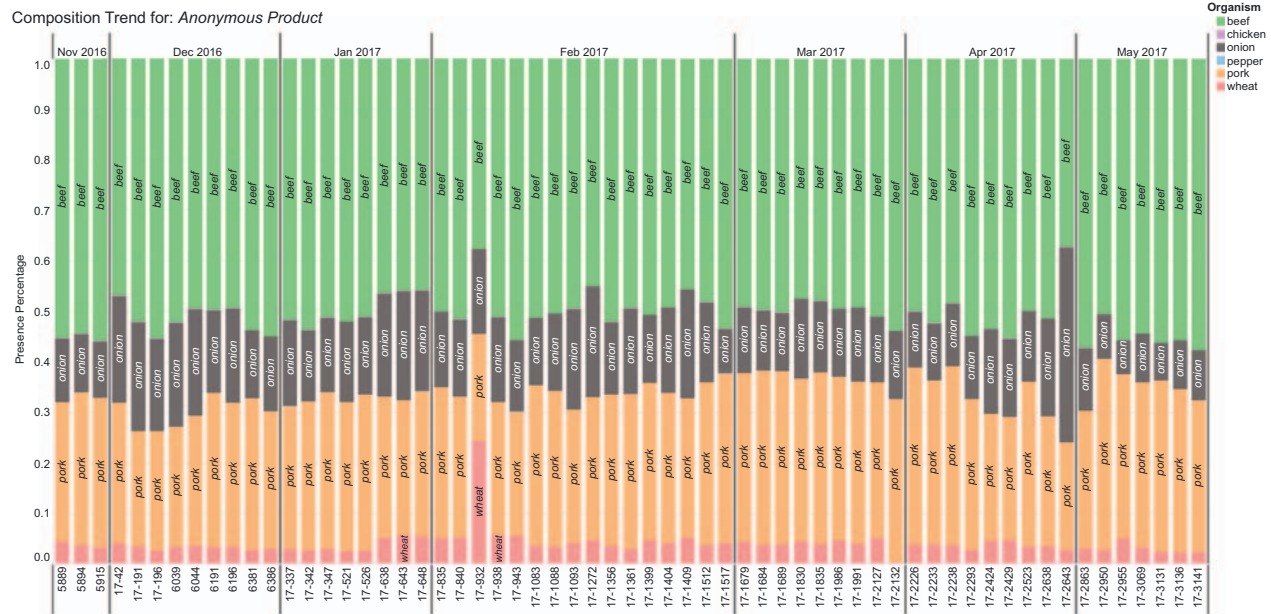
Recent advances in next-generation sequencing (NGS) technologies enable affordable high-throughput sequencing that has never before been available. This level of resolution enables the use of big data analytics to paint a more complete picture of the microbial composition of foods and how those microbial communities are influenced by intrinsic and extrinsic factors. While it is impractical to analyze the microbiome of every food product, it is possible to analyze enough samples to facilitate the development of more accurate predictive models, which use the observed (or expected) microbiome profile of the fresh product and estimate its remaining shelf life. Additionally, microbiome data can be used to measure the effectiveness of control measures such as refrigeration, modified atmosphere packaging (MAP), and preservatives used to extend the shelf life of foods. Furthermore, utilizing food microbiome data for shelf life prediction will also lead to the identification of new and more accurate spoilage indicators.

#### **Predictive Risk Assessment**

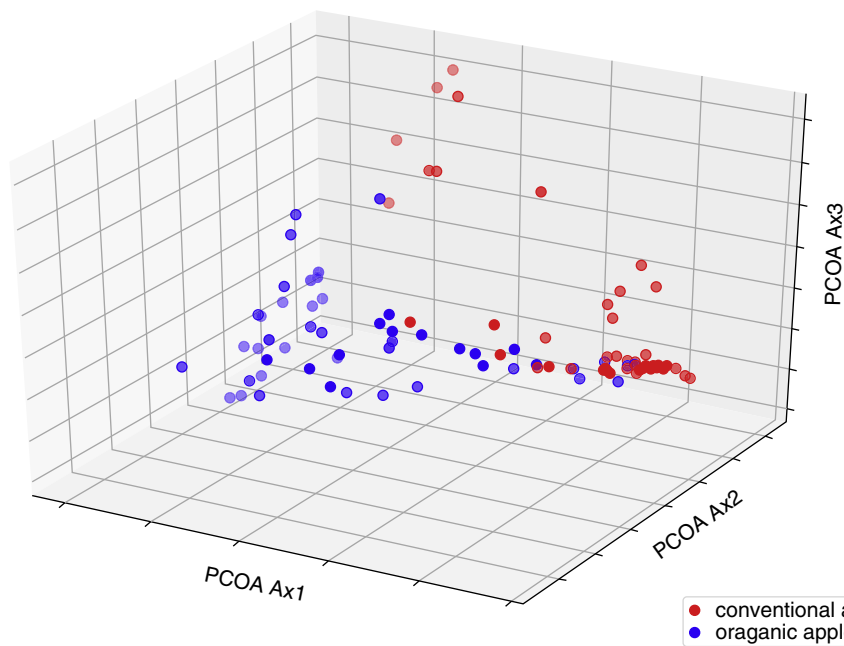
In addition to using microbiome data to predict quality parameters and shelf life of a product, the data can also be used to predict safety parameters and create a predictive risk assessment. Unique signatures can be identified within the microbial composition of products that do not contain a pathogen compared to those that are contaminated with harmful pathogens, which will allow for AI algorithms to assess the risk of a product through the microbial composition. Utilizing metadata derived from contaminated and non-contaminated sources to curate a microbiome database enabling machine learning tools to predict contamination of a product will facilitate a more holistic approach to food safety management. Industry and government will be able to use this big data approach to assess the food safety risk of a product with confidence - the question changes from "Is this pathogen/microbe/toxin present in the sample?" to "what does the microbial community indicate about the safety of this product?". In this way, big data can be used to revolutionize the food safety industry and potentially reduce the number of recalls, foodborne illnesses, and deaths.

#### **Detecting Pesticides and Chemicals**

A different potential for the use of big data is to utilize a large database of microbiome compositions and comprehensive metadata, to detect additives, pesticides, or treatments of a food sample. Distinguishing conventional and organic produce is an example of such problems where traditional tests are not successful. A number of recent studies have shown how microbiome composition can provide such an avenue. A PCOA graph from a small forthcoming study of organic vs conventional apples conducted at Clear Labs is presented in Fig. 4. (For similar results and studies see Abdelfattah et al., 2016 and Shetty et al., 2016). With larger collection of data and sophisticated analysis broad and accurate predictions like this are not very far (Fig. 5).



**Figure 4** Composition trend as means of monitoring product quality.

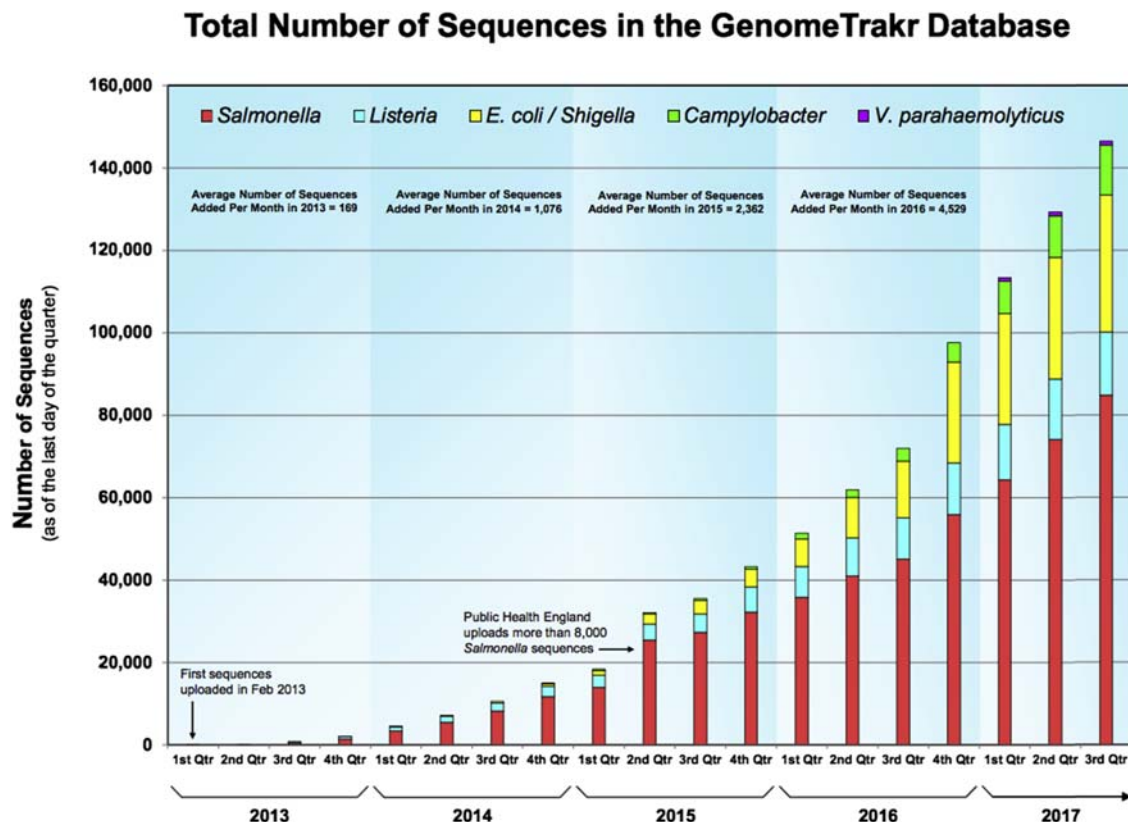


**Figure 5** Spatial view of microbiome data, organic vs. conventional apples (via PCOA to appear in forthcoming Clear Labs study).

## Databases

Reference databases make it possible to use metadata and machine learning tools to build much of the predictive models that have been discussed throughout this chapter. Databases such as GenomeTrakr have been accumulating large collections of data on safety outbreaks. The data has many applications including, but not limited to, the following:

- Trace the source of the outbreak using the geographic information, strain typing, and other metadata that connect groups of reported outbreaks.
- Evaluate risk for each kind of outbreak based on time, location, and matrix and as a result help with resource allocation in facing such incidents.
- Help with establishing preventive measures suggested by identifying factors that contribute to higher rates of outbreaks.



**Figure 6** Total number of sequences in the GenomeTrakr database over the past four years (FDA, 2017).

Other useful databases include National Antimicrobial Resistance Monitoring System (NARMS), Resistome Tracker, PulseNet and FoodNet. NARMS reports resistance genotypes of *Salmonella* isolated from retail meats, food-producing animals, and humans. WGS is then used to further characterize the isolates for bacterial speciation and serotyping. Furthermore, Resistome Tracker is an interactive research and data visualization tool for antibiotic resistance genes in *Salmonella*. Both PulseNet and FoodNet databases are used more for outbreak and foodborne illness surveillance and identifying trends associated with illness occurrences (Fig. 6).

## Future of Big Data

Advances in genomics, data analysis, computer processing and digital technologies are re-shaping the way consumers and producers think about and make decisions regarding food purchases and production practices. The once untapped potential of big data and advanced data analytics applications in food safety and quality is gaining more and more attention as tools become available making it easier to apply these technologies. Big data can be used to drive more informed safety and quality decisions, such as shelf-life prediction, improved sanitation and supply chain decisions (identification of resident vs. transient contamination), and predictive risk assessment among many other applications. However, although the field of food safety and quality has made strides in the use of big data to drive improvements, the possibilities are endless with regards to the potential to revolutionize the entire food system. An important next step in fully utilizing all that big data has to offer the food safety and quality world is to gain a better understanding of how to analyze complex datasets in a way that is unbiased and meaningful.

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# Omics Methods For the Detection of Foodborne Pathogens

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## Foodborne Disease, a Global Burden

Over millennia our food supply has been constantly affected by multiple issues and serious challenges. Many of these challenges would and continue to be the result of key human developments such as agriculture, urbanisation, industrialisation, and leaps in technological innovation. More recently this includes globalisation, whereby advances in worldwide transportation and rapid digital communication technologies have led to food supply chains/networks becoming increasingly more complex, and fragmented; that is to say, the supply chain is rarely horizontal but is a multidimensional network where many participants within that network only have knowledge of their immediate supplier and who they supply. These more recent challenges have included issues such as the intentional adulteration or deliberate misrepresentation of food products for economic gain, so-called food fraud; an issue first highlighted in the scientific literature during the industrial revolution and now again affecting many countries worldwide (Ellis et al., 2012). Of course food fraud falls within the scope of one of the major contemporary challenges of food supply, namely, food security, where serious nutritional disparities exist between developed and developing countries (as well as communities within all countries dependent on socio-economic status), when people do not have constant and sufficient access to a safe and nutritious food supply (FAO, 1996). However, despite these challenges within our food supply system changing over time, one constant and major challenge throughout human history and still affecting all communities worldwide today is the impact and burden of foodborne disease.

Foodborne disease is a serious and largely preventable public health challenge, specifically described as a global burden in the World Health Organisation's (WHO's) first global estimate of the effects of eating contaminated food published in 2015 (WHO, 2015). Illness and deaths caused by foodborne disease have also been described as a constant threat not only to public health but also as a significant impediment to socio-economic development worldwide (Havelaar et al., 2015). Results from the most recent WHO global estimate showed that every year, almost 1 in 10 people across the world become ill following the ingestion of contaminated food, and these numbers are likely to be under reported. Results also showed that 420,000 deaths result from foodborne disease annually, with significant regional variation worldwide, and with children below 5 years of age accounting for 125,000 deaths, almost one-third of the total (Kirk et al., 2015). Some 31 agents are known to cause foodborne disease including bacteria, parasites and viruses, as well as chemicals and toxins. Over half of the fatalities, 230,000, are the result of diarrheal disease, with diarrhoea caused by the ingestion of raw or undercooked fresh produce contaminated either by norovirus, or a range of bacterial pathogens mainly including *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli* 0157, amongst many others (Table 1). Interestingly, foodborne bacterial pathogens have also been observed to have regional variation and different impacts related to socio-economic status: with Africa and South-East Asia having the highest overall incidence and death rates; whilst pathogenic strains of *E. coli* are more prevalent in low-income countries; *Campylobacter* is said to be an important pathogen in high-income countries. By contrast, non-typhoidal strains of *Salmonella* are a major public health concern across all regions and all incomes.

It is these types of information which allows for the targeting of resources for the detection and reduction of these important foodborne pathogens, as well as increasing our knowledge in several areas such as 'pathogenomics' (Hain et al., 2007), food safety, risk monitoring and reduction (Brul et al., 2012), epidemiology, and antibiotic resistance in food chains (Ramos et al., 2016). It is also apparent that there is a constant need to develop new methods, as well as refine existing approaches, for the detection and analysis of foodborne bacterial pathogens and food disease outbreaks, especially so with the emergence and global threat of antibiotic resistant strains (Ramos et al., 2016), in order to reduce the burden of foodborne bacteria. With the largest ever recorded *Listeria monocytogenes* outbreak in South Africa, multi-State foodborne outbreaks of *Salmonella* in the USA, *Salmonella* found in baby formula in France, and continued concerns over *Campylobacter* in Europe, reinforcing this view. Here, in this short overview we offer a brief background introduction to the potential role of omics approaches and look at some of the recent research undertaken using these methods for the analysis of some of the most important foodborne bacterial pathogens.

## Omics, a Short Introduction

We define the neologism omics as a suffix to a molecular target in order to suggest that many of the potential molecules within that molecular target class are collectively characterized or measured. The development of various omics approaches and advancements in their technologies and potential applications (Ellis and Goodacre, 2016; Ellis et al., 2016) has enabled the high-throughput detection and quantification of various bio-molecules at different cellular levels, which has provided us with a deeper understanding of different biosystems and bioprocesses, including of course those directly related to and involving pathogenic foodborne bacteria (Bergholz et al., 2014) and food microbiology in general (Walsh et al., 2017). These can generally be divided into four main areas, namely: genomics, proteomics, metabolomics, and lipidomics. It is perhaps also worth noting at the onset, that no single omics analysis alone can fully reveal and characterize the complexity of a biological system. Thus, application of

**Table 1** A range of important foodborne bacterial pathogens and their known symptoms and potential impact

Bacteria	Symptoms and potential impacts
<i>Campylobacter jejuni</i>	Responsible for most campylobacter-related infections. Common symptoms include inflammatory watery diarrhoea which maybe bloody sometimes, abdominal pains, nausea and vomiting and fever. Symptoms usually manifest between 2 and 7 days following ingestion of food contaminated with <i>C. jejuni</i> and may last for a week. In immunocompromised people (e.g., those living with HIV/AIDS), the pathogen may cause fatal bacteremic conditions.
<i>Clostridium botulinum</i>	The spores of this soil bacterium produce a highly toxic biological chemical called botulinum which is usually transferred to vegetables and intestines of fish, birds and other herbivores which feed on vegetation. Symptoms may appear between 6 and 36 hours, but may sometimes delay until 10 days after eating contaminated food. Botulinum affects the nervous system leading to neurological complications such as slurred speech, dry mouth, blurred vision and muscle weakness. May paralyse muscles and even lead to death if no therapeutic strategies (antitoxins) are administered in the early stages of the symptoms.
<i>Clostridium perfringens</i>	Within 6–24 hours of clostridium infection nausea, diarrhoea and strong abdominal cramps appear. Immunocompromised people are at higher risk of developing serious health complications following infection which may even lead to death if it is left untreated.
<i>Cryptosporidium</i>	Nausea, mild to severe watery diarrhoea leading to body dehydration and weight loss, vomiting and stomach cramps appear within 10 days of infection and may persist for 14 days. Usually children under 5 years old and adults with weak immune system are at higher risk of serious illnesses.
<i>Escherichia coli</i> O157:H7	Most people infected with this pathogen start feeling sick after 7 days following ingestion of contaminated food or drinks. Early symptoms may appear in 3–4 days which include severe watery diarrhoea (often bloody), vomiting, weight loss and abdominal cramps. Prolonged exposure causes potentially life-threatening health complications including haemolytic uremic syndrome (HUS). HUS may cause kidney failure if no treatment is administered on time.
<i>Listeria monocytogenes</i>	Common symptoms include fever, diarrhoea; muscle ache and nausea are reported from 1 to 4 weeks after swallowing infectious dose of the pathogen. Pregnant women, elderly and people with weak immune system are at higher risk. In addition to common symptoms pregnant women experience fever, fatigue and if the pathogen spreads beyond the gut it may lead to premature birth, miscarriage or stillbirth. People other than pregnant women suffer severe convulsions, headache, stiff neck and loss of balance. Ultimately may lead to death if no timely therapeutic intervention is made.
<i>Salmonella</i> (over 2300 types)	Diarrhoea, stomach cramps, fever, and life-threatening body dehydration. Symptoms develop between 12 and 72 hours after eating contaminated food. People with healthy immune system recover within 4 to 7 days without any treatment. The elderly and immunocompromised (e.g., cancer, AIDS) people may develop life-threatening illnesses.
<i>Shigella</i> (over 30 types)	Watery diarrhoea, abdominal pain, fever and tenesmus appear in 1–2 days after becoming infected and last for 5–7 days in people with healthy immune system. If the pathogen enters the digestive system, it would lead to HUS which is usually accompanied by bloody diarrhoea.
<i>Staphylococcus aureus</i>	Early symptoms include diarrhoea, severe nausea, vomiting and abdominal pains. Symptoms appear as early as 30–60 minutes after swallowing food infected with <i>S. aureus</i> but disappear after 1–3 days in healthy immune system. People with weak immune system and those who have had severe skin trauma are at higher risk for invasive staphylococcal infections. If left untreated it may lead to severe and life-threatening illnesses including septic arthritis, pneumonia and sepsis.
<i>Vibrio vulnificus</i>	Symptoms vary depending on the immune system; healthy individuals experience diarrhoea, vomiting and abdominal pains whilst people with weak immune system have fatal illnesses including dangerously low blood pressure, blistering skin lesions, sudden chills and fever which may lead to death within 2 days of infection on average.

Adapted from the USDA, Food Safety and Inspection Service (USDA, 2013).

multi-omics approaches (Burnum-Johnson et al., 2017) may provide a clearer picture, and allow for a more precise conclusion to be made. A typical omics workflow is depicted in Fig. 1.

Genomics can be described as the study and assessment of variability and function of DNA sequences (Aebersold and Mann, 2016; Stefanovic et al., 2017). During the past two decades, the unprecedented advancements in DNA sequencing technologies and computational-based annotation (bioinformatics) techniques has revolutionised the fields of genomics and transcriptomics (Andjelkovic et al., 2017; Valdes et al., 2013). These fast evolving technologies provide detailed information on genome structure, gene function, and various metabolic networks and pathways. However, unlike the genome which is considered more static, and does not show significant response to short-term external changes, the transcriptome allows for the detection and quantification in gene expression levels in a dynamic manner (Zhang et al., 2010).

Proteomics (Martinovic et al., 2016) is the study of the structure, function and abundance of different proteins and peptides (or complexes) in a system. Proteins are important parts of cells and are involved in various processes and regulatory mechanisms. The proteome is also considered highly dynamic, which under different environmental or physiological conditions will result in changes in its properties, such as: protein abundance, structure, localization, synthesis, degradation and modification (Larance and Lomond,



**Figure 1** A typical omics workflow from experimental design to biological interpretation.

2015). Therefore, understanding the changes in the proteome requires the identification and detection of changes in protein abundance, interactions, structure and properties. Subdivisions of proteomics include the fields of peptidomics (Giacometti and Buretic-Tomljanovic, 2017; Giacometti et al., 2013; Korte and Brockmeyer, 2017) and secretomics (He et al., 2015; Sibbald et al., 2006). Many proteins are also modified by post-translational modifications (PTM) including (for example) glycosylation (50% of the proteins in man contain glycans) and phosphorylation which are known to change the function of the protein. It is therefore also important to have information about the protein as well as its PTM.

Metabolomics (Dunn and Ellis, 2005; Ellis et al., 2007) is the comprehensive and systematic study of low molecular weight compounds (metabolites) in a system. The metabolome generally refers to the complete set of small molecules involved in metabolism (metabolites) present in a cell that contributes to metabolic reactions which are required for the maintenance, growth and normal function of a cell in a particular physiological or developmental stage (Fiehn, 2002). The metabolome is considered one of the main components within systems biology (Patti et al., 2012), linked to other cellular processes such as: gene expression, post-transcriptional and translational events and physiological changes. This field encompasses a range of approaches such as metabolic profiling (Li and Zhu, 2017), metabolic fingerprinting (Ellis et al., 2007, 2012; Muhamadali et al., 2016a), metabolic footprinting (Kaderbhai et al., 2003; Kell et al., 2005) and multiple technologies such as a range of mass spectrometry (MS) (Singhal et al., 2015; Ouyang and Cooks, 2009), NMR (Garcia-Perez et al., 2017; Wright et al., 2009), and vibrational spectroscopy (Ellis et al., 2017; Black et al., 2016). The field of fluxomics (Brul et al., 2012; Yadav et al., 2018), which uses isotope tracing to monitor fluxes, is a subdivision of metabolomics.

Lipidomics (German et al., 2007; Jain et al., 2007) is a growing area of study, also originally considered a subdivision of metabolomics, and can be described as the study of pathways and networks of different lipid species in a system. Lipids are considered highly complex and crucial metabolites with important and diverse functions in biological systems, such as extremely important components of cellular membrane and barriers, signalling, and interconnecting various metabolic pathways (Rolim et al., 2015). However, detection and quantification of such a diverse and complex class of compounds requires robust analytical tools. So-called hyphenated methods, where MS (usually tandem and MS<sup>n</sup>) is/are coupled to chromatographic separation-based techniques for example, such as liquid chromatography (LC), and are considered the dominating analytical platforms in the field of lipidomics (Cajka and Fiehn, 2016).

### Recent Omics Studies of Foodborne Pathogens

At the time of writing, the largest recorded outbreak of foodborne bacterial disease is underway in southern Africa with a total of 973 victims and 183 deaths to date (March 2018). Caused by *L. monocytogenes*, this record outbreak is thought to involve ready-to-eat meats such as polony, a regionally very popular form of low-cost sausage typically made from highly processed meat. The ST6 sequence strain has been confirmed at production facilities in South Africa, and the outbreak is beginning to spread to neighbouring countries such as Namibia (reported this month in (Heiberg, 2018; Rosa, 2018)). It is large-scale and lethal foodborne disease outbreaks such as these, that bring the potential effects of pathogenic bacteria present within highly industrial modern food supply chains sharply into focus. Here we very briefly describe a selected range of recent omics studies involving some of the most well-known foodborne bacteria and also direct readers to several reviews of interest in this important area of food safety.

So-called pathogenomic studies of *Listeria* spp. have been undertaken for some considerable time in order to explore data from multiple omics approaches (genomics, transcriptomics, proteomics) to understand better the genomic diversity and evolution, and

the physiological aspects related to the metabolomics, of this deadly genus when these bacteria are exposed to and grow in diverse environments (Hain et al., 2007). Furthermore, this particular genus has recently gained its own omics suffix with the publication of listerionics (Becavin et al., 2017), as well as already being a distinct area of study within microbiology termed listeriology (Lebreton et al., 2016). In addition, one could state that a commonality between many, if not all, of the omics approaches is that they are data rich, generating and subsequently requiring the analysis of large and very highly complex datasets (Gromski et al., 2015), with them once being described as “producing bounteous data floods” (Goodacre et al., 2003) (more on this below).

The listerionics project appears to be no exception, with this very recent study comprising an eponymous web-based platform integrating the complete genomes (>80), transcriptomes (~350) and proteomes (25) published to date, with various tools for omics data analyses (Becavin et al., 2017). These multiple tools are said to allow for an integrative systems biology (Kell, 2004) approach, which the interested reader can find here: <https://listerionics.pasteur.fr/Listerionics/>. This platform includes an interactive genome viewer allowing for the display of gene expression arrays, tiling arrays, sequencing, proteomics, and genomics data sets; a protein and expression atlas to connect genes, RNA, or proteins with the most relevant omics data; a protein conservation exploration tool; and a co-expression network tool. Allowing for what the authors state to be the ability to study and browse multiple mechanisms in this important pathogen such as host–pathogen interactions, RNA regulation and this organisms adaptations to stress (Becavin et al., 2017). Another computational-based and related, if less comprehensive, approach is termed the *Salmonella* food-borne syst-omics database (SalFoS), <https://salfos.ibis.ulaval.ca/>. This consortium-led database hopes to improve food safety via the reduction of salmonellosis. With an analytical pipeline of genomic and phenotypic metadata on genome evolution, antibiotic resistance and virulence, its aims are to improve the accuracy of diagnostic methods, identify prognostic markers to aid surveillance and epidemiology, as well as develop in-field control methods (Emond-Rheault et al., 2017).

Any further development of in-field control methods is very encouraging, as large-scale outbreaks of foodborne disease involving pathogenic bacteria, such as the devastating South African Listeriosis outbreak, always have the potential to remain undetected for some considerable time. Whilst the specific source(s) of a foodborne outbreak remain undetected, this can obviously result in an increase in the incidence and spread of disease through communities and increased mortality rates. Therefore there is an obvious and urgent requirement to be able to attribute the source of foodborne disease rapidly and on-site/in-field, which in itself has been said to be leading towards a trend for more so-called culture-independent diagnostic tests (CIDs) (Forbes et al., 2017), which can link clinical cases both to each other and of course directly to food products. These can include metagenomics approaches (also referred to as environmental genomics) such as next-generation sequencing (NGS) which is increasingly being used for whole genome sequencing (WGS), as well as other omics methods and single cell technologies which have been stated to have the potential to revolutionize the fields of food safety and public health (Forbes et al., 2017).

Some envision that data from genomics and related omics tools could lead to a paradigm shift in contemporary and future food safety similar to those already underway in human medicine (Kambouris et al., 2018; Chen et al., 2012; Wishart, 2016) and the veterinary field (Van Borm et al., 2015). With precise pathogen detection, characterization and identification, leading to highly accurate risk assessments and the foundation for evidence-based food safety surveillance and monitoring decision making, a truly systems level approach (Capra and Luisi, 2014) which Kovac and co-workers term “precision food safety” (Kovac et al., 2017). Others agree, and also that a transition is underway at the molecular level, from traditional molecular biology-based methods currently seen as gold standard, to omics technologies, such as WGS, NGS tracking, as well as high resolution technologies such as CRISPR-based typing methods which constitute practical and powerful alternatives and will provide valuable insights into problematic food-associated bacterial pathogens (Barrangou et al., 2016).

Others have investigated the role of multiple omics methods and in particular the growing role of proteomics for the detection of specific microbial toxins (Martinovic et al., 2016; Josic et al., 2017). Microbial toxins can be placed in foods deliberately of course as a form of bioterrorism (Wein and Liu, 2005), and one recent study investigated the potential of proteomics methods in detecting toxins predominantly isolated from known bacterial food pathogens, namely *Clostridium perfringens*, *Staphylococcus aureus*, *Shigella dysenteriae*, enterohemorrhagic *E. coli* strains, and cytotoxic distending toxin from *Campylobacter jejuni*. With the goal being to develop an antibody-free proteomics assay to improve the multiplexing capacity of foodborne toxins. Antibody-free sample preparation was followed by LC-MS/MS, a targeted proteomics approach also known as LC-SRM (selective reaction monitoring), with highly specific detection and quantification enabled through the use of isotopically labelled protein references spiked into food matrices. The sensitivity of the assay for multiple toxins was lower than the oral LD<sub>50</sub> likely to be used to contaminate the food supply, and whilst initially developed to improve food defense (Manning et al., 2005; Manning and Soon, 2016), this antibody-free proteomic assay could also be applied to food quality control and public health monitoring (Gilquin et al., 2017). For a recent review of sensors used to detect specific pathogens and identify toxin-contaminated foods and beverages the reader is directed to Alahi and Mukhopadhyay (Alahi and Mukhopadhyay, 2017).

Metabolomics has grown exponentially as a field over the last decade with what has been said to be exciting applications across a wide range of biosciences (Putri et al., 2013), including multiple applications to food (Ellis et al., 2012; Cevallos-Cevallos et al., 2009; Nychas et al., 2008; Scalbert et al., 2014; Vrhovsek et al., 2012) and foodborne bacterial pathogens (Pinu, 2016; Cevallos-Cevallos et al., 2011; Xu et al., 2010; Ellis et al., 2002). With far less lipidomics studies relating to food (Hyötylainen et al., 2013; Murphy and Nicolaou, 2013) and even less so to foodborne bacterial pathogens (Dubois-Brissonnet et al., 2016), it is not surprising to find very recent food-related research which integrates both metabolomics and lipidomics approaches (Trivedi et al., 2016).

Interestingly, one of these integrated approaches specifically involved species of bacteria belonging to the well-known foodborne pathogen *Campylobacter*. This study primarily used metabolic fingerprinting approaches utilizing Raman and high-throughput



Fourier-transform infrared (FT-IR) spectroscopies, and matrix-assisted laser desorption ionisation-time of flight-mass spectrometry (MALDI-TOF-MS), with confirmation by 16S rRNA gene sequencing, for the differentiation of 11 strains of *Campylobacter* from six species. These six species included the most common foodborne pathogens from this genus, *C. jejuni* and *C. coli*, as well as *C. lari*, *C. hyointestinalis*, *C. fetus*, and *C. consisus*. Analysis of data from these three methods not only showed successful differentiation of all isolates, but also discrimination of two phylogenetically very closely related subspecies of *C. fetus* by FT-IR and MALDI-TOF-MS, which was found to be partly as a result of information from the lipid region of each rapid method. Subsequently, targeted lipidomics using LC-MS was used to further explore this and confirmed the findings from both fingerprinting methods, revealing major differences in the intensities of several classes of lipids between the two subspecies of *C. fetus* including phosphatidylcholine, phosphatidylethanolamine and phosphatidic acids. These results were said to show the potential of these rapid high-throughput techniques for the simultaneous detection and differentiation of different *Campylobacter* spp. down to subspecies levels (Muhamadali et al., 2016b).

Furthermore, as studies such as those by Muhamadali et al. (2016b) involve rapid methods; they also have the potential to be undertaken with field deployable instrumentation, or on/at-line within food production facilities. Field deployable instrumentation has much promise for the future. With methods such as handheld Raman (Ellis et al., 2017) and other forms of optics and spectroscopy (Crocombe, 2018), as well as developments in miniature MS (Ouyang and Cooks, 2009; Gao et al., 2006) and other sensors (Seo et al., 2016), which can be deployed on-site and/or coupled with smartphone technology (Gallegos et al., 2013) for rapid detection of bacteria on food, as recently demonstrated by Pearson et al. (Pearson et al., 2017, 2018).

## Outlook

Here, we have very briefly discussed the global burden of foodborne disease, provided a short background to the main omics approaches, and showed the potential of these approaches applied to the study of a range of common bacterial food pathogens. The reader will note that the final applied section began with two large data analytical studies, on *Listeria* and *Salmonella* respectively, using web-based platforms, followed by examples from genomic, proteomic, metabolomic and lipidomics approaches and their respective technologies. As already inferred above and by many others previously, it is important to highlight the data rich nature of all omics approaches and therefore the pivotal and indeed crucial role of a range of computational methods employed to analyse highly complex datasets generated by the omics.

What Kell once termed “data floods” over a decade and a half ago (Goodacre et al., 2003) could now quite accurately be described as data reservoirs. Data reservoirs which can not only store but are also able to analyse the massive volumes of data captured from multiple omics sources, with a range of built-in tools and functionality (as per listerionomics above). With these data reservoirs populated with primary data generated by omics, now having the additional potential to be accessed anywhere in the world to be utilised, analysed, or augmented by researchers in the field during and following food disease outbreaks. In order to influence decision-making, reduce the potential risk and impacts, or aid the rapid, accurate, on-site analysis of foodborne pathogens within food production facilities as well as in communities.

Omics can be said to have much potential for the detection of foodborne pathogens, as well as monitoring contamination and disease outbreaks, particularly when integrated with each other but especially so when incorporated within emerging and vitally important current and future technologies and resources such as the Internet of Things (IoT) (Seo et al., 2016; Badia-Melis et al., 2015) and Cloud-based computing (Gorelick et al., 2017; Tzounis et al., 2017).

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# New Analytical Frontiers in Mycotoxin Research

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## Introduction

Mycotoxins are one of the major food contaminants, causing important losses on the agricultural market, as well as being a great concern for food safety due to their toxicity. Indeed, more than 400 mycotoxins with widely different chemical structures have been identified so far, and their number is expected to increase further due to climate changes. The Food and Agriculture Organization (FAO) estimates that 25% of the world's food crops are affected by mycotoxin-producing fungi. Faced with this threat, a legal framework is gradually being set up to establish standards defining the maximum acceptable level of mycotoxin in foodstuffs.

Currently, only maximum levels for aflatoxins (AFs), deoxynivalenol (DON), zearalenone (ZEN), ochratoxin A (OTA), fumonisins (FBs), and patulin in various foodstuffs have been set in the EU countries. Recently, the European Commission fostered the release of two guidance documents concerning the identification criteria for mycotoxins and relating to the estimation of the limit of detection (LOD) and quantification (LOQ) for several classes of contaminants. Moreover, the European Food Safety Authority CONTAM Panel set a group tolerable daily intake (TDI) based on relative potency factors, calculated for the parent mycotoxins and its modified forms. Following their indications, the contamination should be expressed as the overall amount of parent compound and its modified forms. Using the established group, TDI would overestimate any risk of modified forms, structurally altered forms of the parent mycotoxins by plants and other living organisms (i.e., fungi, bacteria, mammals).

As a result, enforcing such standards requires appropriate analytical tools to detect and quantify mycotoxins and to identify new hazardous metabolites. In this frame, it should be mentioned that determination of mycotoxins is a challenging task because of their low occurrence levels and the complexity of the matrices.

Rapid, sensitive, and accurate analytical methods for determination of these mycotoxins in unprocessed cereals and cereal-based products are highly required to properly assess both the relevant toxicological risk for humans and animals, as well as to ensure that regulatory levels fixed by the European Union are met.

Several analytical techniques have been developed for different purpose, having different accuracy and sensitivity. The most widely used methods to monitor mycotoxins are chromatographic and immunological methods. Both approaches are useful when gathering surveillance data to determine the overall amount of legislated mycotoxins. However, they meet the current demands coming from two different stakeholders. Indeed, ongoing developments in the field of mycotoxin analysis polarized between the two approaches on opposite sides of the analytical spectrum to meet the demands of both regulatory authorities and research scientists for mycotoxin detection.

The first has been the development of rapid screening methods for a variety of analytes based on immunochemical techniques, such as ELISA or lateral flow devices. The second is the development of highly advanced multianalyte methods based on liquid chromatography coupled with high-resolution mass spectrometry for identification and simultaneous quantification of a wide range of contaminants.

Rapid screening methods based on ELISA system are user friendly, enable testing of large numbers of samples, and thus potentially represent a powerful tool for point of sampling in all parts of the food processing chain providing semiquantitative results. By contrast, the multicontaminant mass spectrometric methods require highly skilled laboratory staff, and enabling quantification with confirmation of the analytes of interest is spreading rapidly as a promising technique for simultaneous screening, identification, and quantitative determination of a large number of mycotoxins. Taking advantage of the improvement offered by high-resolution mass spectrometry instruments (HRMS), in the past few years, mycotoxin analysis has been moving from the targeted analysis of individual mycotoxins to the analysis of hundreds of contaminants in one run. In addition, omics strategies started to be exploited for the mycotoxin issue, and their applications have been recently reviewed. The increasing number of successful metabolomic approaches suggests that this new omics could soon play a major role in many aspects of food safety.

## Rapid Screening Methods

For farmers and small industrial producers, mainly fast screening tools for monitoring regulated mycotoxins are of interest. This is both due to the need of high-throughput analysis and due to the lack of resources to own expensive instruments and hire highly skilled laboratory staff. On this account the availability of portable devices is highly preferred to permit rapid on-site analysis by importers, traders, and companies. Hence, one of the major challenges is to minimize the detecting instruments to move detection methods from the lab to the field.

Rapid methods based on immunochemical reactions in various arrangements with different types of signal detection continue to attract commercial interest because they are cost-effective, rapid, and easy-to-handle. The most well-known and widely used methods are enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassays (LFIA). Immunoassays rely on the reaction between the specific antibodies and the analyte and therefore present such advantages as high sensitivity and selectivity.

An upcoming trend appears to be the development of immunoassays, which are able to determine several mycotoxins. For instance, the use of an ELISA kit as a promising group detection tool for the accurate evaluation of deoxynivalenol (DON) and

its modified forms, expressed as sum of DON, DON-3Glc, and 3Ac-DON, in soft wheat and maize, has been investigated (Righetti et al., 2016). Recently, a membrane-based flow-through test was presented, which was able to accurately screen zearalenone (ZEN), DON, aflatoxin B1 (AFB1), and ochratoxin A (OTA) in a variety of cereal-based feedstuffs.

The cross-reactivity phenomenon is one of the critical issues of immunochemical methods. However, if it is strictly under control and related to, modified mycotoxins or other similar mycotoxins might be desirable. Indeed, these kits may be helpful in the routine practice for a mycotoxin-group detection, as recommended by the recent EFSA opinions.

Despite the availability of several commercial ELISA there is still a lack of rapid screening methods, which require minimal sample preparation and can be used for on-site monitoring. In this frame, analytical methods using spectroscopy-based system, routinely used for the detection and identification of molecular constituents, are nowadays started to be applied to mycotoxin analysis. Their applications for rapid analysis for mycotoxin-contaminated commodities have been recently reviewed. Rather than to quantify a single mycotoxin, these methods are useful to classify commodities as compliant or noncompliant against a decision limit fixed by the European Union. Therefore, multivariate data evaluation and classification strategies (i.e., chemometrics) are fundamental for establishing appropriate classification and quantification schemes. Successful applications of NIR spectroscopy have been employed to predict DON and fumonisins levels in corn. Hyperspectral imaging allowed to discriminate between contaminated grain for OTA as well as OTA-producing *Penicillium verrucosum* strains.

A step toward a spectroscopy-based system intended to be used as the first-line-control method was described by Sieger et al. (2017). The authors proposed an on-site analysis by using infrared laser spectroscopy to classify peanuts as compliant or noncompliant for levels of AFB1 ( $8 \mu\text{g kg}^{-1}$ ). Furthermore, biosensor array is also a good option for fast screening of mycotoxins. The field of microfluidics holds great promise for the development of simple and portable lab-on-a-chip systems. Very recently, a capillary chip portable device has been tested, enabling the simultaneous detection of OTA, AFB1, and DON. Results were acquired using a standard smartphone camera and analyzed with a simple gray scale quantification procedure.

This scenario is moving fast toward a lab-on-mobile-device platform, and it is also facilitated by the launch from many companies of mobile reading app that have excellent potential for allowing performance of analysis and interpreting results. Despite this technology is still in the early development stage, results are promising. Sensitive and high accurate measurements are reported and also compared to HPLC data.

Mobile phones are now so widespread and accessible that this technology has been proposed for mycotoxins monitoring in developing countries. Smartphone rapid diagnostic readers have been tested in field trials in Africa to control and reduce aflatoxin contamination.

Proposals for rapid analytical methods for legislated mycotoxins, based on chemical molecular recognition, represent still a major fraction of newly published articles. The reduction of instrumental complexity and the development of user-friendly devices for point-of-need analyses allowed the spread of these methods. Miniaturization to foster on-site analysis is the key trend, and multianalyte detection is highly preferred. However, the main drawbacks are the number of false positives (because of cross-reactivity and matrix-dependence) or false negatives (because of low sensitivity), in comparison with chromatographic methods. Therefore, these methods are insufficiently precise to satisfy the legal requirements for making decision.

Future work will be required also on detection and quantification of modified mycotoxins, whose importance is discussed in the recent published EFSA opinion on modified mycotoxins.

## Liquid Chromatography–Mass Spectrometric Methods

### Low- and High-Resolution Mass Spectrometric Methods

In the first section, the many possibilities of rapid methods for mycotoxin analysis were reviewed. Immunological methods can detect most of the mycotoxins, but they are mainly used for screening.

However, mycotoxin analysis is also frequently achieved by chromatographic techniques. Primarily liquid chromatography (LC) coupled with ultraviolet/diode array detection and fluorescence detection was used.

Subsequently, in the last two decades, LC–MS/MS became the method of choice, in particular, for its ability to allow the development of multiresidue and multiclass methods. The first low-resolution multitoxin method was developed in 2006 for the quantitative determination of 39 parent and modified mycotoxins.

Despite having become a well-established technique, the triple quadrupole (QqQ) method setup is time-consuming when aimed at determining a large number of substances. Likewise, this technique presents limitation on the number of compounds that can be analyzed in one run. In addition, only targeted analytes can be detected, making necessary the use of an analytical standard, which is a critical issue in the modified mycotoxin field.

With new development in mass spectrometric instrumentation and with the introduction of benchtop high-resolution mass spectrometry instruments (HRMS), such as Time-of-Flight (ToF) and Orbitrap, full-scan techniques started to be investigated as complementary approach for the QqQ-based methods on the basis of increased resolution power and detectability. While the use of HRMS for the quantification of one or few analytes does not pose any significant advantage compared with MRM-based methods, multicontaminant methods seem to be the most promising approach for food and feed surveillance in the coming years. In particular, the availability of sensitive LC–MS instruments that are less prone to matrix effects allows to oversimplify the sample preparation and to avoid cleanup steps. Thus, the general tendency is to avoid any cleanup steps exploiting the selectivity and

sensitivity of MS technique. Dilute and shoot and the injection of raw extract, without any cleanup, is now commonly performed in multiresidue LC–MS analysis.

The enhanced selectivity and sensitivity provided by HRMS allowed the development of methods that cover a wide range of co-occurring contaminants (i.e., pesticides, mycotoxins, plant toxins) with different physicochemical properties.

The major advantage of using HRMS over MS/MS techniques for the targeted analysis is actually due to the possibility to perform retrospective data analysis, thus enabling the possibility to reconsider analytical results for stored data. The measurement of accurate MS and MS/MS spectra (resolution <5 ppm) allows the determination of compounds without previous compound-specific tuning, to carry out retrospective analysis of data and to perform structural elucidation of unknown or suspected compounds. This is particularly worth noting when modified mycotoxins are considered.

Besides the quantitative analysis, HRMS' major benefit is related to the qualitative analysis and it is due to the possibility to perform structural elucidation of newly discovered mycotoxins and phase I and phase II metabolites. As an example, the identification of new trichothecene conjugates such as FUSX-glucoside and NIV-glucoside, or acetyl-T2, was allowed on the basis of accurate mass measurements. LC coupled to Quadrupole Orbitrap MS was also adopted for the identification of acetylated metabolites of ZEN in wheat and of neosolaniol-glucoside (NESGlc) and diacetoxyscirpenol-glucoside (DASGlc) in maize.

From the abovementioned examples and application, it is clear that HRMS is gaining increasing interest in both research and routine laboratories. Targeted and routine quantification as well as qualitative research analysis can be performed with the same instrument. This situation is also facilitated by the launch from many MS companies of the latest generation of HRMS instrumentation designed for routine analysis and equipped with user-friendly dedicated data processing software.

### Advanced High-Resolution Ion Mobility Spectrometry Applications

The great increases in sensitivity and selectivity of LC–MS instruments have made a significant contribution in qualitative and quantitative determination of mycotoxins in food commodities. However, matrix effects, isobaric interference, and maintaining confidence in the assignment of identity are still the major limitations for LC–MS methods used for the quantification and identification of mycotoxins.

In this frame, the ion mobility–mass spectrometry (IM-MS) is explored as a new promising approach that can overcome the abovementioned HRMS limitation, making it an ideal candidate for improving confidence in the identification and separation of structurally closely related isomers. IMS is a gas-phase electrophoretic technique that provides a new dimension (3D) of separation based on size, shape, and charge of ions. Comprehensive reviews addressing, in detail, the various ion mobility techniques have been presented.

In addition, IMS allows the measurement of collision cross-section values (CCS), which is a unique orthogonal molecular descriptor that, when used in addition to retention time and mass-to-charge ratio ( $m/z$ ), offers the opportunity to further improve and support the identification process. This allows CCS to be used alongside the traditional molecular identifiers.

At first, this interest was primarily oriented in separating structurally related isomers. As an example, DON-oligo-glucosides were analyzed using drift tube ion mobility spectrometry (DTIMS) enabling the detection of one more DON-di-Glc and two more DON-tri-Glc peaks separated by their drift time, compared with LC–HRMS results. These additional peaks were tentatively attributed to the linkages, 1–4 or 1–6, between the sugar moieties and the mycotoxin, as the bounding position was confirmed by HRMS-MS/MS. Therefore, IMS-MS allowed the detection and subsequently the characterization of new isomeric DON-oligo-Glc, also increasing confidence in results.

Apart from the isomeric separation, IMS is starting to be successfully employed in routine screening analysis. Indeed, CCS values can be routinely measured as an integrated part of LC–HRMS experiments and used alongside the traditional molecular identifiers (i.e., precursor ions, accurate masses, fragment ions and isotopic patterns, retention times) to reduce the number of both false positive and false negative of targeted analysis. Recently, the potential of CCS as an additional identification point (IP) for residue analysis has been discussed, in consideration of the precision and the robustness of the CCS measurement. Their reproducibility across multiple samples and instruments has been demonstrated by analyzing more than 100 CCS values of parent and modified mycotoxins. The measurements showed high reproducibility (RSD <2%) across different instrumental settings as well as several complex cereal matrices (i.e., malt, rye, corn flakes, maize feed, wheat), showing a mean intermatrix precision of RSD <0.9%. The database was applied to the analysis of several spiked as well as naturally incurred cereal-based samples, demonstrating that no matrix effect is interfering with the IMS separation.

Thus, it is an argument in favor of considering CCS as valuable parameter in food safety control. Population of databases with CCS values for mycotoxins is pivotal to implement the CCS comparisons and thus to support the inclusion of CCS values as a new identification point.

CCS values may also be estimated computationally if the 3D structure is known. A comparison of the theoretically and experimentally derived collision cross-sections can be used for the accurate assignment of isomeric metabolites when analytical standards are not available. This approach has been recently applied to elucidate glucuronide metabolites of DON, ZEN, AOH, and AME. Indeed, the authors demonstrated a good correlation between theoretical and experimental CCS, suggesting the potential of the CCS matching in supporting the annotation procedure. The use of theoretical CCS values offers a unique means of characterization to overcome the lack of analytical standards and the limitation of HRMS when fragmentation pattern of two isomers is not useful in discriminating.



IMS is spreading rapidly as a promising technique for simultaneous screening, identification, and quantification of a large number of mycotoxins. As for LC–HRMS, both research and routine analysis can be performed in the same instrument. With this perspective, CCS database must be populated to widen the range of contaminants that can be present in food samples.

### Metabolomics Approaches to Study Mycotoxin Metabolism

In the recent years, the tremendous progress in high-resolution mass spectrometry and software tools facilitate the spread of metabolomics approaches also in the contaminants field. Indeed, novel research in mycotoxin analysis has been moving from the targeted analysis of individual mycotoxins to untargeted analysis to detect not only the mycotoxins itself and their biotransformation products but also all the metabolites that are involved in plant–fungi interactions.

Metabolomics is going to open new avenues into safety evaluation of foods and food components. Two different strategies can be distinguished within this approach: metabolomics profiling, focused on a predefined group of structurally related compounds, and untargeted metabolomics, which aim to record MS features of all detectable compounds.

Metabolic profiling strategy has been in depth applied with the aim to elucidate the biotransformation pathways of a determined mycotoxin in plant. Therefore, the major method used in this area is based on *in vivo* stable isotope  $^{13}\text{C}$  labeling (SIL) and subsequent measurement of biological samples by full-scan high-resolution LC–MS. The spectra comparison and metabolite identification are supported by the use of a dedicated software able to monitor pairs of corresponding nonlabeled and labeled precursor. This powerful approach simplifies the identification of novel unknown compounds (i.e., feruloyl-T2) or novel mycotoxin metabolites. So far, the abovementioned approach has been employed to understand the metabolic fate of HT-2 toxin and T-2 toxin in barley and oats and DON in wheat.

Not only profiling but also untargeted metabolomics starts to be widely applied. This approach represents the golden tool for understanding the biological pathways involved in mechanisms of plant resistance against fungal attack.

The main advantage provided by the nontargeted nature of the method is based on the possibility of detecting new metabolites involved in the resistance mechanisms helpful in deciphering new pathways, enabling the detection of hundreds of small molecules in one run. Such an approach requires advanced analytical instruments as well as strong knowledge of chemometric tools.

On the other hand, the main constraint is related to the complexity of the plant metabolome. Seeking for all plant metabolites simultaneously is extremely challenging because of the high chemical diversity. Currently no extraction protocol combined with a single analytical technique allows considering the entire metabolome, and consequently, the data delivered by metabolomic studies only cover a fraction of the metabolome.

This promising method allowed to improve our limited understanding of the roles of plant pathogen cross-talk at the molecular level. Most of the recent findings are deeply described in a comprehensive review.

The advent of “omics” in the mycotoxin research field is also stressed by the inflationary use of this term. “Mycobolome” and “Xenobolomics” have been used to refer to the whole set of fungal metabolites, while “Maskedome” includes all the possible modification. With the aim to estimate the human exposure toward toxic compounds, also the “exposome” has been defined. Although risk assessment is commonly based on the targeted quantification of mycotoxin urinary biomarkers (i.e., glucuronides and sulfate metabolites), the use of untargeted HRMS will theoretically enable the investigation of the whole individual exposure, linking dietary exposure to the outcome of specific diseases.

### Summary and Future Outlook

Over the last few years, the mycotoxin scientific community has experienced a shift from classical LC–MS analysis based on QqQ to the use of HRMS, in particular, for multianalyte applications. The analytical potential of high resolution, accurate mass, and acquisition in full scan permits a retrospective analysis using extensive databases of hundreds of analytes. In addition, studies based on untargeted approaches are becoming more popular, especially for the analysis of biomarkers of exposure, under a “human exposome” approach. Therefore, HRMS is undoubtedly going to redefine the analytical scene for quantitative and qualitative mycotoxin analysis. On the other hand, simple and rapid tools for screening are becoming the golden standard for qualitative routine analysis, when a clear cut-off for acceptance or withdrawal of batches can be set. In this field, the scenario is moving fast toward multitoxin systems, biosensors able to return a response in terms of “cocktail,” and ICT-driven solutions that can be easily managed in remote with cloud data storage.

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# Next-Generation Sequencing

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## Glossary

**Amplicon sequencing** Sequencing of targeted PCR products (e.g. 16S rDNA sequencing).

**Next-generation sequencing** Also called high-throughput sequencing; high-throughput nucleotide sequencing technologies that can sequence many DNA strands in parallel.

**Read** Sequenced base pairs generated from a DNA fragment.

**RNA sequencing (RNA-Seq)** Sequencing of an entire transcriptome, using cDNA synthesized from RNA.

**Sanger sequencing** Also referred to as first-generation sequencing; a low-throughput nucleotide sequencing method which relies on the incorporation of fluorescently-labelled, chain-terminating dideoxynucleotide triphosphates (ddNTPs) by DNA polymerase during the elongation step of PCR. The resulting strands are separated using capillary gel electrophoresis, and the emission spectra of the fluorescently-labelled ddNTPs are determined using laser excitation.

**Shotgun metagenomic sequencing** Untargeted sequencing method that employs NGS technologies to sequence all DNA extracted from an environmental sample, including food or other complex matrices.

**Shotgun metatranscriptomic sequencing** Untargeted sequencing method that employs NGS technologies to sequence cDNA synthesized from RNA extracted from an environmental sample, including food or other complex matrices.

**Whole-genome sequencing (WGS)** Sequencing of an entire genome of a single organism.

## Nomenclature

**List of nomenclature used including all definitions and units.**

**16S rDNA** 16S ribosomal deoxyribonucleic acid

**bp** base pairs

**cDNA** complementary deoxyribonucleic acid

**cgMLST** core genome multi-locus sequence typing

**DNA** deoxyribonucleic acid

**Kb** kilobase pairs

**MLST** multi-locus sequence typing

**NGS** next-generation sequencing

**PCR** polymerase chain reaction

**PFGE** pulsed-field gel electrophoresis

**RNA** ribonucleic acid

**RNA-Seq** ribonucleic acid sequencing

**RT-qPCR** quantitative reverse-transcription polymerase chain reaction

**SBL** sequencing-by-ligation

**SBS** sequencing-by-synthesis

**SLST** single-locus sequence typing

**SMRT sequencing** single-molecule real-time sequencing

**SNP** single-nucleotide polymorphism

**wgMLST** whole genome multi-locus sequence typing

**WGS** whole-genome sequencing

## Overview

*Next-generation sequencing* (NGS) encompasses sequencing technologies that are capable of sequencing many DNA strands in parallel, resulting in higher throughput than can be achieved using *Sanger sequencing*. As NGS has become cheaper and more accessible, it has been used to address an expanding range of biological problems, including many relevant to food safety and quality.

## Next-Generation Sequencing Technologies

Contemporary NGS sequencing platforms employ either a (i) short-read, or (ii) long-read sequencing approach (Table 1). Short-read sequencing approaches typically yield read lengths of up to 700 base pairs (bp), which tend to be shorter than those produced by Sanger sequencing (Goodwin et al., 2016; Liu et al., 2012). Currently, sequencing-by-synthesis approaches (SBS) to NGS are the dominant paradigm in short-read sequencing. These approaches (e.g. Illumina sequencing, Roche 454 pyrosequencing, Ion Torrent semiconductor-based sequencing) rely on the use of DNA polymerase in their respective methods (Goodwin et al., 2016). SBS approaches to short-read sequencing can be contrasted with the sequencing-by-ligation (SBL) approach employed by the SOLiD (Small Oligonucleotide Ligation and Detection) platform, which employs DNA ligase to join fluorescently-labelled probe and anchor sequences to a DNA strand (Goodwin et al., 2016). Among the SBS approaches and short-read sequencing methods as a whole, Illumina sequencing has emerged as the dominant technology (Goodwin et al., 2016), in which fluorescently-tagged nucleotides are added in complement to amplified strands of DNA. Upon the addition of a single nucleotide, the fluorescent dye is imaged, and the identity of the corresponding base is recorded (Goodwin et al., 2016).

While short-read sequencing technologies have been the workhorse of NGS, they are not without limitations; many genomic features, such as long, repetitive regions or copy number variations, cannot be readily resolved using short reads (Goodwin et al., 2016). Long-read sequencing technologies have been able to bridge the literal gaps that their short-read counterparts have been unable to resolve, relying on either (i) synthetic long-read approaches or (ii) single-molecule long-read sequencing approaches (Pacific Biosciences and Oxford Nanopore) (Goodwin et al., 2016). Synthetic long-read sequencing approaches employ existing short-read sequencing platforms, but use barcoding during library preparation to link fragments (Goodwin et al., 2016). Single-molecule long-read sequencing approaches, however, yield “true” long reads that can span kilobases, with the approach most commonly employed as of late 2017 being the Pacific Biosciences (PacBio) single-molecule real-time (SMRT) approach (Goodwin et al., 2016). SMRT sequencing uses a DNA polymerase fixed to the bottom of a well in a specialized flow cell through which a DNA strand is passed (Goodwin et al., 2016). Upon the incorporation of a single, fluorescently-labelled nucleotide by the polymerase, light is emitted and recorded by a camera to determine the identity of the nucleotide (Goodwin et al., 2016). This can be contrasted with the aforementioned short-read SBS approaches, which rely on DNA polymerase traversing the DNA template to which it is bound (Goodwin et al., 2016). In addition to the PacBio platform, the small and highly portable MinION platform from Oxford Nanopore Technologies also employs a single-molecule long-read sequencing approach, during which a strand of DNA is passed through a protein pore along with an electric current (Goodwin et al., 2016). As different combinations of nucleotides are passed through the pore, shifts in the electric current are recorded (Goodwin et al., 2016).

Long-read sequencing is becoming increasingly popular for many applications, including gap closure in reference genomes, characterization of long genomic structures, and the generation of closed chromosomes or transcriptomes (Goodwin et al., 2016). A notable consideration when comparing short-read and long-read sequencing methods is the relatively high error rates of long-read sequencing platforms (Goodwin et al., 2016). For example, the PacBio RS II, which yields average read lengths of 10–15 Kb, has an error rate as high as 15% for a single pass through a molecule of DNA (Goodwin et al., 2016). However, this error rate can be reduced to one that rivals that of Sanger sequencing by increasing sequencing coverage through multiple passes; after 30 passes (i.e. at 30X coverage), the accuracy of the consensus is greater than 99.999% (<http://www.pacb.com/smart-science/smart-sequencing/accuracy/> and <https://www.pacb.com/uncategorized/a-closer-look-at-accuracy-in-pacbio/>) (Goodwin et al., 2016).

**Table 1** Overview of next-generation sequencing technologies discussed in this article<sup>a</sup>

Sequencing technology	Sequencing mechanism	Read length <sup>b</sup>	Error rate (type of error)
<b>Sequencing-by-ligation (SBL)</b>			
SOLiD	Ligation; 2-base encoding	50–75 bp	≤0.1% (AT bias) <sup>c</sup>
<b>Sequencing-by-synthesis (SBS)</b>			
454	Pyrosequencing	Up to 1000 bp	1% (indel) <sup>d</sup>
Illumina	Illumina SBS	25–300 bp; can be 100 Kb if synthetic long-read library preparation is used	0.1% to 1%, depending on platform/output (substitution)
Ion Torrent	Hydrogen ion detection	Up to 400 bp	1% (indel)
<b>Single-molecule long-read</b>			
Oxford Nanopore	Nanopore	Up to 200 Kb	12% (indel)
Pacific Biosciences	Single-molecule real-time sequencing	8–20 Kb	13% for a single pass (indel)

<sup>a</sup>Summarized from reviews of NGS technologies by Goodwin et al., Liu, et al., and Glenn (Goodwin et al., 2016; Liu et al., 2012; Glenn, 2011).

<sup>b</sup>bp, base pairs; Kb, kilobase pairs.

<sup>c</sup>AT, adenine and thymine.

<sup>d</sup>indel, insertion/deletion.

## NGS Data Analysis

Processing and analysis of NGS data is dependent on the sequencing technology used, as well as the experimental goals. Regardless of sequencing method or experimental design, the first steps in the analysis of NGS data usually involve an assessment of read quality, using metrics such as the total number of reads, the distribution of read lengths, sequence quality scores, etc. This can be followed by trimming of adapters and/or low-quality bases, filtering out low-quality reads, and filtering of contaminant DNA, steps for which a number of programs are available (Breitwieser et al., 2017). After these pre-processing steps, data analysis can be carried out according to the goals of the experiment, with possible food science-relevant applications discussed below (Table 2).

## NGS Applications: Whole-Genome Sequencing of Microbial Contaminants

Traditionally, microbial contaminants isolated from food undergo various organism-specific phenotypic or biochemical tests (e.g. testing for motility, toxin production, growth at various temperatures) to elucidate or confirm their identity (FDA, 1998). These tests may be supplemented with additional typing methods, such as serotyping, pulsed-field gel electrophoresis (PFGE), Sanger sequencing of a single taxonomic marker gene or genomic region (i.e. single-locus sequence typing; SLST), or Sanger sequencing of multiple loci used in a multi-locus sequence typing (MLST) scheme (Kovac et al., 2017; Sabat et al., 2013). However, the per-isolate cost of *whole-genome sequencing* (WGS) has decreased to the point at which it is comparable, and even below, the price of many of these traditional subtyping methods (Kovac et al., 2017), making it an increasingly popular method for characterizing microbial contaminants isolated from food matrices, food-associated environments (e.g. farm environments, processing environments), and, in the case of pathogenic microbes, from hosts (e.g. in human- or animal-clinical settings) (Kovac et al., 2017). Furthermore, many of these typing methods (e.g. serotyping, SLST, MLST) can be performed *in silico* using WGS data, with the advantage that one can query the majority of a microbial genome from a single data set, rather than just a small fraction of it (<0.01% for a traditional 7-gene MLST scheme) (Kovac et al., 2017). In addition to *in silico* subtyping, WGS data from microbial contaminants can be used to predict functional characteristics of isolates, query genes or genomic elements of interest within a genome (e.g. plasmids, bacteriophage, and genes contributing to antimicrobial resistance or virulence), and, in the case of pathogenic microorganisms, detect and track outbreaks (Kovac et al., 2017).

After sequencing the genomic DNA and pre-processing the resulting reads from a microbial isolate (see “NGS Data Analysis” section above), possible analysis steps that may be taken include (i) *de novo* genome assembly of the reads into contiguous stretches of sequence (contigs) (Giordano et al., 2017; Liao et al., 2015; Ekblom and Wolf, 2014), (ii) mapping reads back to a reference genome, (iii) identifying single-nucleotide polymorphisms (SNPs), insertions, and deletions (indels) in NGS data through variant calling (Olson et al., 2015), (iv) constructing phylogenetic trees to assess the evolutionary relationship of multiple isolates, (v) assigning allelic types at a genomic scale using core genome or whole genome multi-locus sequence typing (cgMLST and wgMLST, respectively), and (vi) locating genes and features in NGS data via genome annotation (Richardson and Watson, 2013;

**Table 2** Overview of food science-relevant next-generation sequencing applications discussed in this article

Next-generation sequencing application	Number of organisms queried	Nucleic acid extracted/sequenced	Genomic elements queried	Current food science-relevant applications
Whole-genome sequencing (WGS)	1	DNA/DNA	Entire genome	Characterization of food-relevant organisms at the genomic level
RNA sequencing (RNA-Seq)	1	RNA/cDNA reverse-transcribed from RNA	Entire transcriptome	Characterization of food-relevant organisms at the transcriptional level
High-throughput amplicon sequencing (e.g. 16S rDNA sequencing, DNA-barcoding)	≥1	DNA/DNA	Selected amplicon(s) present in sample (usually 16S rDNA for bacterial/archaeal communities; other loci for eukarya)	Taxonomic characterization of food-relevant microbial communities (usually bacterial/archaeal communities); authentication of eukaryotic food matrices (e.g. seafood, meat products)
Shotgun metagenomic sequencing	>1	DNA/DNA	All genomes present in sample	Characterization of food-relevant communities at the genomic level (queries eukarya, bacteria, archaea, and viruses)
Shotgun metatranscriptomic sequencing	>1	RNA/cDNA reverse-transcribed from RNA	All transcriptomes present in sample	Characterization of food-relevant communities at the transcriptional level (queries eukarya, bacteria, archaea, and viruses)

Mudge and Harrow, 2016; Yandell and Ence, 2012). These data can be used to characterize isolates at high resolution, making it possible to compare isolates geospatially and temporally at the whole-genome scale.

WGS is becoming an increasingly valuable tool for characterizing microbial contaminants—particularly pathogens—isolated from food and food processing environments. A notable example of the utility of WGS can be seen in the multi-agency collaboration in the US to sequence all *Listeria monocytogenes* isolates from human patients, food, and the environment (Jackson et al., 2016). Since its implementation in 2013, the WGS-based surveillance program detected more listeriosis clusters and solved more outbreaks each year, relative to the previous year (Jackson et al., 2016). Similar findings have been seen for *Salmonella enterica* serotype Enteritidis (*S. Enteritidis*); retrospective sequencing of 55 *S. Enteritidis* from clinical and environmental sources allowed isolates from known outbreaks to be differentiated from sporadic isolates at greater resolution than PFGE (Taylor et al., 2015). These examples showcase how WGS can be used to not only characterize foodborne pathogens at high resolution, but also the outbreaks associated with them.

### NGS Applications: RNA Sequencing (RNA-Seq) of Food-Relevant Organisms

While WGS can be used to characterize the genome an organism at unprecedented resolution, it offers no information on whether a genomic element of interest is being actively transcribed or not. This is particularly important within a food safety context; for example, the mere isolation of a pathogen from a food matrix does not necessarily mean that particular isolate is viable, or that it is transcribing the genes necessary to cause infection or intoxication in a human host. Traditionally, quantitative reverse-transcription PCR (RT-qPCR) has been employed to quantify or detect shifts in transcript levels of loci of interest. For this method, reverse-transcription PCR (RT-PCR) is used to obtain complementary DNA (cDNA) from a RNA template, and the resulting cDNA can be quantified using quantitative PCR (qPCR). In a food science context, RT-qPCR has been proposed as a method for detecting viable microorganisms, quantifying virulence, toxin, or stress response gene transcription, and quantifying microbial growth in food matrices (Postollec et al., 2011). Studying transcription at a genome-wide scale, however, was made possible with cDNA microarrays, which have been used to study the stress responses of various foodborne pathogens, as well as their transcription of toxin and virulence genes (Postollec et al., 2011; Roy and Sen, 2006; Rasooly and Herold, 2008). As NGS has become more feasible, however, it is now possible to query the transcriptome of an organism in its entirety at low cost: RNA sequencing (RNA-Seq) employs NGS technologies to sequence cDNA reverse-transcribed from RNA that has been extracted from an organism of interest (Wang et al., 2009). RNA-Seq allows one to quantitatively survey transcribed regions of an entire genome, improving upon microarrays in both cost and flexibility (i.e. the ability to characterize any organism that can be sequenced, rather than relying on the availability of an array for a particular organism), which is particularly valuable for studying organisms or genomic regions that may not be well-characterized.

After employing NGS to sequence cDNA from an organism of interest, and determining that the quality of sequencing reads is adequate, reads are usually aligned to a reference genome or an assembled transcriptome (McClure et al., 2013; Conesa et al., 2016). After assessing mapping quality and determining that it is appropriate, reads mapping to various genes or genomic regions can be quantified and normalized, taking into account biases such as gene length (Conesa et al., 2016; McClure et al., 2013). After quantification and normalization, analyses can be carried out according to the experimental goals (e.g. differential transcription under various conditions). Within the realm of food safety, RNA-Seq has been applied to pathogenic and toxin-producing microorganisms to identify differentially-transcribed genes during growth in various food matrices (Tang et al., 2015; Deng et al., 2012; Galia et al., 2017), after exposure to various stressors (e.g. acid, starvation, or antimicrobial stressors) (Zhang et al., 2014; Casey et al., 2014; Butcher and Stintzi, 2013; Jia et al., 2017), and during the infection of a host (Avraham et al., 2016).

### NGS Applications: High-Throughput Amplicon Sequencing

WGS and RNA-Seq have allowed food-associated microorganisms to be characterized at unprecedented resolution. However, these methods typically require the microorganism in question to be in pure culture or isolated via culture-based methods, a process which involves the use of organism-specific enrichment media, selective media, and isolation protocols (Kovac et al., 2017). Metagenomics, which involves sequencing DNA directly from an environmental sample, attempts to bypass the isolation step, making it possible to survey an entire community simultaneously (Kovac et al., 2017).

Until recently, NGS-based metagenomic methods have primarily involved high-throughput *amplicon sequencing*. Also referred to as “metataxonomics”, “meta-genetics”, or “marker gene metagenomics”, high-throughput amplicon sequencing employs NGS technologies to sequence targeted PCR products (amplicons) to characterize particular communities. When surveying bacterial and archaeal communities, the 16S ribosomal DNA gene (16S rDNA) is usually the amplicon of choice, as it is present in all bacterial and archaeal species. 16S rDNA sequencing has been used to survey the microbiota of various foods (De Filippis et al., 2017; Kergourlay et al., 2015; Ercolini, 2013), including fermented foods (De Filippis et al., 2017) and food matrices subjected to pathogen-specific enrichments (Jarvis et al., 2015; Lusk et al., 2012), as well as to monitor bacterial community shifts in food processing environments (Stellato et al., 2016; Hultman et al., 2015).

One of the strengths of 16S rDNA amplicon sequencing is that there are many open-source bioinformatics tools and pipelines available for data analysis and visualization of results (e.g. QIIME, Mothur). A typical workflow for analyzing NGS data from high-throughput 16S rDNA experiments may include pre-processing of the raw reads, clustering of sequences into operational



taxonomic units (OTUs) based on sequence similarity, and taxonomic assignment of sequences using a database of 16S rDNA genes (e.g. RDP, Greengenes, SILVA) (Oulas et al., 2015; Siegwald et al., 2017).

In addition to querying bacterial and archaeal communities, the same principals of amplicon sequencing can be applied to characterize eukarya. DNA-barcoding, a practice in which a specific region of a genome is sequenced, is a commonly-used method for food matrix authentication along the food supply chain (Ellis et al., 2016; Galimberti et al., 2013). For this approach, a genetic marker (i.e. a “barcode”) present in a range of taxa, but variable enough to be capable of discriminating between taxa of interest, is sequenced (Galimberti et al., 2013), similar to the way the 16S rDNA gene is used to survey bacterial/archaeal communities. When querying animal DNA in a matrix (e.g. for seafood or meat authentication), the cytochrome *b* (*cytB*) and cytochrome *c* oxidase subunit 1 (*COI*) genes are common amplicons of choice. For fungi, the internal transcribed spacer (ITS) region of the genome is the locus of choice (Schoch et al., 2012), while a number of loci have been proposed for querying plant DNA present in a matrix (Hollingsworth et al., 2011, 2016). The sequences of these genes are then compared to the barcodes of known taxa, such as those found in the Barcode of Life Database (BOLD) (Ratnasingham and Hebert, 2007) or the National Center for Biotechnology Information’s (NCBI) GenBank database (Benson et al., 2013). Applications of DNA-barcoding within the realm of matrix authentication and contaminant detection along the food supply chain have included authentication of and contaminant detection in seafood (Carvalho et al., 2015; Armani et al., 2015; Pardo et al., 2016; Kim et al., 2015; Chang et al., 2016; Carvalho et al., 2017), meat (Kane and Hellberg, 2016; Hellberg et al., 2017; Naaum et al., 2018), poultry (Hellberg et al., 2017), dairy products (Galimberti et al., 2013), olive oil (Kumar et al., 2011), and spices (Swetha et al., 2017; De Mattia et al., 2011; Galimberti et al., 2013).

Until recently, DNA-barcoding was limited by the low-throughput that Sanger sequencing provides; however, NGS has emerged as a low-cost, high-throughput alternative (Ellis et al., 2016; Shokralla et al., 2014) that has been used for characterizing both raw ingredients and processed foods (Galimberti et al., 2013). In this high-throughput approach, sequencing reads are mapped to sequences in an appropriate database (often BOLD or GenBank) after determining that read quality is appropriate. The proportion of reads mapping to a particular species in the database corresponds to the proportion of that particular species in the matrix. A notable example of the application of high-throughput sequencing for food matrix authentication is provided by Carvalho et al. (Carvalho et al., 2017), in which mislabelled cod products in Brazilian stores and restaurants were identified by targeted sequencing of the *cytB* and *COI* genes present in processed cod products using NGS (Carvalho et al., 2017). In addition to identifying mislabelled products, the composition of blended products composed of multiple fish species could be determined by sequencing the selected loci (Carvalho et al., 2017).

## NGS Applications: Shotgun Metagenomic and Metatranscriptomic Sequencing

Although high-throughput amplicon sequencing has offered a higher-resolution glimpse into food and food-associated microbiomes, it has numerous limitations that are particularly relevant within the realms of food safety and food quality, perhaps most notably the inability to query organisms that do not possess the amplicon of choice (e.g. eukarya in a community cannot be queried if 16S rDNA amplicon sequencing is performed; see “NGS Applications: High-Throughput Amplicon Sequencing” section above). For 16S rDNA amplicon sequencing of bacterial/archaeal communities, additional drawbacks include (i) difficulty achieving species-level resolution (Janda and Abbott, 2007; Rossi-Tamisier et al., 2015) and reliably distinguishing pathogenic bacteria from non-pathogenic species (e.g. *L. monocytogenes* from *Listeria innocua*, human pathogens *Bacillus anthracis* from *Bacillus cereus* and biopesticide *Bacillus thuringiensis*), (ii) PCR amplification and primer bias (Brooks et al., 2015), and (iii) inability to query functionally-relevant genomic elements directly, such as virulence or antimicrobial resistance determinants (Kovac et al., 2017).

An increasingly-popular alternative to amplicon sequencing is *shotgun metagenomic sequencing*, an approach in which all DNA present in a sample is sequenced, rather than solely an amplicon. By sequencing all DNA present in a sample, the amplification bias and low taxonomic and functional resolution issues which plague amplicon sequencing can typically be bypassed (Kovac et al., 2017). In addition to sequencing all of the bacterial and archaeal DNA present in a sample, all viral and eukaryotic DNA is sequenced; this is particularly relevant when the community of interest is derived from a eukaryotic matrix (e.g. from a host or from food), as the majority (as much as 99%) of DNA will come from the eukaryotic matrix itself (Noyes et al., 2016; Kovac et al., 2017). While large quantities of host DNA may not be a problem if the experimental goal is to assess the composition of the food matrix itself, it may hinder the sequencing and detection of many microbial species. As a result, when extracting DNA from a matrix containing high amounts of host DNA, additional steps may be taken to deplete any background DNA originating from the matrix itself to increase the proportion of microbial DNA that is sequenced (Kovac et al., 2017). After sequencing the extracted DNA, analysis of the resulting sequencing reads is carried out according to the experimental goals, which may include taxonomic assignment (Sharpton, 2014), metagenomic assembly, functional annotation (Sharpton, 2014), and/or conducting a metagenome-wide association study by associating community data with a particular phenotype (Wang and Jia, 2016; Lynch and Pedersen, 2016).

As with all genomic approaches, shotgun metagenomic methods can offer insight into the genomic composition of a community, but cannot offer information as to which genes are being transcribed and possibly translated and expressed as protein products (Kovac et al., 2017). Similar to the way RNA-Seq can be used to complement WGS of a bacterial isolate, metagenomic approaches can be supplemented with *shotgun metatranscriptomic sequencing*, which involves sequencing cDNA reverse-transcribed from RNA (typically messenger RNA) extracted from an entire community (Kovac et al., 2017).



Analysis of shotgun metagenomic and metatranscriptomic data usually begins with pre-processing steps such as assessing read quality and trimming adapters (Breitwieser et al., 2017). This can be followed by (i) assembly of the reads into contigs, or (ii) taxonomic or functional classification directly from sequencing reads (Breitwieser et al., 2017). For a review of methods for metagenomic data analysis, see Breitwieser et al. (Breitwieser et al., 2017).

The use of shotgun metagenomic and metatranscriptomic approaches to survey communities in foods has been undertaken only recently (De Filippis et al., 2017). Goals of these studies have included characterization of the microbiomes of various foods in the presence of foodborne pathogens and/or spoilage organisms (Jarvis et al., 2015; Ottesen et al., 2013), tracking foodborne pathogens and antimicrobial resistance genes along the food supply chain (Yang et al., 2016; Noyes et al., 2016), characterizing eukaryotic food matrices composed of multiple species (Ripp et al., 2014), and characterizing the microbiomes of various food matrices during processes such as fermentation (De Filippis et al., 2017; Kergourlay et al., 2015; Alkema et al., 2016; Valdés et al., 2013; Lessard et al., 2014; De Filippis et al., 2016; Monnet et al., 2016). A notable example of the application of shotgun *meta*-omics approaches to identify the cause of a food quality anomaly is provided by Quigley et al. (Quigley et al., 2016); using high-throughput 16S rDNA sequencing followed by shotgun metagenomic sequencing, *Thermus thermophilus* was proposed (and later confirmed) to be the cause of a pink discoloration defect in Continental-type cheeses (Quigley et al., 2016).

## Conclusions

NGS technologies are being employed increasingly in food science-relevant realms, with applications ranging from surveying microbial communities involved in food processing, to rapidly characterizing bacterial isolates from foodborne outbreaks. As sequencing costs continue to decrease, it is likely that whole-genome and *meta*-omics approaches will be applied routinely at various points along the food supply chain.

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- <https://www.illumina.com/> – Illumina sequencing.
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- <https://nanoporetech.com/> – Oxford Nanopore Technologies.
- <http://www.pacb.com/> – Pacific Biosciences.
- <https://www.thermofisher.com/us/en/home/life-science/sequencing/next-generation-sequencing/solid-next-generation-sequencing/solid-next-generation-sequencing-systems-reagents-accessories/solid-next-generation-sequencing-chemistry.html> – SOLiD Sequencing.

# Dioxins and Dioxin-like PCBs in Feed and Food

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## Introduction

### Dioxins

Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are two classes of organic environmental contaminants that are often together termed “dioxins”. They have no use, were never produced industrially, but are formed as unwanted and often unavoidable by-products in a number of thermal and industrial processes. Dioxins are generally not formed as single compounds but as complex mixtures which can give valuable hints on the generating sources. Although they were already formed thousands of years ago, especially during forest fires, their levels in the environment substantially increased in parallel with the start of the growing chlorine industry in the 1940s.

Depending on the number and position of the chlorine atoms at the two rings, 75 PCDDs and 135 PCDFs, termed “congeners” can be distinguished (Fig. 1). They are resistant against acids, show low water solubility but high lipophilic properties. The best studied dioxin is 2,3,7,8-tetrachloro-dibenzo-p-dioxin (2,3,7,8-TCDD) which exhibits also the highest toxicity of the 210 congeners. Well-known pollution episodes are the spraying of the defoliant Agent Orange in the Vietnam War and the release of 2,3,7,8-TCDD in 1976 in Seveso/Italy, both causing high contamination in the environment and the humans living in the affected areas. Agent Orange was composed of the two herbicides 2,4 D and 2,4,5-T. The latter contained 2,3,7,8-TCDD as a by-product from the synthesis of the intermediate chlorinated phenol. The synthesis of chlorinated phenols was also the reason for the release of huge amounts of 2,3,7,8-TCDD in Seveso when a reaction vessel burst in a chemical factory.

From a toxicological point of view, those congeners that are 2,3,7,8-chlorine substituted are toxic and thus are of special concern. At high concentrations following poisoning or exceptional accidental or occupational exposure, they may cause chloracne. At much lower levels, *inter alia* effects on the immune and reproductive system in humans were reported. The biological half-life of TCDD in humans is 7–10 years and even longer for some higher chlorinated congeners.

### PCBs

In contrast to dioxins, PCBs had found wide-spread industrial use. It is estimated that more than 1.5 Million tons were produced since their first commercial introduction in 1929. They were generally manufactured as technical mixtures containing 40%–60% chlorine, and were marketed under trade names, such as Clophen, Arochlor, Phenoclor, Fenclor and Kaneclor. PCBs have excellent dielectric properties, are thermally stable, and resistant against acids, bases and oxidation. Due to these favourable properties, they were widely used as insulating material in electric equipment, such as capacitors and transformers, and as hydraulic fluids in mining. Although these applications are considered as “closed” systems, PCBs were released into the environment due to leakages, accidents and/or destruction of the electrical equipment. Besides the application in “closed” systems, PCBs were also used in “open” systems. This encompassed addition to lubricants and putty, use as plasticizers, paper surface coatings, flame retardants, carrier for pesticides and others. The application in “open” systems is banned in the Western World since the late 1970s, and also the use in “closed” systems was strictly regulated in the past. Nevertheless, it is estimated that several ten thousand tons of PCBs still remain in the environment today.

Depending on the number and position of the chlorine atoms at the two rings, 209 different PCBs can be distinguished, which are also termed “congeners” (Fig. 2). Those congeners that carry none or one chlorine in the ortho-position of the two rings (2, 2', 6, 6') can adopt a spatial orientation comparable to dioxins, exhibit similar toxic properties and thus are termed “dioxin-like PCBs (DL-PCBs)”. PCB congeners with two or more chlorine atoms at the ortho-position show different toxic properties and are called “non dioxin-like PCBs (NDL-PCBs)”. Within the subgroup of NDL-PCBs, the congeners with IUPAC numbers 28, 52, 101, 138, 153, and 180 are considered as “indicator-PCBs”. While the PCBs 28, 52 and 101 are rapidly metabolized in mammals, and thus would indicate a fresh contamination if determined in a food sample, the other three PCBs are highly persistent and generally contribute the most to the sum of PCBs in food stuffs of animal origin and in humans.

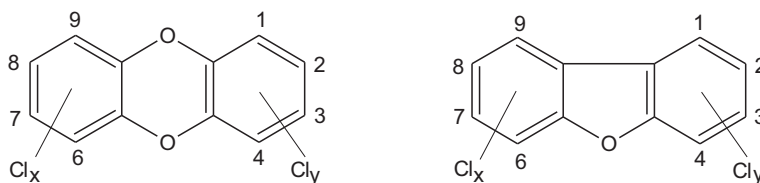
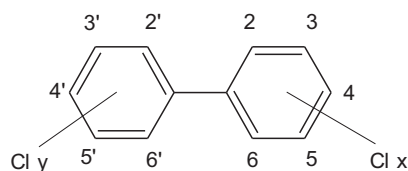


Figure 1 Structure of PCDDs (left) and PCDFs (right); Cl<sub>x</sub> + Cl<sub>y</sub> = 1–8.



**Figure 2** Structure of PCBs;  $Cl_x + Cl_y = 1-10$ .

### Toxic Equivalency Factors (TEFs)

Within the group of dioxins, the 2,3,7,8-chlorine-substituted congeners are of special importance due to their high toxicity in animals and humans. As the DL-PCBs exhibit similar toxic properties as the above mentioned dioxin congeners, both groups of environmental contaminants are generally considered together. Although the toxicity of the individual congeners varies substantially, the toxic effects of all these compounds are mediated through the aryl hydrocarbon receptor (AhR) in the cells. Based on the results of *in vivo* and *in vitro* studies, the diverse toxicity of the individual congeners can be expressed in terms of the most toxic dioxin, 2,3,7,8-TCDD which arbitrarily is assigned a toxic equivalency factor (TEF) of 1. The TEFs for the other toxic congeners are  $\leq 1$ . By summing up the concentrations of the analytically determined congeners multiplied with their individual TEFs, one obtains the toxic equivalent (TEQ) of a sample, which provides the total toxic potency in terms of 2,3,7,8-TCDD.

An important prerequisite of the TEF system is persistence and accumulation of the compounds in the food chain, additivity of effects, binding to the Ah receptor and eliciting AhR mediated toxic responses. Several different TEF systems have been established by national and international organizations in the past decades. The derivation of a TEF underlies the progressive scientific knowledge. New studies e.g. performed with highly purified reference standards may lead to a re-evaluation of the factors and consequently to updated TEFs. From this, it becomes clear that a meaningful comparison of analytical data can only be performed when they are based on the same TEF system. **Tables 1** and **2** show the TEFs that were established by WHO 1998 and 2005 (Van den Berg et al., 1998, 2006). TEQs derived with the WHO<sub>2005</sub>-TEFs are currently the basis for the legal appraisal of dioxins and DL-PCBs in feed and food.

### Health-Based Guidance Values

In 1998, the World Health Organization (WHO) established a Tolerable Daily Intake (TDI) of 1–4 pg WHO<sub>1998</sub>-TEQ/kg body weight. Based on the WHO 1998 assessment, the Scientific Committee on Food (SCF) published an opinion in 2000 (SCF, 2000) which was updated in 2001 (SCF, 2001). The SCF found it more appropriate to express the health based guidance value (HBGV) on a weekly rather than on a daily basis, and established a Tolerable Weekly Intake (TWI) of 14 pg WHO<sub>1998</sub>-TEQ/kg body weight. This TWI is still valid in the European Union. In 2001, the FAO/WHO Joint Expert Committee on Food Additives

**Table 1** Toxic equivalency factors (TEFs) for dioxins according to WHO 1998 and 2005

Congener	WHO-TEF <sub>1998</sub>	WHO-TEF <sub>2005</sub>
<b>PCDDs</b>		
2,3,7,8-TCDD	1	1
1,2,3,7,8-PeCDD	1	1
1,2,3,4,7,8-HxCDD	0.1	0.1
1,2,3,6,7,8-HxCDD	0.1	0.1
1,2,3,7,8,9-HxCDD	0.1	0.1
1,2,3,4,6,7,8-HpCDD	0.01	0.01
1,2,3,4,6,7,8,9-OCDD	0.0001	0.0003
<b>PCDFs</b>		
2,3,7,8-TCDF	0.1	0.1
1,2,3,7,8-PeCDF	0.05	0.03
2,3,4,7,8-PeCDF	0.5	0.3
1,2,3,4,7,8-HxCDF	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01
1,2,3,4,6,7,8,9-OCDF	0.0001	0.0003

**Table 2** Toxic equivalency factors (TEFs) for DL-PCBs according to WHO 1998 and 2005

Congener	WHO-TEF <sub>1998</sub>	WHO-TEF <sub>2005</sub>
<b>Non-ortho PCBs</b>		
PCB # 77	0.0001	0.0001
PCB # 81	0.0001	0.0003
PCB # 126	0.1	0.1
PCB # 169	0.01	0.03
<b>Mono-ortho PCBs</b>		
PCB # 105	0.0001	0.00003
PCB # 114	0.0005	0.00003
PCB # 118	0.0001	0.00003
PCB # 123	0.0001	0.00003
PCB # 156	0.0005	0.00003
PCB # 157	0.0005	0.00003
PCB # 167	0.00001	0.00003
PCB # 189	0.0001	0.00003

(JECFA, 2001) re-evaluated the earlier risk assessments on dioxins and DL-PCBs and concluded that the tolerable intake should be assessed over a period of at least one month, and thus established a Provisional Tolerable Monthly Intake (PTMI) of 70 pg WHO<sub>1998</sub>-TEQs/kg body weight. The US-EPA published its reanalysis of dioxin toxicity in 2012 (US-EPA, 2012) and established an oral reference dose (RfD) of 0.7 pg TCDD/kg bw per day. The oral RfD is defined as “an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily oral exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime”. Currently, the European Food Safety Authority (EFSA) is performing a re-evaluation of dioxins and DL-PCBs. The outcome of this risk assessment is expected for summer 2018.

Exposure estimates performed around the year 2000 indicated that the exposure of a considerable part of the European population exceeds the established HBGVs. This triggered numerous measures, including *inter alia* intensified identification and reduction of source emissions, installation of filters in incinerators, and also setting of legal limits for feed and food in order to withdraw commodities with the highest contamination from the market.

## Legal Limits for Dioxins and PCBs in Feed and Food

### Feed

Maximum levels (termed maximum content) for dioxins, the sum of dioxins and DL-PCBs, and the sum of the six NDL-PCBs 28 + 52 + 101 + 138 + 153 + 180 are laid down in Annex I, Section V of Directive 2002/32/EC (EC, 2002/32) on undesirable substances in animal feed. All levels are expressed relative to a feed with a moisture content of 12% (88% dry matter, DM). The current legal limits for dioxins and the sum of dioxins and DL-PCBs are based on WHO<sub>2005</sub>-TEFs. While the legal limits for dioxins range between 0.75 and 5 ng WHO<sub>2005</sub>-TEQ/kg with the highest level for fish oil, the maximum contents for the sum of dioxins and DL-PCBs are between 1 and 20 ng WHO<sub>2005</sub>-TEQ/kg, both relative to a moisture content of 12%. The maximum content for NDL-PCBs is based on the sum of the six indicator-PCBs and ranges between 10 µg/kg and 175 µg/kg with the highest limit for fish oil, each relative to a moisture content of 12%. Maximum contents in feed are neither based on toxicological considerations nor deduced from feeding habits, but are derived from the frequency distribution of dioxins and PCBs in the respective feed commodity following the principle “strict but feasible”. In general, the maximum contents were set around the 90th percentile of the respective frequency distribution. Feed products have to comply with all three legal levels.

As a peculiarity, all legal limits for dioxins and PCBs are set as upper bound concentrations (UB). These are calculated on the assumption that all values of the different congeners below the limit of quantification (LOQ) are equal to the numerical value of the LOQ.

According to Article 5 of Directive 2002/32/EC, Member States shall prescribe that products intended for animal feed containing levels of an undesirable substance that exceed the maximum level fixed in Annex I may not be mixed for dilution purposes with the same, or other, products intended for animal feed.

In addition to maximum contents, also action thresholds are set separately for dioxins and DL-PCBs, respectively in Annex II of Directive 2002/32/EC. The action thresholds also refer to products relative to a moisture content of 12%. Action thresholds are meant as an early warning tool. They are set at around 2/3 of the corresponding maximum content. If a sample exceeds the action threshold but is still below the maximum content, the product can be marketed. Nevertheless, an exceedance of action thresholds is an indication of an increased contamination level substantially above background. Consequently, the competent authorities of the Member States, in cooperation with economic operators shall carry out investigations to identify the sources of the contaminants in order to reduce and eliminate the emissions.



## Food

Maximum levels (MLs) for dioxins, the sum of dioxins and DL-PCBs, and the sum of the six NDL-PCBs are laid down in Commission Regulation (EC) No 1881/2006 ([EC, 1881/2006](#)), as amended. The MLs for dioxins and the sum of dioxins and DL-PCBs are both based on the application of the WHO<sub>2005</sub>-TEFs, and are given as pg WHO<sub>2005</sub>-TEQ/g. MLs for the NDL-PCBs are set as the sum of the concentrations of the six indicator-PCBs. The derivation of the MLs based on frequency distributions follows the same approach as for feed. All MLs for food represent upperbound values. Except for certain fish and fish products, liver of fish and terrestrial animals, and foods for infants and young children, all other MLs for dioxins, the sum of dioxins and DL-PCBs, and NDL-PCBs are given on a fat basis. The MLs for dioxins and the sum of dioxins and DL-PCBs expressed on a fat weight basis are not applicable for foods containing < 2% fat. These have to be calculated on a product basis, assuming a fat content of 2%. Depending on the foodstuff, the MLs for dioxins range between 1 and 2.5 pg WHO<sub>2005</sub>-TEQ/g fat, and 0.1 to 3.5 pg WHO<sub>2005</sub>-TEQ/g wet weight, respectively. The MLs for the sum of dioxins and DL-PCBs are between 1.25 and 6 pg WHO<sub>2005</sub>-TEQ/g fat, and 0.20–20 pg WHO<sub>2005</sub>-TEQ/g wet weight, respectively. For the sum of the six NDL-PCBs, the MLs are set between 1 and 300 ng/g wet weight and at 40 ng/g fat for all commodities which are based on fat weight.

According to Article 3 of Regulation (EC) No 1881/2006, foodstuffs not complying with the MLs shall not be used as food ingredients, and foodstuffs complying with the MLs shall not be mixed with foodstuffs which exceed the MLs. Article 7 of the Regulation stipulates that by way of derogation, Finland, Sweden and Latvia may authorize the placing on their market of specific fish species originating in the Baltic region and intended for consumption in their territory with levels of dioxins and/or DL-PCB higher than those set out in the Regulation. These derogations apply only provided that a system is in place to ensure that consumers are fully informed of the dietary recommendations with regard to the restrictions on the consumption of the respective fish species by identified vulnerable sections of the population in order to avoid potential health risks.

As for feed, the legislation for food also includes an early warning tool which is termed “action levels (ALs)”. The separate setting of ALs for dioxins and DL-PCBs as well as their enforcement in case of exceedance follows the same approach as described for the action thresholds in the feed legislation. The most recent amendment of ALs for dioxins and DL-PCBs in food was stipulated by Commission Recommendation 2014/663/EU ([EU, 2014/663](#)).

## Sampling and Analysis

Because of the low levels of dioxins and DL-PCBs which are mostly in the fg/g or pg/g range, powerful analytical methods are required to not only separate the toxic congeners from the non-toxic ones but also to unequivocally quantify the concentrations at the legal limits.

Unlike in the US, in the European Union there are no fixed standard methods for the determination of dioxins and PCBs in feed and food. Instead, the EU follows the “criteria approach”. Strict analytical performance criteria are set by the EU Commission. As long as these criteria are fulfilled which has to be demonstrated in a traceable manner, the analysts can use their method of choice. In addition, the fitness of purpose of the analytical methods have to be substantiated by successful participation in proficiency tests which is controlled by the accreditation bodies.

Detailed requirements for methods of sampling and analysis for the control of levels of dioxins and PCBs in foodstuffs and feed are laid down in Commission Regulation (EU) No 2017/644 ([EU, 2017/644](#)) and in Commission Regulation (EU) No 2017/771 ([EU, 2017/771](#)), respectively. Besides provisions concerning methods of sampling depending on the size of the lot, packaging, transport, storage, and handling of the samples in the laboratory, the two Regulations also stipulate the analytical performance criteria, such as precision, addition of isotope-labelled standards, gas chromatographic separation, and maximum tolerances for retention times, as well as provisions for the interpretation of results and requirements for assessing the compliance of a lot or sub-lot with the legislation.

The analysis of dioxins and PCBs in feed and food is either performed by bioassays or by mass spectrometric based methods. While bioassays can be considered as semi-quantitative screening methods allowing a high sample throughput, gas chromatography coupled to high resolution mass spectrometry (GC-HRMS) and GC coupled to tandem mass spectrometry (GC-MS/MS) are assigned as confirmatory methods.

The Chemical Activated Luciferase gene eXpression assay (CALUX) is the most commonly used bioassay in dioxin and DL-PCB analysis. It mimics the mode of action of dioxins and DL-PCBs in mammals. Rat and mouse hepatoma cells are genetically modified by embedding a DNA fragment from the firefly encoding for luciferase in the “dioxin responsive elements” (DREs) in the DNA ([Sanderson et al., 1996](#)). After exposure of the cells with the sample extract and addition of luciferin, the resulting concentration dependent light reaction can be measured by a luminometer. As the CALUX is sensitive to all compounds that act through the Ah receptor, it requires some efforts to remove potential disturbing co-extracts. This can best be done by including a sulphuric acid containing step in the clean-up process which destroys labile compounds but does not affect dioxins and DL-PCBs. As NDL-PCBs do not bind to the Ah receptor they cannot be measured by CALUX.

Another bioassay that is occasionally used is the EROD assay ([Kennedy et al., 1993](#)). It finally measures the deethylation of ethoxyresorufin to resorufin following exposure of mammalian cell lines to dioxin and DL-PCB containing extracts.

Bioassay results are given as “Bioanalytical Equivalents (BEQs)”. As there are some differences between the relative response of dioxins and DL-PCBs in the bioassays and the WHO-TEFs, non-compliant and suspect results that are above a certain cut-off-level

have to be confirmed by GC-HRMS or GC-MS/MS. The advantage of the latter methods is the ability of a congener-specific analysis which does not only give a “yes/no” result, but also valuable hints of the potential source of the contamination derived from the analysed congener profile. Moreover,  $^{13}\text{C}_{12}$ -labelled standards can be added at the very beginning of the analysis giving information on the losses of the analytes in question. Nowadays, it is good practice in confirmatory feed and food analysis to add all seventeen  $^{13}\text{C}_{12}$ -labelled dioxin congeners with 2,3,7,8-chlorine substitution and all twelve  $^{13}\text{C}_{12}$ -labelled DL-PCBs as internal standards, i.e. each congener of interest is quantified on its corresponding internal standard. At the last step before the chromatographic separation a further  $^{13}\text{C}_{12}$ -labelled standard is added, which is used to calculate the recovery of the various congeners.

## Levels in Feed and Food

### Feed

Feed materials of plant origin and compound feed of plant origin are generally low contaminated with dioxins and PCBs. The levels are in most cases near the ubiquitous background contamination. Somewhat higher concentrations can be found in roughages and grass samples because of their long growing period and the expression of their contaminant levels relative to a moisture content of 12%. If the feedingstuffs contain feed material of animal origin, especially derived from fish, the dioxin and PCB levels are generally higher than in feed commodities of plant origin.

In their 2012 report where EFSA evaluated the occurrence data in feed submitted by 26 European countries, they reported that the feed group with the highest average level of dioxins and DL-PCBs was “fish oil”, followed by “feed for fur animals, pets and fish” and “fish and other aquatic animals, their product”. Some high levels could also be observed in a few samples of “other feed additives” and “vegetable oils and their by-products”.

Due to natural formation under high pressure and temperature, dioxins can also be formed and determined in some clay materials (Holmstrand et al., 2006). Some of these contaminated clays, such as Mississippi ball clay (Hayward et al., 1999) and kaolinite clay (Jobst and Aldag, 2000) caused incidents because of their use in animal feed. Hoogenboom et al. (2010) investigated the use of kaolinite to help to remove poor quality potatoes which led to an incident with milk from dairy cows and meat from ruminants fed the contaminated potato peel. Dioxins have also been detected in other minerals used in animal feed, such as zinc oxide (Kim et al., 2011) and feed supplements containing copper sulphate (Ferrario et al., 2003).

The contamination of feed with dioxins and/or PCBs was often the initial incident that resulted in deterioration of food of animal origin. Prominent examples are *inter alia* pentachlorophenol (PCP) contaminated saw dust as carrier in pre-mixed choline chloride in 2000, use of waste wood for drying of bakery leftovers in 2003 and introduction of fatty acids from biodiesel production intended for industrial uses into feed for food producing animals. Malisch and Kotz (2014) summarize these incidents and illustrate the consequences for the contamination of food of animal origin.

### Food

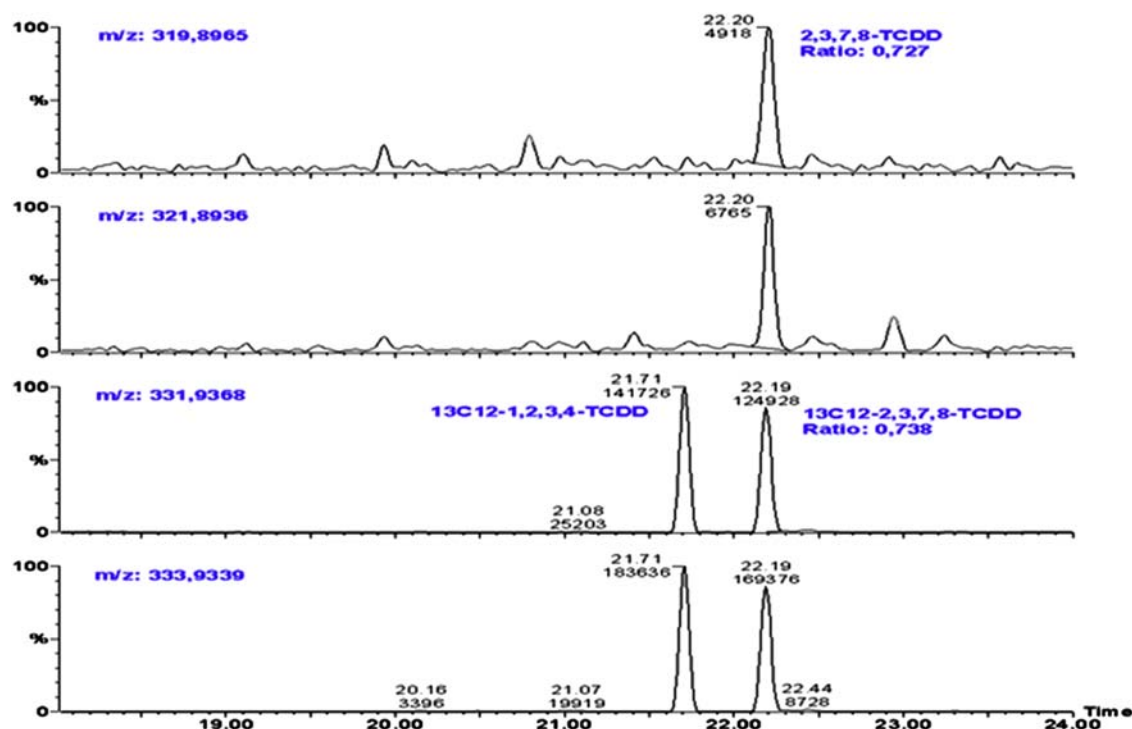
Performing a pathway analysis, Fürst et al. (1992) concluded that diet represents the main route of exposure to dioxins. While more than 90% of the total daily intake was estimated to be derived from food, exposure via air and soil was considered to contribute less than 10%. Because of the lipophilic properties, persistence and accumulation in the food chain, food samples of animal origin are of special importance. Those food commodities, especially from mammals, generally only contain 2,3,7,8-chlorinated congeners at measurable level, unless they are affected by an extraordinary contamination. Other non-toxic congeners are to the greatest possible extent metabolized. This is illustrated by a typical analysis of a cow's milk sample (Fig. 3). It shows the GC-HRMS determination of 2,3,7,8-TCDD at a concentration of 0.25 pg/g fat.

While the upper two chromatograms show the ion traces for the native analyte with retention time, area and ratio of the two measured fragments characteristic for TCDDs, the two lower chromatograms display the fragments measured for the  $^{13}\text{C}_{12}$ -labelled internal standards. Although there exist 22 different TCDD isomers, solely 2,3,7,8-TCDD, the only TCDD congener with 2,3,7,8-chlorine substitution can be determined and quantified against its isotope labelled standard.  $^{13}\text{C}_{12}$ -labelled 1,2,3,4-TCDD was added at the end of the clean-up just before injection into the GC to calculate the recovery of the various congeners.

A different situation can be seen for food samples of plant origin. These samples are generally affected by airborne contamination and in contrast to animals and humans, plants lack the possibility of metabolising the contaminants. As a result, plant samples do not only contain the toxic 2,3,7,8-chlorinated congeners but also a number of non-toxic congeners. This is demonstrated in Fig. 4 which shows the GC-HRMS analysis of tetrachloro-dibenzofurans (TCDFs) in a kale sample.

While the upper two chromatograms show the ion traces of the native tetrachloro-dibenzofuran analytes, the two lower chromatograms present the fragments of the isotope labelled 2,3,7,8-tetrachloro-dibenzofuran (2,3,7,8-TCDF). The upper two chromatograms illustrate that in addition to the toxic 2,3,7,8-TCDF a number of other non-toxic TCDFs are present in the sample. A total of 38 different TCDF isomers are theoretically possible. This fact demands thorough skills from the analyst as the toxic congeners have to be separated from the non-toxic ones in order to avoid false positive results which may have severe consequences for the food business operators.

Plants are generally not able to uptake considerable amounts of dioxins from the soil. Exceptions are zucchini and pumpkin. Hülster et al. (1994) studied the uptake of dioxins by zucchini and related plant species from two contaminated soils. The



**Figure 3** Determination of 2,3,7,8-TCDD in cow's milk by GC-HRMS.

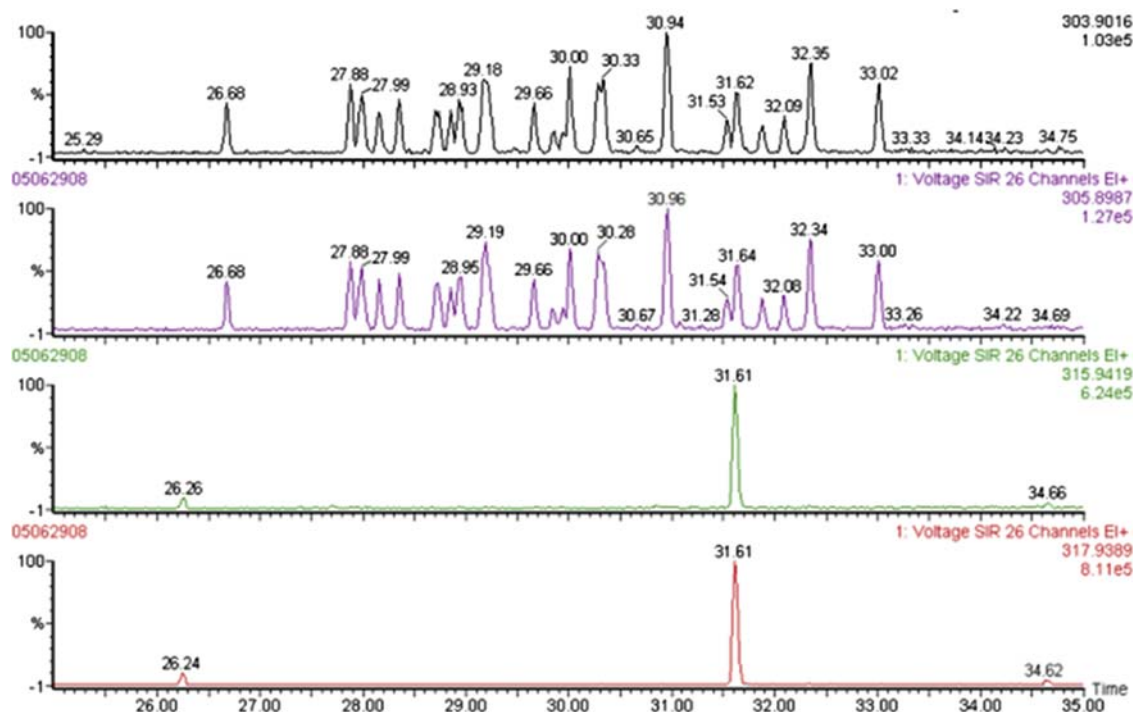
experimental design allowed the discrimination of several uptake pathways and an evaluation of their contribution to the total dioxin contamination of the plants. For zucchini and pumpkin, both belonging to the genus *Cucurbita*, root uptake of dioxins and subsequent translocation to the shoots and into the fruits was found to be the main contamination pathway. In contrast, cucumber plants (*Cucumis sativus* L.) are mainly contaminated by deposition of airborne dioxins and thus show much lower dioxin concentrations. In fruits of zucchini, the dioxin levels were approximately two orders of magnitude higher than in other fruits and vegetables analysed.

The surveys on the contamination of food with dioxins and PCBs revealed some food commodities that generally contain elevated contamination levels. Bruns-Weller et al. (2010) analysed sheep liver and reported that around 90% of the samples exceeded the European maximum levels. In the corresponding meat of the sheep only low concentrations of dioxins and DL-PCBs were determined, all below the MLs in force. The results did not show any significant differences in contamination between female or male sheep. Moreover, no correlation was found between the age of the animals and the dioxin and DL-PCB levels in their liver. EFSA (2011) assessed the risk to public health related to the presence of high levels of dioxins and DL-PCBs in liver from sheep and deer and identified a lower activity of CYP1A enzymes in sheep compared to cattle and other ruminants as a possible reason for higher dioxin and DL-PCB levels in sheep liver. Furthermore, EFSA concluded that on individual occasions, consumption of sheep liver could result in high intakes exceeding the TWI, and that the frequent consumption of sheep liver, particularly by women of child-bearing age and children, may be a potential health concern.

Another product that is generally highly contaminated with dioxins and PCBs is fish liver. This is especially true for fish liver originating from the Baltic proper. Also certain fish species from this area are often contaminated, especially with dioxins. One possible reason is the release of effluents from the pulp and paper mills in the past. As some of the concerned countries could demonstrate that the benefits of fish consumption is superior to the possible impairment caused by dioxins and PCBs, they got exceptions from the EU Commission under specific pre-conditions to place certain fish species originating in the Baltic region that exceed MLs on the market in their territory.

Eggs from laying hens kept on ground or outdoors often contain higher dioxin and PCB levels than conventionally produced chicken eggs. This was shown especially for organic eggs where numerous samples exceeded the existing MLs. DeVries et al. (2006) reviewed the possible factors which influence the elevated levels. Ingestion of soil, uncontrolled intake of excrements and usage of litter produced from pentachlorophenol (PCP) treated wood may be possible sources. PCP which is generally contaminated with substantial amounts of dioxins from the synthesis, was a widely used wood preservative but its application is meanwhile banned.

EFSA periodically evaluates occurrence data on dioxins, PCBs and other contaminants submitted by European countries. The aim is not only to generate a data base on occurrence of contaminants but also to combine these data with consumption surveys to perform exposure estimates for the European population, and to monitor a potential decline of the contaminants in food and feed. The latest evaluations were performed in 2010 and 2012 (EFSA, 2010, 2012). In summarizing the occurrence data submitted



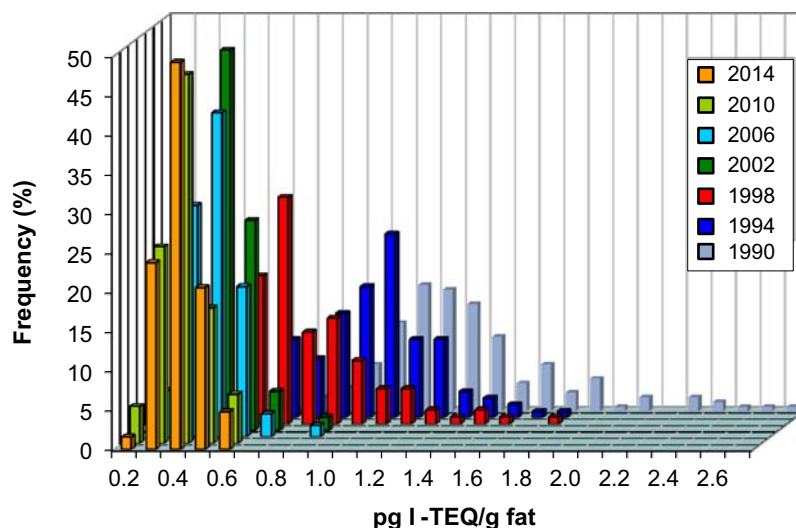
**Figure 4** Determination of tetrachloro-dibenzofurans in a kale sample by GC-HRMS.

from 26 European countries, EFSA concluded that “meat from eels” and “fish liver and derived products” contained the highest average contamination levels for both dioxins and PCBs. The non-ortho PCBs were the main contributor to total WHO<sub>2005</sub>-TEQs, making up between 21.0% and 74.5%, followed by PCDDs and PCDFs which together represented between 12.4% and 73.2% of the total TEQ level. Moreover, a decrease in the contamination levels of dioxins and PCBs was observed over the years in the three food groups taken into consideration for the time trend analysis: “raw milk and dairy products”, “hen eggs and egg products” and “muscle meat from fishes other than eels”. However, EFSA stated that it cannot be excluded that the heterogeneity in the foods constituting the groups, in the countries of origin covered and targeting strategies between the years, has influenced the observed trends.

More meaningful is an analysis of the contamination trend over time if the food products are collected from the same area and are analysed with the same analytical equipment. Fürst studied the dioxin and PCB contamination of dairy products from all dairies in North Rhine-Westphalia/Germany with own milk delivery every four years between 1990 and 2014. In order to investigate whether seasonal trends exist, each dairy was sampled four times per year. Meanwhile, more than 600 individual samples were analysed. Fig. 5 shows the frequencies of the dioxin levels determined in the samples analysed between 1990 and 2014. In order to allow a comparison with the data generated prior to 1998, all results are expressed as I-TEQ values calculated with the international TEFs proposed by NATO/CCMS in 1988, and not with the WHO-TEFs which were only published in 1998 for the first time. The concentrations calculated as I-TEQs only differ marginally from the results calculated with the WHO<sub>2005</sub>-TEFs.

The Figure clearly illustrates that with each survey the dioxin levels tend to lower values and the range of contamination becomes smaller. This demonstrates that the measures to reduce dioxin emissions and to close known sources show positive and beneficial effects. However, the last two surveys conducted in 2010 and 2014 revealed similar results. This may be an indication that the reduction is levelling off. In any case, the mean dioxin concentration in the analysed dairy products has decreased by 75% since 1990. A similar decrease of 70% was estimated for DL-PCBs since 1998 when they were included in these surveys for the first time.

The EFSA (2012) report on the “update of the monitoring of levels of dioxins and PCBs in food and feed” also estimated the dietary exposure for the European population based on the occurrence levels submitted by 26 European countries and the different dietary surveys collated in the EFSA Comprehensive European Food Consumption Database, representing seven age classes (infant, toddler, other children, adolescent, adult, elderly and very elderly). As a result, EFSA stated: “Chronic dietary exposure to the sum of dioxins and DL-PCBs was estimated to be on average between 0.57 and 2.54 pg WHO<sub>2005</sub>-TEQ/kg b.w. per day and at the 95th percentile between 1.2 and 9.9 pg WHO<sub>2005</sub>-TEQ/kg/kg b.w. per day depending on the population group. Between 1.0 and 52.9% of individuals were estimated to exceed the TWI of 14 pg TEQ/kg b.w. The major contributor to total exposure was milk and dairy products for almost all infant and toddler groups, whereas it was fish and seafood products for most of the adolescents, adults, elderly and very elderly groups. Meat and meat products also contributed significantly to total exposure. A general decrease in exposure to the sum of dioxins and DL-PCBs was observed between 2002–04 and 2008–10, estimated to be between 16.6% and 79.3% according to the different population groups.”



**Figure 5** Dioxins in dairy products from North Rhine-Westphalia 1990–2014.

Surveys performed in the past decades have indicated that due to numerous measures the emissions of dioxins and PCBs are reduced, their occurrence in the environment and thus the exposure of the European population has substantially decreased. However, due to criminal or grossly negligent actions of individuals, a number of incidents occurred in the past two decades causing massive feed contamination and subsequent food adulteration which foiled the successful measures to reduce human exposure with dioxins and PCBs. Prominent examples are the Brazilian citrus pulp pellet case 1997, the Belgian PCB incident 1999, the Irish pork and beef incident 2008, the German biodiesel case 2011, and others. Pentachlorophenol related incidents causing considerable dioxin contamination, such as the Dutch wood-shaving litter case 2004, the Indian guar gum pollution 2008, and continuously non compliances of chicken eggs from small holdings are further examples. [Malisch and Kotz \(2014\)](#) give a comprehensive overview on these incidents and the consequences for human exposure. As these incidents do not only have severe financial consequences but most notably spoil the numerous efforts to minimize human exposure, vigilance remains necessary also in the future to identify hitherto unknown sources and consider dioxins and PCBs in food and feed as a continuous matter of concern.

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## Modified Mycotoxins: A New Challenge?

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### Glossary

**Mycotoxins** Secondary metabolites of filamentous fungi.

**Modified mycotoxins** This term summarized all modified forms of mycotoxins that differ in their chemical structure from the parent toxin.

**Masked mycotoxins** This term is used in older literature for plant metabolites of mycotoxins and should no longer be used.

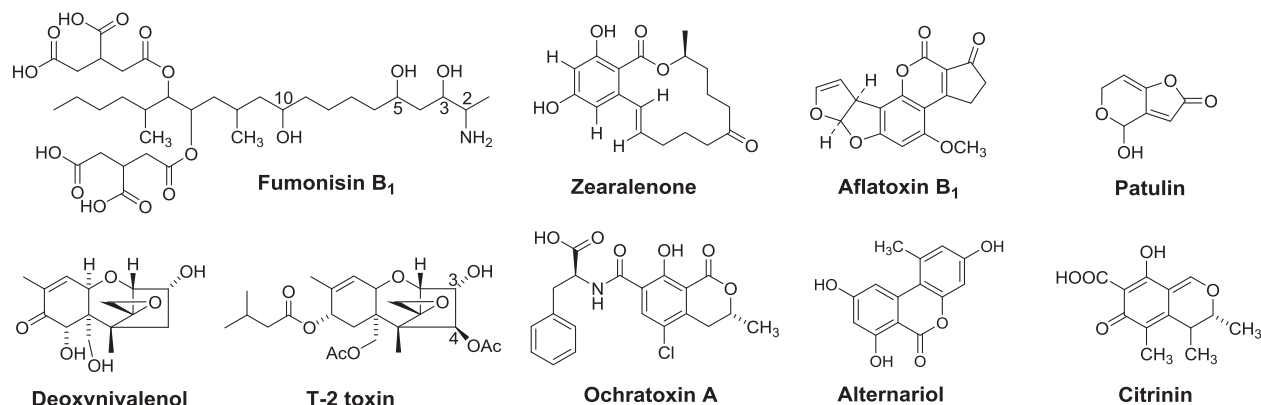
### Nomenclature

AcDON acetyl-deoxynivalenol  
AFB<sub>1</sub> aflatoxin B<sub>1</sub>  
AFM<sub>1</sub> aflatoxin M<sub>1</sub>  
AME alternariol monomethyl ether  
AOH alternariol AOH  
DOM deepoxy-deoxynivalenol  
DON deoxynivalenol  
DON3Glc deoxynivalenol-3-O- $\beta$ -glucoside  
DONS deoxynivalenol-sulfonate  
FB<sub>1</sub> fumonisin B<sub>1</sub>  
HFB<sub>1</sub> hydrolyzed fumonisin B<sub>1</sub>  
HFB<sub>2</sub> hydrolyzed fumonisin B<sub>2</sub>  
HT2 HT-2 toxin  
NCM-FB<sub>1</sub> N-(carboxymethyl)-fumonisin B<sub>1</sub>  
NDF-FB<sub>1</sub> N-(1-deoxy-D-fructos-1-yl)-fumonisin B<sub>1</sub>  
NIV nivalenol  
norDON thermal degradation products of DON  
norNIV thermal degradation products of NIV  
OTA ochratoxin A  
Sa sphinganine  
So sphingosine  
T2 T-2 toxin  
ZEN zearalenone  
ZEN14Glc zearalenone-14-O- $\beta$ -glucoside  
ZEN14Sulf zearalenone-14-sulfate  
 $\alpha$ -/ $\beta$ -ZEL  $\alpha$ -/ $\beta$ -zearalenol  
 $\alpha$ -/ $\beta$ -ZEL14Glc  $\alpha$ -/ $\beta$ -zearalenol-14-O- $\beta$ -glucoside

### Introduction

Mycotoxins are toxic secondary metabolites of filamentous fungi, which are produced under suitable temperature and humidity conditions. Mycotoxins are well known as contaminants of food and feed and due to their toxic potential they may pose a potential health risk for humans and animals. Mycotoxins are in most cases low molecular weight compounds and they have no biochemical significance in fungal growth and development.

Numerous mycotoxins have been characterized to date, the most relevant are aflatoxins such as aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) besides deoxynivalenol (DON), fumonisins e.g. fumonisin B<sub>1</sub> (FB<sub>1</sub>), zearalenone (ZEN), ochratoxin A (OTA), T-2 Toxin (T2), alternariol (AOH), citrinin and patulin (Fig. 1). These toxic fungal secondary metabolites, also named “parent” mycotoxins, can occur as “free” compounds in infested food and feed, but can also be converted in various ways into products with altered chemical structures



**Figure 1** Structures of relevant mycotoxins.

with different physico-chemical, chemical and biological properties. Such modified forms of the “free” parent mycotoxins can be formed in fungi, or in plants and animals used for food and feed production, as well as during food and feed processing. As such “modified” forms are more and more detected in food and feed they should be taken into account for risk assessment, as they may substantially contribute to the overall toxicity of mycotoxins.

### Examples and Structures of Modified Mycotoxins

As several different terms such as “bound”, “hidden” or “masked” mycotoxins have been used in the past, Rychlik et al. (2014) proposed a new definition of modified mycotoxins, which is summarized in Fig. 2 with representative examples. According to this definition mycotoxins can be classified on the first level in “free”, “modified” and “matrix-associated” mycotoxins.

#### “Free” Mycotoxins

The term “free” includes all “unmodified” or parent mycotoxins such as ochratoxin A, aflatoxin B<sub>1</sub>, deoxynivalenol or fumonisins as shown in Fig. 1, which are produced as secondary metabolites of various fungi. Based on this definition intermediates in the biosynthetic pathway such as 3- and 15-acetyl-deoxynivalenol (AcDON) also belong to “free” mycotoxins. However these compounds can also be classified as modified mycotoxins when they are formed in plants.

#### “Matrix-Associated” Mycotoxins

“Matrix-associated” mycotoxins include mycotoxins which are covalently or non-covalently bound to matrix compounds such as proteins or starch. Non-covalent interactions, also described as “physical entrapment”, are mediated by hydrogen, ionic or any other kind of non-covalent binding. Such physical entrapment is especially described for fumonisins and is regarded as analytical issue as it leads to low recovery rates and an underestimation of fumonisin levels. One possibility to overcome this unspecific entrapment is a chemical hydrolysis of fumonisins and the quantification of hydrolysed fumonisins as described in a recent review by Dall’Asta and Battilani (2016).

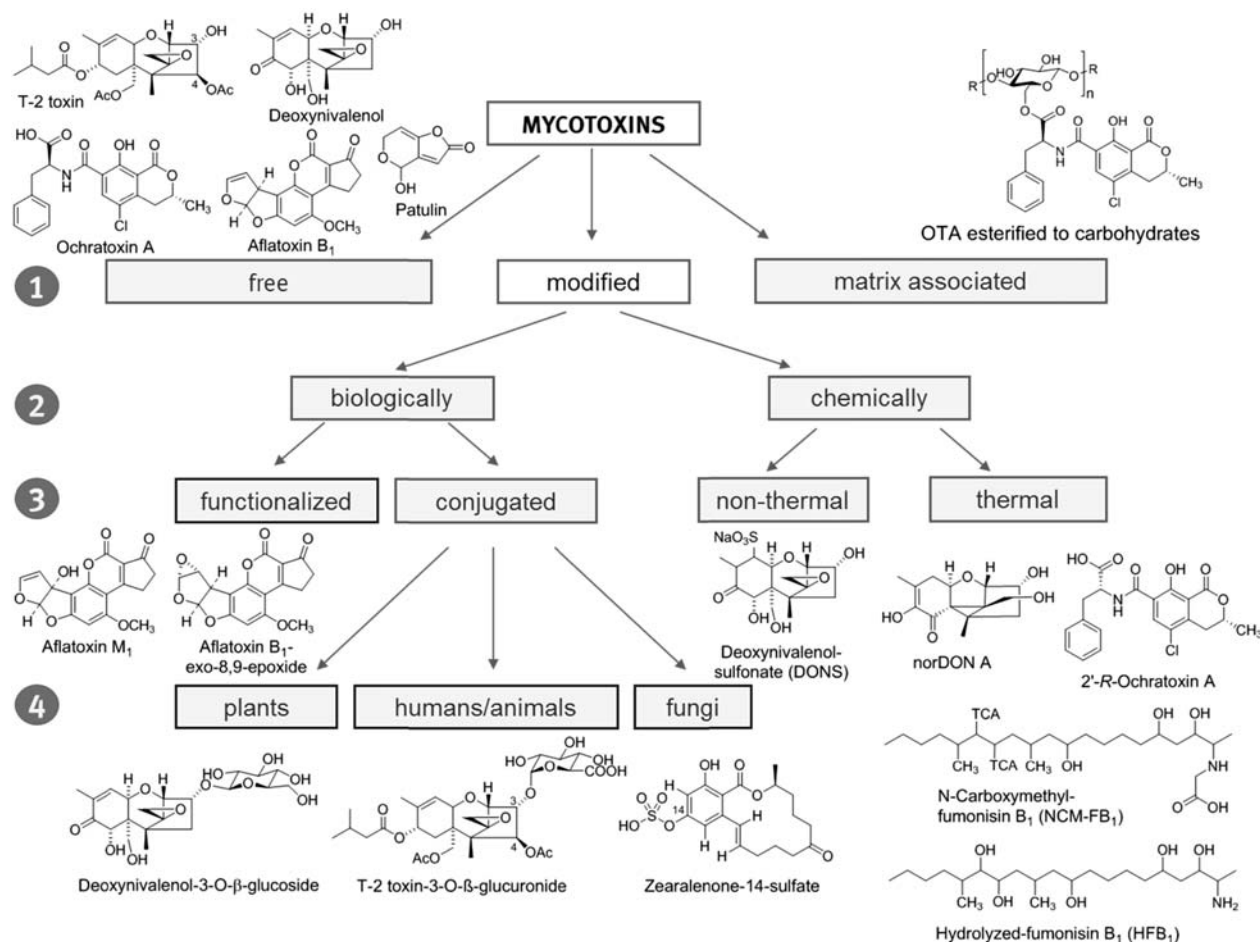
Covalently bound forms of mycotoxins have been proposed for the first time by Shier (2000) based on experiments with radio-labelled fumonisins and later confirmed by Seefelder et al. (2003) in model experiments. During food processing the tricarballic acid side chains of fumonisins can react with polysaccharides and proteins leading to covalently bound forms of fumonisins (Seefelder et al., 2003). Such covalently bound forms of fumonisins can be quantified with the above mentioned chemical hydrolysis method leading to hydrolysed fumonisins. Other covalent binding reactions of mycotoxins with polysaccharides have been described for OTA by Bittner et al. (2013) and for DON by Zachariasova et al. (2012). Ochratoxin A esterified to carbohydrates such as cellulose (see Fig. 2) could be identified in model heating experiments by high resolution mass spectrometry (Bittner et al., 2013).

### Modified Mycotoxins

On the next level (level 2, Fig. 2) modified mycotoxins can be subclassified as “biologically” or “chemically” modified forms.

#### Chemically Modified Mycotoxins

Chemically modified mycotoxins can be further divided in level 3 into forms generated by “thermal” and “non thermal” reactions. Thermal degradation reactions have been described for several mycotoxins and are of relevance as thermal processing methods



**Figure 2** Definition and examples of “free”, “matrix bound” and “modified” mycotoxins. The different hierarchic levels are indicated by numbers 1–4 (definition according to Rychlik et al., 2014).

including baking, roasting, frying and extruding belong to the main food processing technologies. Examples are *N*-(1-deoxy-D-fructos-1-yl)-fumonisins B<sub>1</sub> (NDF-FB<sub>1</sub>) and *N*-(carboxymethyl)-fumonisins B<sub>1</sub> (NCM-FB<sub>1</sub>) which are formed in a Maillard-type reaction between fumonisins and reducing sugars (Humpf and Voss, 2004). During this reaction NDF-FB<sub>1</sub> is formed in the first step as unstable intermediate, which is further converted to NCM-FB<sub>1</sub> (Fig. 2). The latter is detectable in processed corn samples in the µg/kg range (Seefelder et al., 2001). Other examples are norDON A-F (Fig. 2) and norNIV A-C as thermal degradation products of DON and NIV (Bretz et al., 2005, 2006a). As example of a non thermal chemically modified mycotoxin DON-sulfonate is shown in Fig. 2. This reaction product is formed when animal feed is treated with sodium bisulfite to reduce the DON levels in contaminated feed. The structure was described by Young et al. (1986) and later confirmed by Beyer et al. (2010) and Schwarz et al. (2013). In the latter study further DON sulfonates have been identified in model experiments.

However it should be mentioned that some modified mycotoxins, e.g. hydrolysed fumonisins B<sub>1</sub> (HFB<sub>1</sub>) (Fig. 2), can be classified in different categories. HFB<sub>1</sub> is “chemically modified” as it is formed during nixtamalization, which is a traditional alkaline cooking process of corn to produce masa and tortilla chips (Humpf and Voss, 2004). However HFB<sub>1</sub> can also be classified as “biologically modified” as it was identified together with partially hydrolysed FB<sub>1</sub> as intestinal metabolite of FB<sub>1</sub> in piglets (Fodor et al., 2008).

### Biologically Modified Mycotoxins

According to Fig. 2 biologically modified mycotoxins can be further divided in level 3 into “functionalized” and “conjugated” forms. One example of the first group is the highly reactive aflatoxin B<sub>1</sub> metabolite aflatoxin B<sub>1</sub>-exo-8,9-epoxide, which is functionalized in the liver of humans and animals by cytochrome P450 and is responsible for the formation of DNA adducts (Guengerich, 2005). Another prominent example is aflatoxin M<sub>1</sub> (AFM<sub>1</sub>), the hydroxylation product of aflatoxin B<sub>1</sub>, which is detectable in milk of animals and humans exposed to aflatoxin B<sub>1</sub> (Masri et al., 1967). The conjugated biologically modified mycotoxins can be further subclassified in level 4 into plant, fungal and human/animal conjugates (Fig. 2).

## Plant Conjugates

Examples for plant conjugates are phase II metabolites such as deoxynivalenol-3-O- $\beta$ -glucoside (DON3Glc) (Fig. 2), zearalenone-14-O- $\beta$ -glucoside (ZEN14Glc) or T2/HT2-3-O-glucoside (Sewald et al., 1992; Engelhardt et al., 1988; Busmann et al., 2011). For a detailed literature overview of plant modified mycotoxins see recent review articles (Freire and Sant'Ana, 2018; Rychlik et al., 2014; Berthiller et al., 2013). Concerning the sugar configuration of naturally occurring T2 and HT2-glucosides contradictory results have been published as summarized in Schmidt et al. (2018). Such glucosides as well as other plant metabolites have been termed in the past "masked mycotoxins" as they are not detectable with routine analytical methods due to structural changes (Gareis et al., 1990; Berthiller et al., 2013). However as several structures of plant conjugates of mycotoxins have been elucidated and analytical methods are also available the term "masked mycotoxins" should be not longer used as proposed by Rychlik et al. (2014). Other modified forms such as (Malonyl)-glucosides of *Alternaria* mycotoxin alternariol (AOH) have recently been described in tobacco plant cells (Hildebrand et al., 2015). Besides glucosides other phase II metabolites such as DON-3/15-sulfate have been identified in wheat artificially contaminated with *Fusarium graminearum* (Warth et al., 2015).

## Fungal Conjugates

Fungi are also able to modify the chemical structure of mycotoxins produced by other fungal species. Typical fungal conjugates are zearalenone-14-sulfate or *N*-*O*-acyl fumonisins, which are produced by *Fusarium* spp (Plasencia and Mirocha, 1991; Bartok et al., 2013).

## Human/Animal Conjugates

Conjugation reactions by humans and animals are phase II reactions such as the glucuronidation of mycotoxins. Examples are the formation of HT2-3/4-glucuronides (Fig. 2) or DON-3/15-glucuronides (Welsch and Humpf, 2012; Uhlig et al., 2013). Specific glucuronidases are responsible for the formation of these conjugates, resulting in species dependent differences in the mycotoxin glucuronide pattern. As an example, for HT-2 toxin, glucuronidations at the 3- and 4-hydroxy-group are possible. Incubation studies with microsomes from different species showed that pig liver microsomes can indeed form HT2-3- and HT2-4-glucuronide whereas in all other tested species (rat mouse, human) only HT2-3-glucuronide was detectable (Welsch and Humpf, 2012).

## Analysis of Modified Mycotoxins

The first evidence that mycotoxin analysis needs considering modified forms was reported by Gareis et al. (1990), who quantitated zearalenone-14-O- $\beta$ -glucoside (ZEN14Glc) in wheat by an indirect enzymatic approach. The latter authors released "masked" ZEN after treatment with  $\beta$ -glucosidase and measured ZEN by HPLC-FLD in comparison with untreated samples. Among indirect methods enzymatic treatments are one approach besides chemically releasing the free toxin by acidic or alkaline hydrolysis. These indirect methods are still in use, but restricted, on the one hand, to glucoside or sulfate conjugates as only these can be cleaved by easily available enzymes. On the other hand, chemical treatment as the other approach may lead to degradation of the free toxin thus rendering the method inaccurate. Indirect variants are the method of choice when the modified toxin is not available as analytical standard. However, as the indirect methods principally do not guarantee a complete cleavage, direct methods are generally considered more accurate. For a review of analytical methods, one has to differentiate between approaches to detect modified mycotoxins and validated methods for quantitation. In this chapter, only the latter ones will be covered (Table 1), as only these are a solid basis for calculating occurrence data and allow a subsequent risk assessment.

Most of the methods are targeted at modified DONs such as DON-3-glucoside (DON3Glc), acetylated DONs and thermally formed norDON modifications. For ZEN, LC-MS/MS methods are applied for simultaneous quantitation of ZEN, ZEN14Glc, ZEN-14-sulfate (ZEN14Sulf),  $\alpha$ -zearalenol ( $\alpha$ -ZEL),  $\beta$ -zearalenol ( $\beta$ -ZEL), and their respective phase II metabolites. In case of fumonisins, as already mentioned above, the "hidden" forms can be hydrolysed by alkaline treatment to the hydrolysed fumonisins HFB<sub>1</sub> and HFB<sub>2</sub>, the latter of which are also generated from FB<sub>1</sub> and FB<sub>2</sub> during this treatment. HFB<sub>1</sub> and HFB<sub>2</sub> are also formed by nixtamalization as a traditional processing of corn and may occur also in these products. Therefore, these two toxins have also to be considered as modified forms along with NCM-FB<sub>1</sub> mentioned before. A direct LC-MS/MS-method for FB<sub>1</sub>, HFB<sub>1</sub>, and NCM-FB<sub>1</sub> with [<sup>2</sup>H<sub>6</sub>]-FB<sub>1</sub> as internal standard has been developed by Seefelder et al. (2001) and an indirect method for "free, hidden and total" fumonisins by Oliveira et al. (2015). However, the term "hidden" is likewise ambiguous as the term "masked" and should be replaced by "matrix-associated". For the thermally generated modifications of OTA, namely 2'-OTA and 2'-decarboxyOTA, Cramer et al. (2008) developed a stable isotope dilution assay by using deuterated internal standards. Recently, Walravens et al. published LC-MS/MS methods for modified *Alternaria* toxins by using the labelled free toxin [<sup>2</sup>H<sub>4</sub>]-AME as internal standard for tomato products (Walravens et al., 2016) and cereal products (Walravens et al., 2014). For the first described modified mycotoxin, aflatoxin M<sub>1</sub>, a lot of methods already exist mainly due to its early discovery and due to existing regulatory maximum limits in many countries.

**Table 1** Compilation of validated methods for quantitation of modified mycotoxins

Compound	Method	Matrix	LOD	Reference
DON3Glc, AcDON	direct LC-TOFMS	malt, beer	1 µg/kg	Kostelanska et al. (2011)
DON3Glc	direct LC-MS/MS with [ <sup>13</sup> C <sub>6</sub> ]-DON3Glc as internal standard	beer	3 µg/L	Habler et al. (2016)
"Total DON"	indirect ELISA after hydrolysis with trifluoromethanesulfonic acid	corn	100 µg/kg	Tran et al. (2012)
norDON A, B, C	direct LC-MS/MS with ZAN as Internal standard	cereal products	1–2.5 µg/kg	Bretz et al. (2006a)
DON3Glc, AcDON, ZEN14Glc, ZEN14Sulf, α-ZEL, β-ZEL, α-ZEL14Glc, β-ZEL14Glc, HFB <sub>1</sub> , Aflatoxin M <sub>1</sub>	Direct LC-MS/MS	nuts	5–110 µg/kg	Varga et al. (2013)
DON3Glc, AcDON, ZEN14Glc, ZEN14Sulf, α-ZEL, β-ZEL, α-ZEL14Glc, β-ZEL14Glc	Direct LC-MS/MS with DOM and ZAN as internal standards	cereal products	5–13 µg/kg	De Boevre et al. (2012)
ZEN14Glc	indirect ELISA after hydrolysis with glucosidase from <i>A. niger</i>	wheat, corn, breakfast cereals, feed	3.0 µg/kg	Beloglazova et al. (2013)
HFB <sub>1</sub> , NCM-FB <sub>1</sub>	direct LC-MS/MS with [ <sup>2</sup> H <sub>6</sub> ]-FB <sub>1</sub> as Internal standard	tortilla chips, nacho chips, corn flakes	10 µg/kg	Seefelder et al. (2001)
Hydrolysed and "hidden" fumonisins	indirect LC-MS/MS after hydrolysis with aqueous KOH	corn	10–40 µg/kg	Oliveira et al. (2015)
2'-R-OTA, 2'-decarboxyOTA	direct LC-MS/MS with deuterated internal standards	coffee	0.3 ng/kg	Cramer et al. (2008)
AOH-3-sulfate, AOH-3-glucoside, AME-3-glucoside; AME-3-sulfate	direct LC-MS/MS with [ <sup>2</sup> H <sub>4</sub> ]-AME as Internal standard	tomato products	0.3–1.0 µg/kg	Walravens et al. (2016)
Aflatoxin M <sub>1</sub>	direct LC-MS/MS	milk	0.6 ng/kg	Campone et al. (2013)

Although many new modified mycotoxins are currently being discovered, the development of validated methods is still restricted by the limited availability of analytical standards and certified reference materials. Commercially available up to now are only DON3Glc, AcDON and aflatoxin M<sub>1</sub>. For the other modified forms to be used in quantitation, chemical syntheses have been reported for norDONs (Bretz et al., 2006a), for modified forms of ZEN, α-ZEL and β-ZEL (Grabley et al., 1992; Mikula et al., 2013a, 2013b, 2014, Michlmayr et al., 2017), for NCM-FB<sub>1</sub> (Seefelder et al., 2001), for 2'-R-ochratoxin A and 2'-decarboxy ochratoxin A (Cramer et al., 2008) and for modified forms of AOH and AME (Walravens et al., 2014). Biotechnological syntheses of modified ZEN were performed by plant cell cultures (Engelhardt et al., 1988), by yeasts (Poppenberger et al., 2006) or by *F. graminearum* (Plasencia and Mirocha, 1991).

Another obstacle for developing accurate methods is the lack of stable isotope labelled internal standards, which are the state of the art for the regulated "free" mycotoxins due to ideal compensation for matrix effects and ionization interferences in LC-MS. The only reported labelled modified mycotoxins up to date are U-[<sup>13</sup>C<sub>17</sub>]-aflatoxin M<sub>1</sub>, [<sup>13</sup>C<sub>6</sub>]-DON3Glc (Habler et al., 2016), [<sup>2</sup>H<sub>3</sub>]-3-AcDON (Bretz et al., 2006b), [<sup>13</sup>C<sub>2</sub>]-3-AcDON, [<sup>13</sup>C<sub>2</sub>]-15-AcDON (Asam and Rychlik, 2007), [<sup>2</sup>H<sub>5</sub>]-2'-R-OTA, [<sup>2</sup>H<sub>5</sub>]-2'-decarboxyOTA (Cramer et al., 2008) as well as dihydrocitrinone, a human metabolite of citrinin (Bergmann et al., 2018).

## Occurrence of Modified Mycotoxins

A view at occurrence data of modified mycotoxins reveals that a representative basis only exists for aflatoxin M<sub>1</sub> and to a lesser extend for DON3Glc.

For aflatoxin M<sub>1</sub> the most recent review on its occurrence has been published by Womack et al. (2016). Contents in milk marketed in the EU are mostly below the EU maximum limit of 0.05 µg/kg, whereas milks from Serbia, India, Mexico and Sudan were reported to contain up to 1.2, 3.8, 7.6 and 6.9 µg/kg respectively.

For DON3Glc, naturally contaminated wheat was found to contain high contents sometimes exceeding 1000 µg/kg (Berthiller et al., 2009). For risk assessment the ratio between DON3Glc and its free form is important as many monitoring data still miss the information on the modified form. The molar ratios were found to be quite variable within ranges depending on the type of cereal and product. In a recent opinion from the EFSA Panel on Contaminants in the Food Chain (Knutsen et al., 2017a) the DON3Glc to DON ratio for grain-based food products (except for beers) was 10% in mean and for malt and beers around 100% in mean. Surveys



from the UK (Vendl et al., 2010), the Czech Republic (Malachova et al., 2011), China (Li et al., 2012) and Canada (Tran et al., 2012) revealed that cereals are contaminated with DON3Glc globally.

In case of norDON modifications, norDON A is reported to be the most abundant form in many cereal products with a maximum of 36 µg/kg in wholemeal crackers, which accounted for 27% of the DON content (Bretz et al., 2006a).

For ZEN and its modifications in cereals and cereal-based foods only few data are available from De Boevre et al. (2012, 2013, 2014) and Vendl et al. (2010). In corn, for the sum of  $\alpha$ -ZEL and  $\beta$ -ZEL a maximum of 7970 µg/kg was found and for the sum of ZEN14Glc, ZEN14Sulf,  $\alpha$ -ZEL14Glc and  $\beta$ -ZEL14Glc a maximum of 9750 µg/kg (De Boevre et al., 2014). For some staple foods like bread and breakfast cereals the sum of the amount of modified forms of ZEN even exceeded the EU maximum limits for ZEN of 50 and 75 µg/kg, respectively.

Among modified fumonisins, HFB<sub>1</sub> and HFB<sub>2</sub> are found in processed corn products after nixtamalization and may exceed the amount of FB<sub>1</sub> as has been reported for tortilla chips, corn flakes and nacho chips. In some of the latter samples, NCM-FB<sub>1</sub> was also detected and in one case of corn flakes also exceeded the amount of FB<sub>1</sub> (Seefelder et al., 2001). Steeping and washing steps in the process usually lead to reductions of up to 80%, e.g. in tortillas as reviewed by Humpf and Voss (2004).

The already mentioned "hidden" fumonisins in corn in several studies have been reported to exceed the free forms by up to 145% (Oliveira et al., 2015) or even by 263% (Falavigna et al., 2012).

For OTA, the thermally formed diastereomer 2'R-ochratoxin A was found in a maximum of 0.63 µg/kg in roasted coffee, which was equivalent to 26% of the content of the natural OTA isomer. In contrast to this, only very low amounts of 2'-decarboxy ochratoxin A were detected (Cramer et al., 2008).

Alternaria toxins are considered to be "emerging" mycotoxins and even for the free toxins only few validated methods are available. Therefore, the methods for modified forms are even more scarce. The only group to report occurrence data on modified *Alternaria* toxins was that of Walravens et al. (2016), who quantified sulfates of AOH and alternariol monomethyl ether (AME) in tomato products. Whereas glucuronides were not detectable, AOH-3-sulfate was found up to maximum contents of 8.7 µg/kg and AME-3-sulfate to 9.9 µg/kg in tomato concentrates, which referred to 43% of AOH and 116% of AME, respectively.

## Toxicity of Modified Mycotoxins

As several forms of modified mycotoxins have been described in the past (Fig. 2) and their occurrence in food samples confirmed, informations concerning toxicity and metabolism are needed. Key question is especially in the case of mycotoxin conjugates or matrix-associated forms of mycotoxins whether they can be converted into the parent mycotoxin during digestion in humans and animals or during food processing, potentially leading to adverse health effects. In the case of chemically modified mycotoxins several in vivo and in vitro studies have been performed. Only a few examples will be described here. Further detailed information concerning the toxicity of modified mycotoxins can be found in the literature (European Food Safety Authority, 2014; Freire and Sant'Ana, 2018; Rychlik et al., 2014).

Howard et al. (2002) evaluated the effect of NCM-FB<sub>1</sub> in a dose-response feeding study in mice for 28 days. NCM-FB<sub>1</sub> in doses up to approximately 140 µM/kg diet had no effect on characteristic parameters (Sa/So ratio, ceramide levels, serum analytes, organ weights, hepatic structure), which were affected by FB<sub>1</sub> (Howard et al., 2002). Other thermally modified mycotoxins mentioned above showed no or much lower cytotoxicity in cell culture experiments. NorDON A showed no cytotoxicity in concentrations up to 100 mM in IHKE cells and 2'R-ochratoxin A was by a factor of ten less cytotoxic compared to OTA (Bretz et al., 2006a; Cramer et al., 2008).

The above described DON sulfonates, which are formed upon treatment of DON-contaminated feed material with sodium bisulfite, are much less cytotoxic compared to DON as was shown in animal and cell culture studies (reviewed in Daenicke et al., 2012).

In the case of phase II metabolites such as sulfates or glucosides several studies have shown that these conjugates are stable in the upper gastrointestinal (GI) tract but effectively cleaved during digestion by the intestinal microbiota (reviewed in Freire and Sant'Ana, 2018). McCormick et al. (2015) showed that 70% of T2-3-O- $\alpha$ -glucoside and T2-3-O- $\beta$ -glucoside are hydrolysed within 24 h of incubation by bacteria found in human feces. T2 and HT2 as well as so far unknown metabolites were detectable in amounts of up to 58% of the initial glucoside concentration. In another recent study the hydrolysis of several mycotoxin glucosides was confirmed. DON3Glc and nivalenol-3-O- $\beta$ -glucoside were hydrolysed within 24 h liberating the parent mycotoxins. T2-3-O- $\alpha$ -glucoside was also hydrolysed (ca. 70% within 24 h) and T2 was further deacetylated to HT2. ZEN14Glc as well as glucosides of  $\alpha$ -/ $\beta$ -ZEL are rapidly hydrolysed (ca. 97% within 4 h), however besides the parent toxins 40%–70% of unknown metabolites were formed (Gratz et al., 2017). Matrix-associated mycotoxins such as OTA-saccharide esters or starch/protein-bound forms of fumonisins (Seefelder et al., 2003; Bittner et al., 2013) might also be cleaved by the gut microbiota or by digestive enzymes. However, in the case of matrix-associated mycotoxins the available data concerning toxicity are rather limited.

These examples clearly show that in the case of mycotoxin conjugates and matrix-associated mycotoxins the parent compounds are released during digestion and contribute to the overall mycotoxin level and potentially increase the toxic effects, assuming that the liberated mycotoxins are effectively absorbed after hydrolysis in the GI tract. For this reason modified mycotoxins should be considered during risk assessment, which has been also highlighted by the European Food Safety Authority assigning group tolerable daily intake values or relative potency factors for modified mycotoxins of DON (Knutsen et al., 2017a), ZEN (EFSA, 2016) as well as T2 and HT2 toxins (Knutsen et al., 2017b).



## Future Perspectives

Modified mycotoxins can be formed in various chemical and/or biochemical reactions and pose a potential health risk for humans and animals. As there are currently many open questions concerning the occurrence, analysis, formation and toxicity of modified mycotoxins research activities have to be intensified in the near future.

Furthermore toxicity data of modified mycotoxins are scarce and limited in many cases to *in vitro* data. Thus until sufficient data are available for risk assessment, modified mycotoxins should be considered as similarly toxic as the parent mycotoxin in order to avoid any adverse effects in humans and animals.

In the case of modified mycotoxins formed during food and feed processing, uncertainties concerning the potential risk for humans and animals can be overcome by setting legislative maximum levels of mycotoxins for raw materials and not only for the end products.

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## Relevant Websites

<https://www.efsa.europa.eu/en/topics/topic/mycotoxins> – EFSA.

<https://www.youtube.com/watch?v=yi46ZQLjMYw> – Youtube.

<http://www.mykotoxin.de/docs/public/home.asp> – Mykotoxin.

## Mycotoxins in Food and Feed: An Overview

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### Glossary

**Aptamer** short single DNA or RNA oligonucleotides, whose 3-D structure can bind specific to other molecules.

***Aspergillus* fungi** filamentous fungi, whose spores are arranged similar to water trickles running out of an aspergil or ewer.

**Endocrine disruptor** substance that interferes with the hormonal system.

**Immunoaffinity** procedure using antibodies raised against a molecular structure with the aim to specifically enrich and/or purify the target molecule.

**Molecular imprinted polymer** synthetic polymers used for molecular recognition

**Mycotoxin** toxin produced by filamentous fungi.

**p53 tumor suppressor gene** important regulatory gene in the initiation of cell apoptosis preventing a cell turning malignant.

***Penicillium* fungi** filamentous fungi, whose spore arrangement is remindful to strands of a brush.

**St. Antoniusfire** Synonym for the disease ergotism, resulting in gangrene, burning sensations, hallucinations and ultimately loss of limbs and death.

**Turkey "X" disease** disease in poultry that marked the research in mycotoxins and led to the discovery of aflatoxins in the 1960s.

### General

Mycotoxins are a chemically diverse group of substances produced by filamentous fungi. The molecular weight of the mycotoxins relevant for food and feed safety ranges approximately from 100 g/mol, such as moniliformin (Fig. 1) or nitropropionic acid (Fig. 2) to about 700 g/mol such as fumonisin B1 (Fig. 3) or beauvericin (Fig. 4). A classification of a substance as mycotoxin is purely done by the fact that it is a secondary metabolite of a filamentous fungus and toxic to humans or animals, while other properties, such pharmaceutical usability as antibiotic (Pal et al., 2017) might be present as well.

The occurrence of mycotoxins is however not limited to agricultural products, as fungi can spoil almost any sufficiently humid organic material. As a result, fungal growth on building materials (Nielsen et al., 1998) may result in the production of similar mycotoxins than those found in food, such as sterigmatocystin or alternaria toxins (Nielsen, 2003; Ren et al., 1998). However not all fungal spoilage results in the production of mycotoxins.

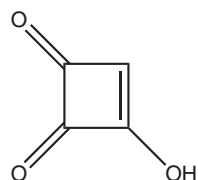
More than a thousand fungal metabolites have been spectroscopically described (Cole and Schweikert, 2003), including the mycotoxins identified as a public health concern.

The reason why fungi produce mycotoxins is not fully understood in all cases. However the biosynthesis has been extensively studied and the typical metabolic pathways were identified. These pathways (also in combination) lead to numerous secondary metabolites, including mycotoxins (Bu'Lock, 1980). In a few cases like the trichothecene deoxynivalenol (DON) its production by the fungi has an evolutionary advantage as DON is a key element in the pathogenicity, allowing the fungi to weaken the plant and causing fusarium head blight (FHB) in wheat (Gunupuru et al., 2017).

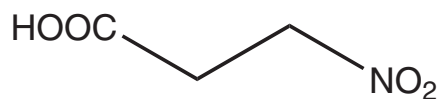
The toxic effects of the mycotoxins differ as widely as they differ in their chemical structure. Aflatoxin B1 is carcinogenic to humans (Group 1 classification by the International Agency for Research on Cancer) and hepatotoxic (International Agency for Research on Cancer, 2012). The action of zearalenone (ZON) and its metabolites is not targeting a specific organ but systemic to humans and animals as they are endocrine disruptors (EFSA Scientific Committee, 2016a). Trichothecenes like DON and T-2 toxin interfere with protein synthesis and consequently result in numerous effects ranging from immune dysfunction to vomiting, which brought DON also the synonym "vomitoxin". Fumonisin show different carcinogenic and pathologic effects depending on the animal species affected (Marasas, 2001), while ergot alkaloids are neurotoxic. As a result health risks of mycotoxins have to be evaluated individually based on their mode of action, their occurrence in food and feed as well as the metabolic pathways for detoxification within an animal species.

### Historical and Regulatory Background

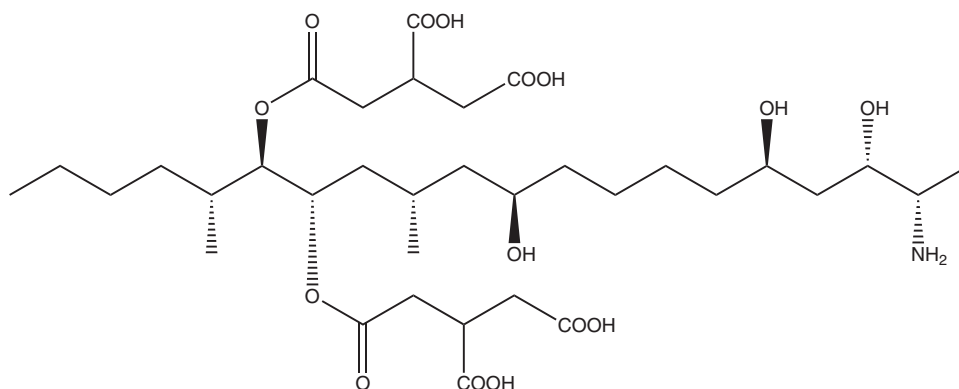
Mycotoxin intoxications are known since centuries in Europe and outbreaks occurred in irregular intervals. The historically most relevant outbreaks are related to *Claviceps purpurea* resulting in ergotism (also called St. Antoniusfire) (van Dongen and de Groot, 1995). Cereals infested with sclerotia of *C. purpurea* contain the toxic ergot alkaloids. Symptoms of the disease inspired the painter



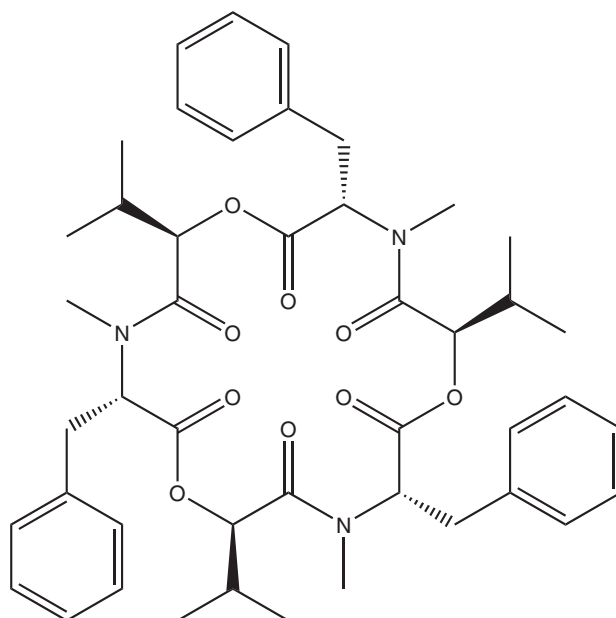
**Figure 1** Moniliformin.



**Figure 2** Nitropropionic acid.



**Figure 3** Fumonisin B<sub>1</sub>.



**Figure 4** Beauvericin.

Pieter Bruegel (the Elder) in his 1568 painting "The beggars". It is remarkable that the outfit of the depicted beggars reflects different societal classes indicating that ergotism was of general public concern (Barker, 1927) already by that time.

Other major mycotoxicosis outbreaks were described in the past, such as "cardiac beriberi" in Asia, which is caused by consumption of *Penicillium citreonigrum* infested rice and the "alimentary toxic aleukia" in the 1940s in former Russia caused by fusarium

toxins in cereals that were left in the field over winter (as result of war time) and affected humans and animals (Pitt and Miller, 2017).

In 1960 more than 100.000 turkeys died in the United Kingdom, after being fed with aflatoxin contaminated peanut meal (Latham, 1964). This incidence, which was the beginning of the awareness for mycotoxins at global scale, was called the turkey "X" disease. Rapidly the causing factor "X" was identified as aflatoxin B1 (and G1) (Wannop, 1961). Initially research focused on the safety of peanuts for animal feed (Latham, 1964), while the global extent of the aflatoxin impact is nowadays addressed (Wild and Gong, 2009; Ostry et al., 2017; Gong et al., 2002; Richard, 2008; Wild et al., 2015). Still nowadays several decades after the turkey "X" disease mycotoxicosis outbreaks occur occasionally like aflatoxicosis in African regions (Azziz-Baumgartner et al., 2005) or cardiac beriberi in Brazil (Rosa et al., 2010).

This finding paved the way to the development of a number of analytical methods based on chromatography for aflatoxins and many other mycotoxins (Betina, 1993a). In the aftermath of the turkey "X" disease the probable involvement of cyclopiazonic acid was discussed (Cole, 1986) while potentially overlooked at that time due to the easier detectable and highly toxic aflatoxins. In the course of instrumental and analytical development a number of mycotoxins were discussed as potential food and feed contaminants (Directorate-General, 1994), however only a fraction has been identified to potentially impose a general public health risk. These are regulated in many parts of the world (van Egmond and Jonker, 2004; van Egmond et al., 2007), with the European Union having the most extensive regulation on mycotoxins in food (European Commission, 2006a) and feed (European Commission, 2002) (European Commission, 2013).

Currently efforts are made by the European Food Safety Authority (EFSA) to evaluate "emerging" mycotoxins and their potential for public and animal health concern. The resulting EFSA scientific opinions address the scientific questions needed by legislators and risk managers and a number of mycotoxins have been addressed in the past like the ergot alkaloids, alternaria toxins, beauvericin and others (EFSA website on Opinions). This is been fostered by recent advances in coupling liquid chromatography with mass spectroscopy, allowing the screening of hundreds of fungal metabolites (Vishwanath et al., 2009; Malachová et al., 2014) with a single method. This technology is currently the most useful tool for generating occurrence data, which is a key challenge for any risk assessors like the EFSA or the U.S. Food and Drug Administration.

The worldwide list of regulations on mycotoxins (van Egmond and Jonker, 2004) demonstrates that almost 100 countries have implemented regulations for mycotoxins in 2003 covering 87% of the world population and the levels for aflatoxins put into national legislation correspond in the majority of cases with those set by the Codex Alimentarius (CODEX excerpt). An overview of the rather extended list of mycotoxins regulated in the European Union given in Table 1.

## Social and Economic Impact of Mycotoxins

The setting of maximum permitted levels by risk managers is linked to the consumption pattern, local availability and production of the produce as well as international trade while respecting the conclusions of the health risk assessment. As a result risk managers may conclude differently how to manage a mycotoxin risk. Such a difference was subject of the 2001 World Bank report (Otsuki et al., 2001) regarding new EU legislation for aflatoxins in food which was thought to have a negative influence on African countries exporting to the European market. This conclusion was updated in 2005 (World Bank, 2005), correcting the estimates on financial losses as highlighted in 2001. These losses did not verify an in contrary a number of exporting countries benefited from the new legislation.

Studies on the economic impact of produce loss due to mycotoxin contamination have improved and are used to help all stakeholders involved on the food/feed production chain estimating the impact due to mycotoxin contamination (Wu, 2015). Especially

**Table 1** Summary of mycotoxin limits in the EU in 2017 (European Commission, 2002, 2006a, 2006b, 2013)

Mycotoxin	Food products (µg/kg) <sup>a</sup>		Products for animal feed (µg/kg) <sup>a</sup>	
	Lowest level	Highest level	Lowest level	Highest level
Aflatoxin B1 (Σ of aflatoxins)	0.1 (–)	5 (10)	5	20
Aflatoxin M1	0.05	0.025	–	–
Deoxynivalenol	50	750	900 <sup>b</sup>	12000 <sup>b</sup>
Fumonisin B1+B2	200	1000	5000 <sup>b</sup>	60000 <sup>b</sup>
Ochratoxin A	0.5	10	10 <sup>b</sup>	250 <sup>b</sup>
Zearalenone	20	200	100 <sup>b</sup>	3000 <sup>b</sup>
T-2 & HT-2 toxin	15 <sup>b</sup>	200 <sup>b</sup>	50 <sup>b</sup>	2000 <sup>b</sup>
Patulin	10	50	–	–
Citrinin	2000	2000	–	–
Ergot sclerotia	500 000 (0.5 g)	500 000 (0.5 g)	1 000 000 (1 g)	1 000 000 (1 g)

<sup>a</sup>Lowest and highest level depends on the food type.

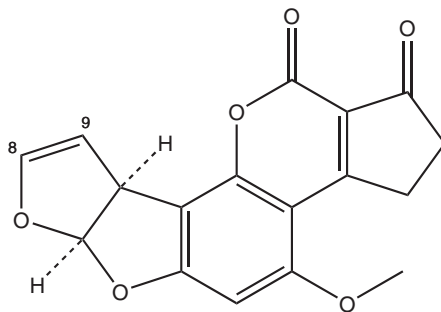
<sup>b</sup>Levels marked with an asterisk are indicative/recommended (maximum) levels.

in the European Union the economic impact on trade becomes apparent by the number of notifications in the European Union Rapid Alert System for Food and Feed. In 2016 mycotoxins accounted for more than 1/3rd of all food/feed border rejections at European ports of entry. These rejections exceeded those resulting by the presence of pathogens, and exceeding limits for heavy metals and pesticides (European Union, 2017).

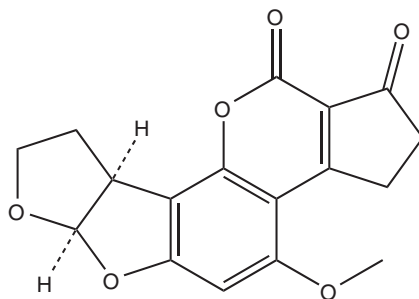
## The Different Mycotoxin Groups

### The Aflatoxins

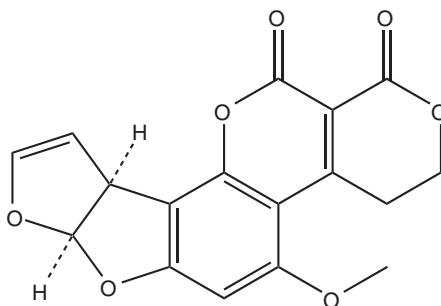
Aflatoxins is the first group of mycotoxins studied extensively after the turkey “X” disease outbreak. In food of plant origin aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> (Figs. 5–8) occur with different pattern, depending on the fungal species (e.g. *Aspergillus flavus* or *Aspergillus parasiticus*) and the fungal growth conditions. The name aflatoxin is derived from (*A*)spergillus (*fla*)vus, however a number of other *Aspergilli* have been identified to produce aflatoxins, too (Ito et al., 2001). The typification B or G is linked to their appearance under ultra violet light (366 nm), namely blue or green, depending on the presence of a cyclic ketone or a lactone in the molecular structure. The classification number refers to a double bond (indexed as 1) or saturated bond (indexed as 2) at the position 8, 9 of the molecule. Due to their native fluorescence they are relatively easy to detect with chromatography, an asset for their discovery in the turkey “X” disease investigation.



**Figure 5** Aflatoxin B<sub>1</sub>.

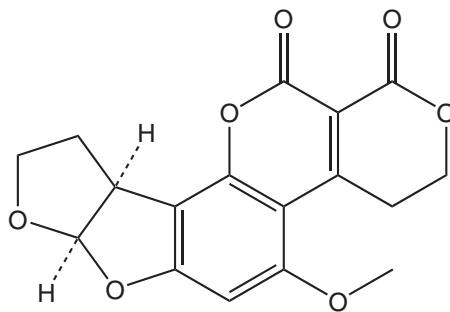


**Figure 6** Aflatoxin B<sub>2</sub>.



**Figure 7** Aflatoxin G<sub>1</sub>.





**Figure 8** Aflatoxin G<sub>2</sub>.

Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is the predominant aflatoxin found and in addition the most potent natural carcinogen known to man. Its toxicity depends on the animal species (Patterson and Allcroft, 1970), resulting in rainbow trout being extremely sensitive, while within an animal species toxicity is generally age dependent.

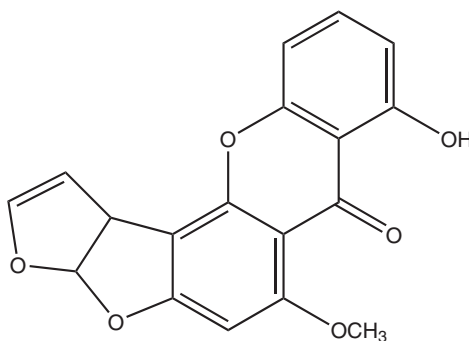
The mode of action leading to its carcinogenicity has been studied in great detail and is result of the formation of a highly reactive epoxide along the 8, 9 position. The enzyme involved is mainly found in the liver. In a following step the epoxide can react with either DNA/RNA or proteins, forming a covalent adduct (Oesch et al., 1999). If such adduct leads to the dysfunction of the tumor suppressor gene p53 it can lead to a malignant cell cycle (Hsia et al., 1992; Bressac et al., 1991). Despite its high carcinogenicity the number of actual cases of primary liver cancer is low in industrialised countries, while in other parts of the world, where chronic hepatitis is prevalent, an increased aflatoxin exposure significantly increases cancer rates (Henry et al., 2002; Kew, 2003). The exposure to aflatoxins in the African continent can be relatively high and occasional aflatoxin outbreaks occurred in recent times in Kenya, resulting in acute toxicities, eventually leading to human casualties (Azziz-Baumgartner et al., 2005).

Aflatoxins are also metabolized to a number of other metabolites of which aflatoxin M<sub>1</sub> (hydroxylated AFB<sub>1</sub>) is the most known. This can be found in milk when AFB<sub>1</sub> is taken up by mammals. The conversion rate is approx. 5% of the AFB<sub>1</sub> ingested (Britzi et al., 2013) and gave reason for setting maximum levels for AFB<sub>1</sub> in animal feed (European Commission, 2002). As a result of this transformation of AFB<sub>1</sub>, aflatoxin M<sub>1</sub> is considered a modified mycotoxin (Rychlik et al., 2014).

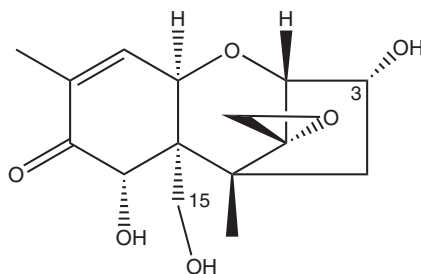
Closely related to the aflatoxins is sterigmatocystin (Fig. 9), a metabolic precursor leading to production of the aflatoxins in the fungal cells. It is produced by *Aspergillus versicolor* and *Aspergillus nidulans* who cannot further metabolise sterigmatocystin to aflatoxins as they lack the needed enzymes (FAO and WHO, 2017a). The occurrence in food and feed is considered low compared to that of aflatoxins according to some studies (Scott, 2004), while it has been observed that about 20% of all analysed sorghum samples contain sterigmatocystin in some regions of the world (FAO and WHO, 2017a). However its genotoxic effect with *in vitro* experiments of human cell lines has shown to be greater than that of AFB<sub>1</sub> (Theumer et al., 2018), while a review of studies come to a slight different conclusion interpreting *in vivo* experiments (FAO and WHO, 2017a).

### The Trichothecenes

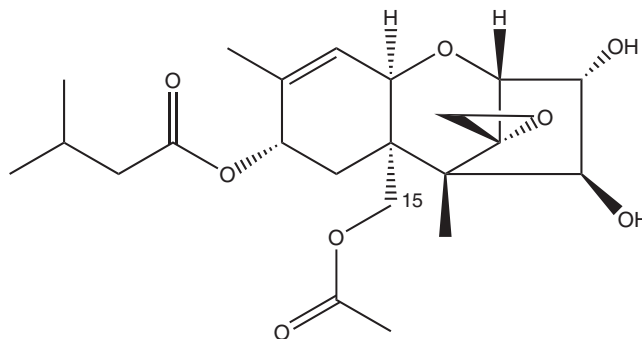
Trichothecenes are produced by a number of different fungi (Wannenmacher and Wiener, 1997) and they have the same core structure, being 12,13-epoxytrichothec-9-ene (FAO and WHO, 2017b). The most known and studied mycotoxin of the trichothecen family for food and feed safety concerns is deoxynivalenol (DON) (Fig. 10). It occurs as result of cereal infestation with *Fusarium graminearum* and *Fusarium culmorum* in the field and can be found in corn, wheat and other cereals grown in temperate climates. Next to DON, some fusarium fungi produce also acetylated forms of DON, namely 3-acetyl and 15-acetyl DON.



**Figure 9** Sterigmatocystin.



**Figure 10** Deoxynivalenol.



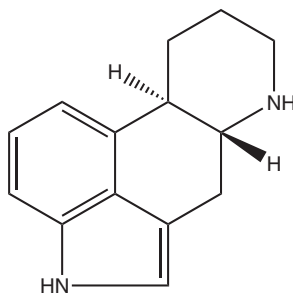
**Figure 11** HT-2 toxin.

Several dozen trichothecenes have been described and are produced by a number of *Fusarium* species, like *Fusarium sporotrichioides*, *Fusarium langsethiae*, however only DON, T-2 and HT-2 toxin (**Fig. 11**) have drawn attention for public health so far. Trichothecenes are classified into different groups as result of structural differences. The type-A trichothecens (T-2 and HT-2 toxins, diacetylscipenol) have a functional group other than a ketone at carbon position 8. They occur less frequently but are more toxic than the type-B trichothecens such as DON and nivalenol, who possess the keto-function ([Directorate-General, 1994](#)). While DON has been reported in a large number of different cereals, T-2 and HT-2 toxin are often associated with oat ([Pettersson et al., 2011](#)). However, their presence, like DON, is found across cereal species and environmental climatic zones ([Morcia et al., 2016](#)).

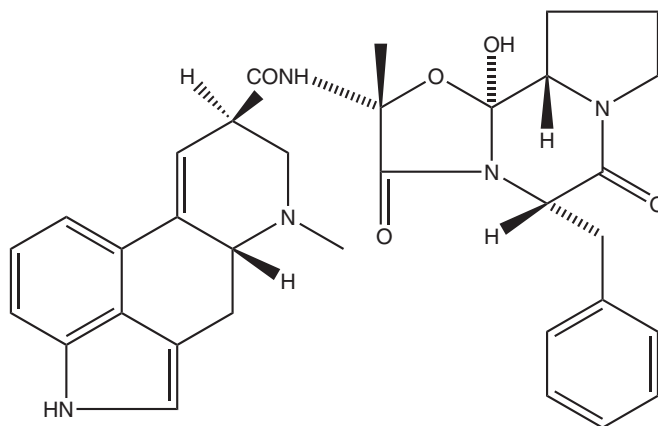
While trichothecenes show gastrointestinal, dermatological and immunotoxic effects, they vary from toxin to toxin and the affected species. This manifests to poultry being comparably tolerant to DON, while T-2 and HT-2 toxins are extremely toxic to cats ([EFSA Scientific Committee, 2017a](#)). The animal susceptibility is also reflected in the levels stipulated for feed in Europe ([European Commission, 2006b](#)).

### The Ergot Alkaloids

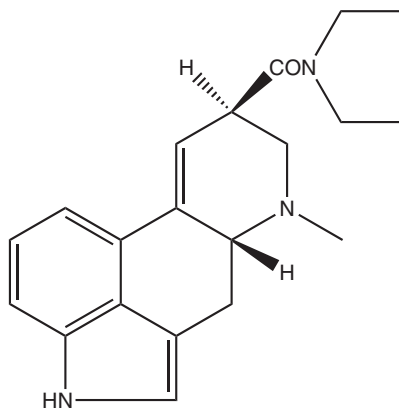
The ergot alkaloids (EA) are a rather large group of roughly 80 closely related compounds sharing a common backbone structure, which is ergoline (**Fig. 12**) of which ergotamine (**Fig. 13**) is the most known one. Sclerotia containing EA were used for medical purposes since centuries. The EA exhibit a number of neurological effects and are used as pharmaceuticals ([Eadie, 2001](#)). Intoxications result mainly in neurological disorders such as numbness of the limbs, convulsions and hallucinations, gangrene and ultimately death.



**Figure 12** Ergoline.



**Figure 13** Ergotamine.



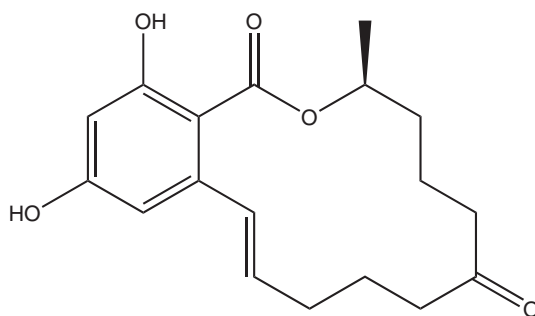
**Figure 14** Lysergic acid diethylamide (LSD-25).

Different from the other mycotoxin producing fungi, the sclerotia of *C. purpurea* can largely be removed mechanically from cereals like rye or wheat. However, for this the sclerotia must sufficiently differ from the cereal kernels with respect to size, density or color (Scott, 2009). Currently ergot is regulated in the European Union by visual/microscopic identification of the sclerotia as “foreign” particles (European Commission, 2006a, 2006b). Taking note that ergot alkaloids vary significantly in sclerotia and that analytical methods are availability for these alkaloids the current strategy focuses on setting limits for individual ergot alkaloids. Relevant for the chemical analytical monitoring of ergot alkaloids is that the different alkaloids act partially synergistically or antagonistically. This aspect makes a food/feed safety evaluation complex (EFSA Scientific Committee, 2012).

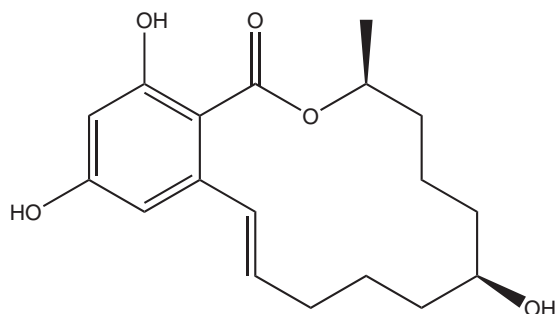
The most known ergot alkaloid derivative is the highly neurologically active lysergic acid diethylamid (LSD) which became a known drug leading to psychotic effects even if taken only at fractions of a milligram. The synthesis of LSD (Fig. 14) was triggered by the success in which nicotinic acid was modified before, leading to the stimulant nikethamid (Coramine). This synthesis approach to modify pharmacologically active substances was known that time by Albert Hofmann who first synthesized LSD in 1943.

### Zearalenone

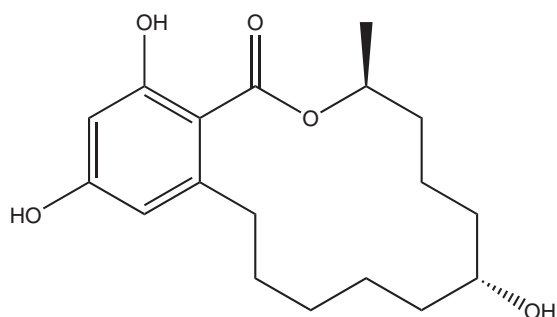
Zearalenone (ZON) (Fig. 15) is a metabolite of *Fusarium graminearum* and *F. culmorum* infesting mainly corn, wheat and barley but also other cereals. ZON acts as an endocrine disruptor and its toxicity is animal species dependent. Concerning the metabolism of ZON in animals, it is important to note that the toxicity of ZON is linked to the way an animal metabolises this mycotoxin. In particular the pathway leading to the modified form  $\alpha$ -zearalenol (Fig. 16), with the keto function reduced to a hydroxyl group, plays a key role for the toxicity as the EFSA concluded a much higher estrogenic potency factor for this metabolite. Other metabolites like  $\alpha$ -zearalanol (also known as zeranol) (Fig. 17) with a saturated bond, are marketed as anabolic agents and growth promoters. As a result, the EFSA addressed the co-occurrence of different metabolites of ZON by publishing a “health based guidance value for ZON and its modified forms” in food and feed (EFSA Scientific Committee, 2016a; 2017b).



**Figure 15** Zearalenone.



**Figure 16**  $\beta$ -Zearalenol.



**Figure 17**  $\alpha$ -Zearalenol.

### The Fumonisin and Moniliformin

The fumonisins, in particular fumonisin B1 (Fig. 4) and fumonisin B2 were the last group of mycotoxins identified that impose great public health concerns. Their discovery in 1988 in South Africa as the chemical agents responsible for equine leukoencephalomalacia (ELEM) in horses and pulmonary edema (PE) in pigs (Marasas, 2001) involved a decade of international research collaboration. Fumonisin are produced mainly by *Fusarium verticillioides* (*Fusarium moniliforme*) infesting mainly corn and relate to different toxicological observations dependent on the animal species. Next to the specific effects known for horses and pigs, rats develop primarily liver cancer and for humans a strong indication exist that fumonisins cause esophageal cancer (Gelderblom et al., 1988; Somdyala et al., 2003).

Fumonisin interfere with the sphingosine metabolism. This is used as a biomarker for exposure (Shephard et al., 2013; Qiu et al., 2001; van der Westhuizen et al., 2010). Next to corn other agricultural produce like asparagus or garlic can be contaminated as well (Seefelder et al., 2002). However, recent data shows that fumonisin contamination in commercial dry garlic is comparably low (Tonti et al., 2017). Besides the initial assumption that only *Fusarium* fungi can synthesize the fumonisins, it was shown that some *Aspergilli* are as well producers of this mycotoxin group (Perrone and Gallo, 2017; Munkvold et al., 2018).

Moniliformin (Fig. 1) is produced by *F. moniliforme* and can co-occur with fumonisins. It is made of a cyclic 4 carbon ring and is considered an “emerging mycotoxin”, reported in Norwegian cereals (Uhlig et al., 2004) as well as in other regions of the world (Jestoi, 2008). Current data showed that the levels found in retail samples of central Europe during 2016–7 were less contaminated than those reported in previous years (Herrera et al., 2017).

## The Alternaria Toxins

The group of *Alternaria* fungi are capable to synthesize a number different of secondary metabolites, however only a few have been identified as toxicologically relevant for public food safety (EFSA Scientific Committee, 2016b). These are tenuazonic acid (Fig. 18), alternariol (Fig. 19), alternariol monomethyl ether (Fig. 20), altenuene and tentoxin (Fig. 21). Alternaria toxins can be found in cereals, oil seeds, like sunflower or canola as well as a number of vegetables (in particular tomatoes and carrots) and fruits. Tenuazonic acid is the most abundant alternaria toxin and can reach levels of several mg/kg in vegetables or fruits with sufficient high water content. Since *Alternaria* fungi grow also at temperatures near the freezing point, even chilled products can become contaminated. Black spots, (next to other types of spoilage) are an indicator for alternaria infestation.

Sunflower seeds are frequently contaminated and levels can differ for shelled and peeled seeds. As a result the way of consumption (habit of dehulling salted seeds in the mouth in some countries) is important when drawing conclusions for the exposure to alternaria toxins from sunflower seeds.

## Ochratoxin A

Ochratoxin A (OTA) (Fig. 22) is known as potential contaminant in a number of food stuffs and has been monitored in the last 2 decades due to the fact that it occurs in products of high market value, such as spices, coffee, cocoa, wine or licorice but also in grapes and cereals, in particular barley. It is produced by some *Penicillium* as well as *Aspergillus* fungi. A mycotoxin often associated with OTA is citrinin, which gained attention in the EU due to the levels occasionally found in “fermented” red yeast rice, a product taken as natural medicine against high cholesterol levels. Both mycotoxins are potent nephrotoxins and are regulated in the European Union. OTA has been associated with Balkan endemic nephropathy, while there is doubt whether other factors, such as the plant toxin aristolochic acid, play a key role. In the body OTA binds tightly to serum albumin (Riley and Voss, 2011). This allows the

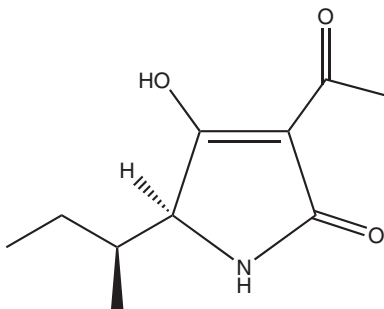


Figure 18 Tenuazonic acid.

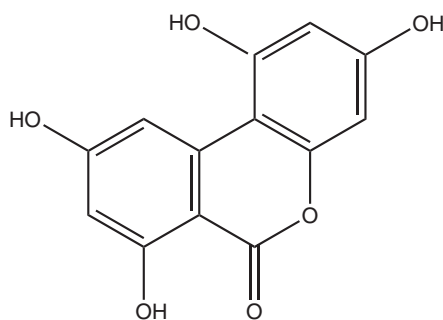


Figure 19 Alternariol.

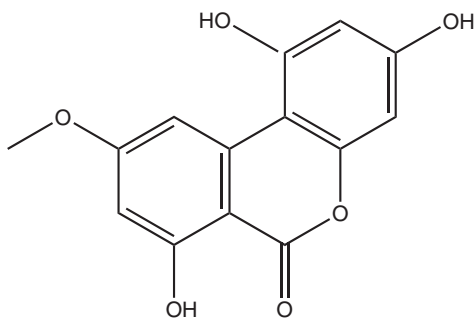
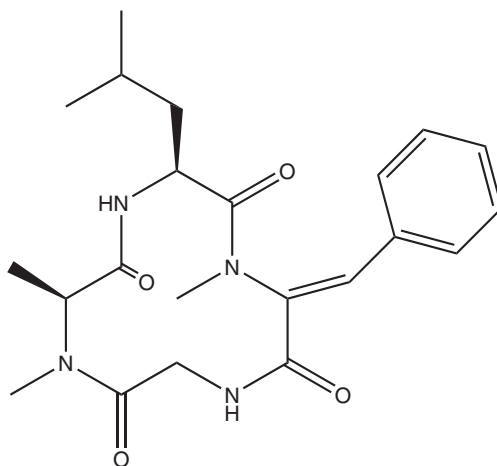
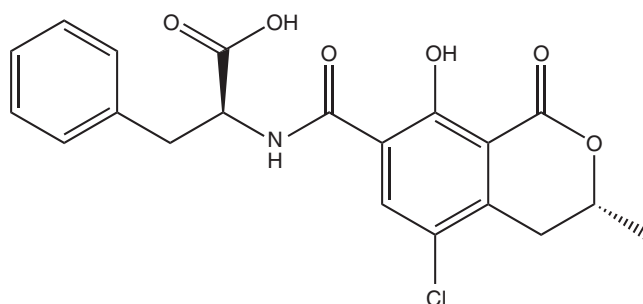


Figure 20 Alternariol monomethyl ether.



**Figure 21** Tentoxin.



**Figure 22** Ochratoxin A.

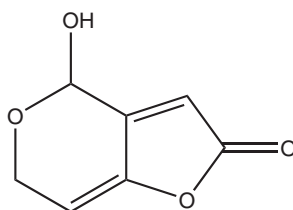
monitoring of long term OTA exposure with ease compared to most other mycotoxins that are either rapidly excreted or metabolized. Due to consumption pattern and the occurrence of OTA it is a frequently found biomarker in humans. Remarkable is that OTA can be used to monitor the consumption of coffee since a specific heat induced degradation product of OTA is only formed during coffee roasting and has been exclusively identified in the blood of coffee drinkers (Cramer et al., 2015).

### Patulin

Patulin (Fig. 23), chemical name (2,4-Dihydroxy-2H-pyran-3-(6H)ylidene)acetic acid, 3,4-lactone or 4-Hydroxy-4H-furo(3,2-c)pyran-2(6H)-on, [CAS: 149-29-1] is one of the mycotoxins with the largest number of synonyms associated with it, namely: clairformin, clavacin, clavatin, claviformin, expansin(e), gigantint, leucopin, mycoin, mycoin C, mycoin C3, patulin(e), penicidin, penantint and terinin.

It is produced by a number of fungi such as *Penicillium*, *Aspergillus* and *Byssoschlamys* and is primarily found in apples and products such as puree or juice thereof. As a result it enters the food chain almost exclusively via rotten fruits.

Modern production technology allows effective elimination of patulin by identifying rotten apples and/or excavating potentially rotten parts with water jet streams leading to apple products of high quality and little concern with respect to patulin. Another effort to reduce patulin is the application of good manufacturing practice (GMP) (Food and Agricultural Organisation, 2003; State of Michigan, 2016).



**Figure 23** Patulin.



Patulin was extensively tested as an antibiotic agent after the discovery of penicillin in mid last century (Stuart-Harris et al., 1943). It has been shown in various tests to be a mutagen and genotoxic. However no conclusion exists to categorize it as carcinogen and therefore it is classified by the IARC in group 3. It has been shown in a self-experiment with isotope labelled patulin, that human metabolism appears to be effective in dealing with moderate levels of patulin (Rychlik, 2003).

Nonetheless as apple products are frequently consumed by infants and young children (Raiola et al., 2015) patulin reduction management is important since *Penicillium expansum* can produce concentrations up to 1 g per liter in apple juice if it is grown to fully cover juice in Petri dishes and in 1995 an outbreak of diarrhea in Denmark was associated with patulin found in blueberries (Berg et al., 1995).

### Beauvericin and the Enniatins

Beauvericin (Fig. 3) and enniatins A, A1, B and B1 are often mentioned in the context of “emerging mycotoxins”. Enniatins (incl. Beauvericin) are cyclic peptides similar to some macrolid-antibiotics like valinomycin. Even though Beauvericin has been described as an antibiotic mycotoxin in 1969, the attention as food contaminant just grew in the last decade of the last century in Scandinavia when weather conditions favored the fungi that produced beauvericin (Logrieco et al., 2002; Uhlig et al., 2007). Beauvericin is produced by a number of *Fusarium* fungi, while it was first discovered as metabolite of *Beaveria bassiana*, a fungus that attacks insects, which led also to studies for its insecticidal use (Wang and Xu, 2012).

In 2013 a multi-screening on 83 samples of food and feed raw materials revealed that beauvericin and enniatins are found more frequently than deoxynivalenol in about 98% of all analyzed samples. However the maximum observed levels were less than 1/10th of that of deoxynivalenol (Streit et al., 2013).

The limited availability of fit-for-purpose reference substances (purified and characterized mycotoxin portions) are one of the reasons for a delayed monitoring of emerging mycotoxins by larger groups of laboratories (Goncalves et al., 2017) and this makes exposure estimation difficult for risk assessors.

### Gliotoxin and Mycotoxins With Lesser Frequency

Gliotoxin (Fig. 24) is not an explicitly regulated mycotoxin in any country. However it is produced by a number of fungi, including *Aspergillus fumigatus*, whose spores are ubiquitous in the global environment (Weidenbömer, 2001). It is further produced by *Byssosclamyces nivea* and *Paecilomyces variotti*, which are patulin producer and the acid-tolerant *Penicillium roqueforti*, the producer of roquefortin C. Gliotoxin is often associated with fungal in-door pollution while it has also been reported in cereals, and dried fruits. It is considered an “important mycotoxin with fewer occurrences” in the same way as citreoviridin, griseofulvin, mycophenolic acid,  $\beta$ -nitropropionic acid, kojic acid, penitrems, penicillic acid, viomellein, vioxantin and xanthomegnin and walleminols (Marroquín-Cardona et al., 2014).

*A. fumigatus* and gliotoxin exposure to animals was studied in recent years in South America (Monge et al., 2012, 2013; Pena et al., 2015) indicating the presence of the fungi but no exposure to the toxin. These results must however be evaluated with care, as the obtained limits of detection reported were relatively high (44  $\mu\text{g/kg}$ ) compared to those of routinely analyzed mycotoxins such as aflatoxin B1 (1.6  $\mu\text{g/kg}$ ), fumonisin B1 (2.4  $\mu\text{g/kg}$ ), resulting in a large fraction of left censored data (=below the limit of detection) (Monge et al., 2012).

*A. fumigatus* commonly grows on decaying organic material (including food or feed waste) and is identified as a frequent fungi in silage (Pettersson et al., 2004). Monitoring silage for gliotoxin, showed that levels up to 20 mg/kg or more can be reached (Keller et al., 2012). This is an important aspect in current ambitions aiming at reducing food waste or re-directing materials that are classified as unfit for human consumption as insect feed (Van Huis et al., 2013). Therefore such new streams in the food and feed chain may require the monitoring of some metabolites like gliotoxin.

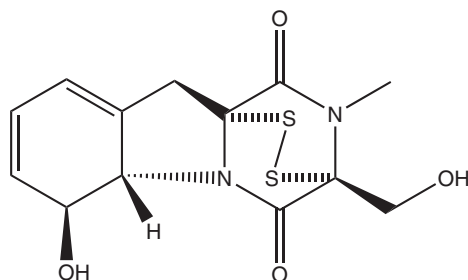


Figure 24 Gliotoxin.

## Spoiled and Cured Food

The growth of mycotoxin producing fungi on food items in the household is a common phenomenon. The main targets for fungal spoilage are fresh fruits like strawberries, vegetables like carrots or semi-dry food like bread or preserves like marmalade/jam. The fungal strains involved can range from *Botrytis cinera* or *Rhizopus stolonifera* on strawberries to *Cladosporium butyris* on fatty products like butter. When products are visibly spoiled they are classified as unfit for consumption. Therefore they are not considered a public health concern and should be properly disposed.

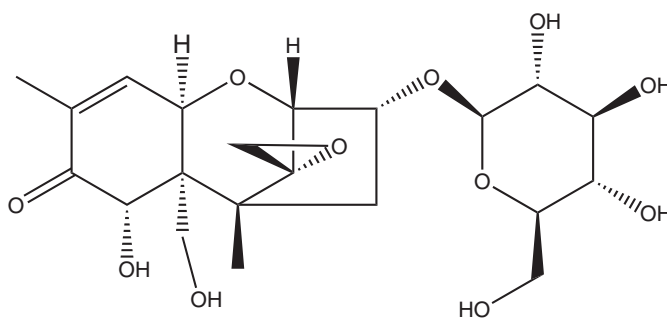
On the other hand a number of fungi are used for curing food like cheese, sausages or other meat like Swiss Luma<sup>®</sup> beef. In Europe *Penicillium* fungi are primarily used and a few of them have shown to be able to produce mycotoxins (ochratoxin A and citrinin) depending on the growth conditions. However others have also shown to be cytotoxic in cell tests. Therefore fungi used for curing should be tested prior selection to conclude on their safe use (Gareis et al., 1999).

## Modified Mycotoxins

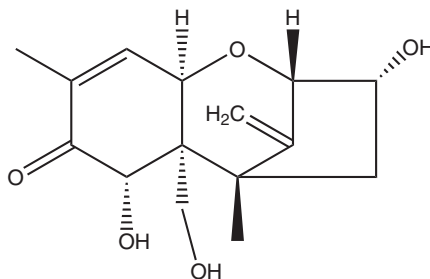
More and more chemically changed mycotoxins, that are the result of different modes of metabolism or chemical reaction have been identified in the last decade. These biological or chemical modification are mainly result of the plant defense mechanism or thermal processes. Both terms, “masked mycotoxins” as well as “modified mycotoxins”, exist in literature and a proper classification to address the differences has been proposed (Rychlik et al., 2014). The biological modification aims at neutralizing or excreting mycotoxins by the organism. Trichothecenes like DON are plant (cereal) pathogens (McCormick et al., 2011) and the metabolism to DON-3-glycoside (Fig. 25) allows the plant cell to expel the compound from the cytosol into the vacuole (Berthiller et al., 2016) reducing the toxicity to the plant. This defence also reduces the overall amount of all DON related compounds in those plants that make efficient use of this principle (Lemmens et al., 2016). As a result cereal varieties that are efficient in this, are more resistant to fusarium head blight.

In general two major pathways are known for the biological modification of mycotoxins, namely the phase-I-metabolism and phase-II- metabolism. Phase-I metabolites are characterized by oxidation, reduction or equivalent alteration of the mycotoxin leading to substances like de-epoxy-nivalenol (Fig. 26) or  $\alpha$ -zearealenol (Fig. 16). Phase-II metabolites are the result of a conjugation reaction such as glucosylation, leading to DON-3-glycoside or ZON-sulphate (Fig. 27).

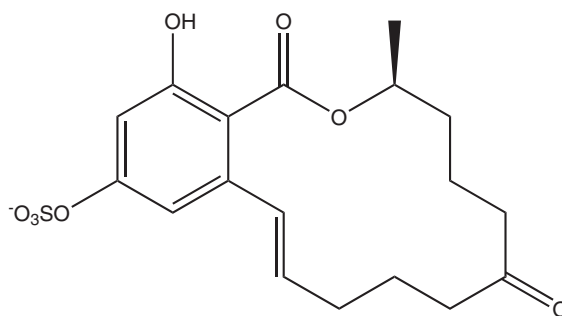
In recent years a number of modified mycotoxins have been identified, either as plant or animal metabolites. Due to their toxicological potential, metabolites of DON and ZON as well as a number of fumonisin derivatives have been described (Berthiller et al., 2012) and it was concluded that their presence can generally contribute to the overall exposure to mycotoxins. One reason is that the toxic potential can be partially regained when the modified forms are “re-metabolized” to the parent mycotoxin, like it has been shown for DON in pigs (Nagl et al., 2014).



**Figure 25** Deoxynivalenol-3-glycoside.



**Figure 26** Deepoxy deoxynivalenol.



**Figure 27** Zearalenone-4-sulphate.

The possible metabolism of ZON to  $\alpha$ -zearalenol and other metabolites as described in the paragraph on ZON is interesting for the safety evaluation of feed and novel-foods when insects are subject to consumption as proposed and authorized (European Union, 2015; Makkar et al., 2014). In 1991 it was shown that  $\alpha$ -zearalenol is the main metabolite in mealworms and is excreted with the feces with a conversion rate of approx. 30% and thought to be a detoxification process prior any public discussion on endocrine disruptors (de Rijk et al., 2015 Hornung, 1991).

Taking into account the estrogenic effect that has been estimated by the EFSA (60-fold compared to ZON) (EFSA Scientific Committee, 2016a) insects fed with ZON levels at currently tolerated limits for animals (European Commission, 2006b) might have the potential to amplify the estrogenic potential compared to that in the feed they grew on themselves if fed further to animals or are eaten by humans without precaution like proper elimination of the metabolites of ZON.

## Analytical Techniques

Prior discussions on analytical methods for mycotoxins, their often inhomogeneous distribution in agricultural products must be addressed (Whitaker, 2010). Especially aflatoxins are heterogeneously distributed (de Rijk et al., 2015), while others like deoxynivalenol are more homogeneously distributed within consignments (Biselli et al., 2008). The reason for a more homogeneous distribution is the usually area-wide fungal infestation of the cereals prior harvest. Aflatoxins on the other hand occur often in “hot-spots” (Fig. 28) and contaminate individual agricultural items such as single peanut/pistachio kernels, or single dried figs at minor frequency but high concentration (Schatzki and Haddon, 2002; Toyofuku et al., 2009; da Gloria, 2011). A tool that tackles this problem and generates sampling schemes with a defined seller and buyer risk has been developed by the FAO (Mycotoxin Sampling Tool). The challenges in preparing a laboratory sample for chemical analysis (at gram size) from an aggregate sample (at kg size) has been investigated and a watery slurry mixing procedure is proposed as reliable solution to reduce the sample size representatively (Spanjer et al., 2006).

Classical analytical techniques for mycotoxins used thin-layer chromatography (TLC) combined with liquid–liquid or column chromatography clean up (Betina, 1993b) and either instrumental (optical) or visual detection. Modern routinely used – confirmatory – methods for mycotoxin monitoring or control make use of liquid chromatography (LC) coupled to mass-spectroscopy (MS) (De Girolamo et al., 2017; Meneely et al., 2011). The introduction of LC-MS technology allows furthermore the simultaneous determination of numerous mycotoxins and fungal metabolites (Vishwanath et al., 2009). The key challenges when quantifying



**Figure 28** Workers removing a fungal “hot spot” in a corn shipment at the port of Dar es Salaam in 1993. Courtesy of Henry Njapau, National Institute for Scientific and Industrial Research, Lusaka, Zambia.

mycotoxins with MS are matrix effects that in the majority of cases suppress the signal in the detector compared to the signal from pure reference materials. Such suppression effects are dependent on the sample analyzed and often require either standard addition or matrix matched calibration if no stable-isotope labelled reference materials are available (Rychlik and Asam, 2008).

Despite instrumental progress with MS technology, TLC is still widely used in many regions of the world. Reason is that local conditions favor methods that are technically easy to control. In addition, despite a considerable shift to mass selective detectors in industrialized countries; fluorescence detection is still one of the most reliable and easy to implement methodologies for those mycotoxins that exhibit a natural fluorescence like the aflatoxins, zearalenone or ochratoxin A. As aflatoxins B1 and G1 undergo fluorescence quenching derivatization is frequently used and different types exist. Only post column derivatization techniques (Papadopoulou-Bouraoui et al., 2002) offer real benefits as pre-column derivatization (usually with trifluoro acetic acid) results in rather polar products that elute usually with matrix peaks. In combination with an immunoaffinity clean-up limits of quantification in the range of 20 ng/kg or lower can be achieved for aflatoxin B1. These reasons often favor the use of fluorescence detection for aflatoxins.

Methods using solely UV absorbance are considered not any more state of the art and are disfavored since the desired selectivity of the signal is questionable (European Commission, 2016). This challenge was demonstrated by the interpretation of chromatograms during a survey on the occurrence of patulin in a number of apple products and during method validation studies (Yurdun et al., 2001).

Parallel to chromatographic methods, the development of antibodies specific to smaller molecules like mycotoxins (Fremy and Usleber, 2003) as well as molecular imprinted polymers (MIPS) led to the development of mycotoxin specific sensor systems (Peltonmaa et al., 2018) and to powerful sample cleanup procedures (Lucci et al., 2017; de Smet et al., 2009). In recent years the advances in the development of aptamers for molecular recognition (Ruscito et al., 2016) open similar aspects similar like antibodies or MIPS.

In particular enzyme-linked immune sorbent assays (Goryacheva et al., 2007) and lateral flow devices (Zheng et al., 2006) are widely applied for mycotoxin screening, while sensor based applications are described either as prototypes or as instruments for end users (Maragos and Busman, 2010; Chauhan et al., 2016; Vidal et al., 2013). A few screening methods have been tested for eligibility in official food control in the EU and the USA (Lattanzio et al., 2016; United States Department of Agriculture, 2018) as their use for official control is regulated in both, in the EU (European Commission, 2014) and in the USA (United States Department of Agriculture, 2015, 2017).

Detection methods for mycotoxin contamination by non-destructive approaches have been proposed using different technologies. Vibrational spectroscopic methods (e.g. infra-red) focus not directly on the presence of the mycotoxin itself, but on co-occurring changes within the fungal infested material (Sieger et al., 2017; Lee et al., 2015; Fox and Manley, 2014). Also sound waves are used to identify specific changes in the kernel structure indicating mycotoxin presence after fungal infestation (Juodeikiene et al., 2014). An important application of spectroscopic instrumentation is its use in machine sorting, which is widely used in cereal and dried fruit (nuts) industry (Stasiewicz et al., 2017).

A comparably simple visual screening method is the so called bright greenish yellow fluorescence (BGYF) induced under ultra-violet light by a mercury lamp with 366 nm wavelength. The observed fluorescence associated with the presence of other fungal metabolites like kojic acid produced from *A. flavus* or *A. parasiticus* indicates the likelihood of aflatoxin presence (da Gloria, 2011; Hrsuka et al., 2017; Shotwell and Hesselstine, 1981).

The development of analytical methods for mycotoxins is annually followed up since more than a decade in a series of reviews critically evaluating new methodologies with respect to their use in the analytical laboratory analyzing food and feed for mycotoxins in routine control and research (Berthiller et al., 2017).

In order to derive sound conclusions from analytical methods for risk assessment as well as legal enforcement of regulatory limits the availability of standardized methods with validated performance characteristics for precision and trueness is a principal requirement.

Standardized methods with such validated performance characteristics are provided by various standardization bodies like the European Committee for Standardization (CEN), the International Standardization Organisation (ISO) and AOAC International and the inventory of methods covers all mycotoxins of public health concern.

Furthermore tools to monitor the implementation of analytical methods within testing laboratories are important (called proficiency tests). They allow to prove the proper implementation of analytical methods and are a cornerstone in the accreditation of testing laboratories according to ISO 17025.

Proficiency test schemes for the determination of mycotoxins in food and feed are provided by a number of organisations focusing on national and international test laboratories and are usually open to all interested testing laboratories.

## **Mycotoxin Prevention/Detoxification**

The main strategy to combat the presence of mycotoxins in food and feed is prevention. Numerous approaches ranging from improved agricultural practices to traditional breeding for resistant plant varieties and genetic engineering (= novel breeding technologies) have been applied with success. Monitoring weather conditions that favor fungal growth were realized by meteorological

data maps, predicting the occurrence of mycotoxin and consequently allowing punctual combat strategies (Schaafsma and Hooker, 2007). The field application of non-toxic competitive fungi is another strategy (Accinelli et al., 2018).

Mycotoxins are fairly stable along the food production chain. However there are processing techniques that can lead to a significant degradation resulting in a lower toxic potential (Estiarte et al., 2018), while not all degradation products are non-toxic *per se*. Fumonisin and aflatoxins undergo massive degradation during nixtamalization, a traditional treatment of corn with lime or ash. It is originated from Latin America and initially applied to improve the processing and sensory properties of corn flour (Voss et al., 2017).

Aflatoxins in feed can be largely reduced at industrial scale with ammonia, while this treatment is however not totally benign as it also lowers the nutritional value of the feed (Hoogenboom et al., 2001; Bailey et al., 1994). In recent years a number of feed additives have been tested for their ability to lower the toxic effect of mycotoxins in feed. Such products target different approaches, like binding mycotoxins such as the aflatoxins and making them unavailable for digestion (Wang et al., 2017) or detoxifying mycotoxins like fumonisins during digestion by enzymatic degradation (Schwartz-Zimmermann et al., 2018). As European legislation requires an authorization for such products several have been authorized in the European Union (European Union, 2013, 2014) and are marketed under tradenames like FumEnzyme® or MycoFix®.

In literature such additives are often referred to as “mycotoxin binders” (Kolossova and Stroka, 2001). An alternative attempt to reduce the toxicity of aflatoxins is chemoprevention, which targets the *in vivo* production of aflatoxin B1 epoxide (Bolton et al., 1993).

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- United States Department of Agriculture – Grain Inspection, Packers and Stockyards Administration (GIPSA), <https://www.gipsa.usda.gov/fgis/mycotoxins.aspx>
- The European Commissions Rapid Alert System for Food and Feed (RASFF), [https://ec.europa.eu/food/safety/rasff\\_en](https://ec.europa.eu/food/safety/rasff_en)
- Society for mycotoxin research, <http://www.mycotoxin.de/docs/public/home.asp>
- The European Union Reference Laboratory for mycotoxins and plant toxins, <https://www.wur.nl/en/Expertise-Services/Research-Institutes/rikilt/Reference-laboratory/European-Reference-Laboratory/EURL-mycotoxins-plant-toxins.htm>
- The European Food Safety Authority, <https://www.efsa.europa.eu/de/topics/topic/mycotoxins>

# Occurrence & Risk of OTA in Food and Feed

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## Introduction

Ochratoxin A (OTA) is a ubiquitous mycotoxin produced mainly by fungal species of the genera *Aspergillus* and *Penicillium*, detected worldwide in various food and feed sources and represents a potential human health hazard. OTA is nephrotoxic and is suspected of being the main etiological agent responsible for human Balkan endemic nephropathy (BEN) and associated urinary tract tumours. International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B). The mycotoxin ochratoxin A (OTA) is the most important and most common member among several structurally related ochratoxins. It is a secondary metabolite produced mainly by the following three fungal species *Aspergillus alutaceus* (formerly known as *A. ochraceus*), *A. carbonarius*, and *Penicillium verrucosum* (EFSA, 2006).

Cereals and cereal by-products constitute a major part of the daily diet of the human and animal populations. The total annual yields of cereals globally add up to more than 2000 million tons. Because they are easy to package and transport, they are used for producing a large variety of highly desirable foods, beverages and feed.

However, an investigation on a worldwide scale showed that 25% to 40% of cereals are contaminated by mycotoxins (El Khoury and Atoui, 2010).

Due to high stability, mycotoxins represent an important problem not only during cereal grain production in field, but also in storage, transport, processing and post-processing steps. This contamination can occur in several times, in the field and/or during storage.

It is especially in the countries with hot and wet climatic conditions (in particular African countries, South Asia and South America) that the growth of toxigenic filamentous fungi is most favoured. Thus, rice, corn, and millet, the basic foods of the populations of these countries, are often contaminated by ochratoxins (Nguyen et al., 2007).

## Occurrence in Food

The main OTA sources included cereals, spices, coffee, tea, cocoa, wine and beer. Thus, worldwide, cereals are considered as being the major source of OTA contamination, where 50% of human daily intake of this mycotoxin is due to the consumption of different cereals derived products including rye-wheat bread, multigrain bread, cereal porridge, fine bakery wares, breakfast cereals, sweet biscuits and muesli. Due several toxicological effects, the analysis of OTA content in foodstuffs is need. The control at all stages of product processing need accurate and sensitive analytical methods (Table 1).

In the recent Spanish study, the OTA occurrence was 0.72 ng/g in corn-based breakfast cereals, 0.29 ng/g in wheat-based breakfast cereals and 0.28 ng/g in loaf bread (Coronel et al., 2012). A Lebanese report show that the food products exhibiting the highest concentration of OTA were cereal-based products, in particularly, "biscuits and croissants" (mean OTA concentration 2.8 ng/g) (Raad et al., 2014). OTA was detected in samples of breakfast cereals collected in France, with OTA in the range of 0.2–8.8 ng/g. The highest concentration was determined in a sample containing dry fruit and bran (Moliné et al., 2005). Regarding contamination of bread, Duarte et al. (2010) compiled the data on the occurrence of OTA in different types of bread worldwide. The mean values of positive samples of wheat bread ranged from 0.07 ng/g in Switzerland to 13 ng/g in Morocco, although they are mostly below 0.50 ng/g (Legarda and Burdaspal, 2001; Zinedine et al., 2007).

Wine, together with cereals, represents the second largest source of OTA human intake (4.7%–29.7%) and several surveys were conducted to assess the relevance of the presence of this mycotoxin in wine and grape products (Silva de Abreu et al., 2016). The presence of OTA in the grape can be shifted from grain grapes to wine during its brewing. Zimmerli first detected OTA in wines (1996), since then, the presence of OTA in wines has been reported from a number of European and other countries wines,

**Table 1** Occurrence in different food and maximum levels of OTA

Cereal and cereal products		≤ 0.01–13 ng/g
Beer		≤ 0.01–0.50 ng/mL
Wine	Dessert wine	≤ 0.01–15.25 ng/mL
	Red wine	≤ 0.01–7.63 ng/mL
Cocoa and cocoa products		0.25–7.8 ng/g
Milk		5–8 ng/L
Spices		≤ 0.01–98.2 ng/g
Coffee (roast and ground)		0–10 ng/g
Herbal teas (licorice root)		1.4–252.8 ng/g

with an apparent increase in levels in wines originating from southern areas of Europe (Remiro et al., 2013; Bellver Soto et al., 2014; Di Stefano et al., 2015a,b).

OTA levels in wine depend on different factors such as vineyard location (latitude), weather (rain, temperature, relative humidity in the vineyards), period of harvest, pesticide treatments and the wine brewing technique employed. Currently, EU legislation permits a maximum of 2 ng/g of OTA in wine and grape juice (EC n°1881/2006); this limit does not apply to liquor or dessert wines with more than 15% alcohol content.

Many recent works highlight the presence of considerable levels of OTA in wines, musts and grape juices (up to 7 ng/mL).

OTA levels usually are higher in red than in rose wines, following by the whites, mostly due to the must maceration with the skin of the grain grape, which could enhance OTA extraction. In sweet or special wine, wine making practices and the temperatures reached promote growth of OTA mould producers.

It is difficult to compare OTA levels in wines from different countries due to the great variety of wines analysed and the differences between the limits of detection used in each analytical method.

However, red and sweet wines seem to be the most contaminated; in particular wine samples from southern regions presented higher incidences and concentrations of OTA, as consequence of the warm temperatures and humid weather conditions, which favour fungus growth and mycotoxin's production. In any case, due to their toxicity, a low but continued dietary exposure to OTA could contribute to a risk to consumers, so its level in wine should be as low as reasonably achievable (Di Stefano et al., 2015a,b).

OTA has also been detected in other beverages such as beer. The origin of OTA mycotoxin in beer comes from contamination of prime material used for its processing as barley, malt, or cereal derivatives. Contaminated barley grains contain ochratoxigenic strains, which are transferred to beer. Currently EU Commission has not yet fixed the maximum admitted level of OTA concentration in beer. Recent monitoring about OTA content in beers was performed and analysed samples showed concentration levels below 0.2 ng/mL (Mateo et al., 2007).

OTA contamination occurs also in cocoa and chocolate-based products. There are only a few publications concerning the analysis of OTA in cocoa and in cocoa products; in one of these studies levels of ochratoxin A in cocoa and cocoa products available in Canada showed an incidence of 100%, with concentrations ranging from 0.25 to 7.8 ng/g (Turcotte and Scott, 2001). Another study of ochratoxin A occurrence was carried out by Serra Bonvehí (2004), where 138 samples of cocoa by-products (cake, mass, shell, nibs, butter and powder) from some cocoa producing countries (Indonesia, Ivory Coast, Ghana, Malaysia, Nigeria, Ecuador, Honduras and Peru) were analyzed. 120 (87%) samples had OTA above the detection limit (0.1 ng/g). The highest contamination was found in shell (11 ng/g, followed by cake (2.6 ng/g) and powder (2.41 ng/g). At the present time, there are no EU limits for cocoa and cocoa products, although these are presently under consideration (EC n°1881/2006). Recently, the European Commission stated that '*on the basis of the information available, it does not appear necessary for the protection of public health to set a maximum level of OTA in ... cocoa and cocoa products ...*' (Commission Regulation No. 105/2010). It is concluded that cocoa does not represent a major source of ochratoxin A in the diet. However, one concern is the fact that chocolate-containing products are widely consumed by children who are more sensitive to the effects of mycotoxins. Thus, it is important that constant monitoring should be carried out of their occurrence and also to find ways to prevent the contamination in the cocoa production chain.

OTA may also occur in milk. However, there is a paucity of information on the rate of transfer of this toxin into milk for dairy cows. In dairy sheep, the carryover is less than 1%. In a survey conducted in the northwest of France in 2003, Boudra et al. (2007) evaluated the presence of OTA in bulk milk from farms based on corn silage and cereal grains. OTA was detected in three milk samples at low levels, 5–8 ng/L, that do not appear to pose any particular risk to milk consumers.

## Risk Assessment

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a Provisional Tolerable Weekly Intake (PTWI) of 100 ng/kg bw/week (equivalent to 14.3 ng/kg bw/day), derived from a lowest-observed-adverse-effect level (LOAEL) of 8 µg/kg bw/day that caused minimal kidney changes in pigs (JECFA, 2001, 2008).

The European Food Safety Authority (EFSA) established a Tolerable Weekly Intake (TWI) of 120 ng/kg bw/week (equivalent to 17 ng/kg bw/day), derived from the same pig LOAEL but applying a slightly different uncertainty factor (EFSA, 2006). It reconfirmed this in 2010 (EFSA, 2010).

Health Canada has established a TDI of 3 ng/kg bw/day based on a benchmark response of 10% above background from the same pig study.

EFSA (2006) estimated total dietary exposures of 2–3 ng/kg bw/day (ca. 15–20 ng/kg bw/week) and 6–8 ng/kg bw/day (ca. 40–60 ng/kg bw/week) for average and high adult consumers (97.5th percentile) respectively. It was noted that exposure could be higher amongst children and certain consumer groups with preferences for particular foods.

RIVM (2009) estimated that >20% of 2- to 6-year-olds in the Netherlands exceed EFSA's tolerable daily intake (TDI) of 17.1 ng/kg bw/day (as derived from the TWI). This was the case in about 40% of 2-year-olds and 11% of children aged 4 and above. At worst the TDI was exceeded by up to four times.



## Regulations (EU)

Within the EU, maximum levels for OTA are defined for several raw materials. For example, in the spices and licorice categories a limit of 20 ng/g for licorice root has been set ([Commission Regulation No. 105/2010](#)); 15 ng/g for white and black pepper, ginger, nutmeg, and turmeric ([Commission Regulation No. 2015/1137](#)); 20 ng/g for dried chilies, chili powder, cayenne, and paprika; and 15 ng/g for mixtures containing one or more of the aforementioned spices.

The Scientific Commission of the European Community have regulated the maximum levels of OTA for unprocessed cereals (5 ng/g), products derived from cereals (3 ng/g) and processed cereal-based foods and baby foods for infant and young children (0.5 ng/g) ([EC n°1881/2006](#)).

## Occurrence in Animal Feed

The feed supply chain is a crucial element for all livestock production systems. Cereals and cereal byproducts constitute a major part of the daily diet of animals and are important ingredients in animal compound feed. Mycotoxins represent a significant risk to animal health and are a significant issue for a safe feed supply chain.

In animal feeding, the contribution of forages to total mycotoxin intake could be significant. Recent studies reported not only that preserved forages, like silage, can be an important source of mycotoxins in ruminant diet, but also that fresh forage and/or pasture can be a route of exposure to these contaminants.

A high incidence of multi-mycotoxin contamination has been reported in feed. Co-occurring mycotoxins may exhibit additive or synergetic toxic effects well documented by many studies and represents an extremely common problem in feed industry world that should be routinely monitored ([Smith et al., 2016](#)).

Moreover, globalized feed grain trade may distribute mycotoxins outside of their natural occurrence geographical areas, complicating the prediction of mycotoxin contamination in compound feed. OTA maximum levels and guidance values in animal feed have been set in Commission Directive 2003/100/EC and Commission Recommendation 2006/576/EC.

In a study carried out in Spain with a large number of ingredients sampled from the same feed mill, were found levels of OTA above the levels recommended by the EU legislation (<50 µg/kg) in a reduced number of samples of maize (up to 225 µg/kg) and barley (up to 90 µg/kg) ([Espada, 2008](#)).

In Brazil, [Rosa et al. \(2009\)](#) described the OTA occurrence in corn, brewers grain and finished swine feed samples collected from different factories. Corn samples (44%) were contaminated with 42–224 µg/kg of OTA. The animal feed (31%) and samples of brewers grain (13%) were contaminated with 36–120 µg/kg and 28–139 µg/kg of OTA, respectively.

Ochratoxin-contaminated feed has its major economic impact on the poultry industry. Chickens, turkeys, and ducklings are susceptible to this toxin. Clinical signs of avian ochratoxicosis generally involve reduction in weight gains, poor feed conversion, reduced egg production, and poor egg shell quality. Economic losses also occur in swine farms, linked to nephropathy and costs for the disposal of carcasses. Toxicity does not seem to constitute a problem in cattle, because OTA is rapidly detoxified by rumen protozoa and bacterial enzymes into less toxic metabolites.

OTA amount in animal feed varies from country to country. The highest amounts have been reported in Northern Europe and North America. Specifically, the highest frequencies were described in Denmark (57.6%), Canada (56.3%) and Yugoslavia (25.7%), showing isolated samples with values of OTA contamination above 5000 ng/g ([WHO, 2002](#)).

The impact of mycotoxins entering the feed supply chain could increase in the future. Most predictions indicate that climate change scenarios, with global warming, could affect agriculture and increase and change the threat of fungal invasion of crops.

## Conclusion

Currently, data on the natural occurrence of Ochratoxin A in various food products and animal feed in different regions of the world are still scarce and make a final risk assessment impossible.

Emerging evidence, however, suggests an effect of climate and, thus, climate change including increases in temperature and other related variables (e.g., humidity/moisture) to the occurrence of mycotoxins in agricultural products.

The scientific community has done a lot in the last years, to identify and quantify the mycotoxins in food, clarify their destination in the plant, during processing or through the metabolism of microorganisms and animals, as well as possible health effects.

More generally, however, the current study reinforces the need for regular surveillance of OTA in agricultural products, as well as investigation of factors which drive OTA contamination.

Data on the possible interactions between different mycotoxins are still limited or non-existent, especially through high consumption food products, and is expected to result in greater human health problems. The need a regular monitoring of highly consumed foodstuff, is suggested.



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## Occurrence & Risk of Aflatoxins in Food and Feed

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### Introduction

Aflatoxins were discovered after the sudden death of turkeys in England in spring 1960. As the cause of the disease was a mystery for some time, it was called "Turkey X-disease". As the number of casualties raised quickly over 100,000 and the mortality rate was almost 100%, thorough investigations were started promptly. The affected turkeys looked to be in good condition, just until right before their sudden death, when they showed nervous behaviour before falling into coma and collapsed. After autopsy some necrotic tissue was discovered in the liver, clearly indicating exposure to poison. Looking for the possible intake of aflatoxin the feed chain was traced back from the affected farms, revealing peanut meal being imported from Brasil to be the most likely source, as it glowed up when exposed to UV light, suggesting contamination by molds.

Detailed investigations lead to the conclusion the peanut meal was contaminated with fungi from the genus *Aspergillus*, specifically the species *Aspergillus flavus* and *Aspergillus parasiticus*. Further investigations revealed the chemical compounds which were responsible for the death of the turkeys. These toxins were named aflatoxins, after the mold *A. flavus*, which was responsible for the most toxic one: aflatoxin B<sub>1</sub> (AFB<sub>1</sub>). Its chemical structure is given in Fig. 1. Related aflatoxins are aflatoxin G<sub>1</sub>, aflatoxin G<sub>2</sub> and aflatoxin M<sub>1</sub>. Aflatoxins B are named after the blue color when seen in fluorescent light, whereas aflatoxins of the G type show greenish-yellow light in similar conditions. Aflatoxin M<sub>1</sub> is a metabolite which is produced in humans and animals. Exposure by aflatoxin B<sub>1</sub> due to consumption of contaminated food or feed leads to formation of the metabolic version aflatoxin M<sub>1</sub>, which is excreted in urine. In the female sex it can be transferred via the placenta to the foetus, thus exposing the unborn generation already with aflatoxin during pregnancy of the mother and thereafter by mother's milk as well.

### Contamination

Aflatoxins are regularly measured in nuts, dried fruits, spices, oil seeds, cereals, corn, rice and milk. Worldwide, aflatoxins are most frequently found in peanuts, pistachios, hazelnuts, Brazil nuts, figs, nutmeg and corn. Contamination can occur before harvest, during the time between harvesting and drying, and in storage. Contamination of milk by aflatoxin M<sub>1</sub> occurs when cows are fed with feed which is contaminated with AFB<sub>1</sub>, for example contaminated corn or peanut cake of poor quality. All contamination of a commodity starts with favorable conditions for mold growth. The crucial factors which primarily cause mold growth are moisture, temperature and pests. Molds can grow over a wide range of temperatures, so as well under field conditions as well during storage. In general, the rate of mold growth will decrease with decreasing temperature and lower

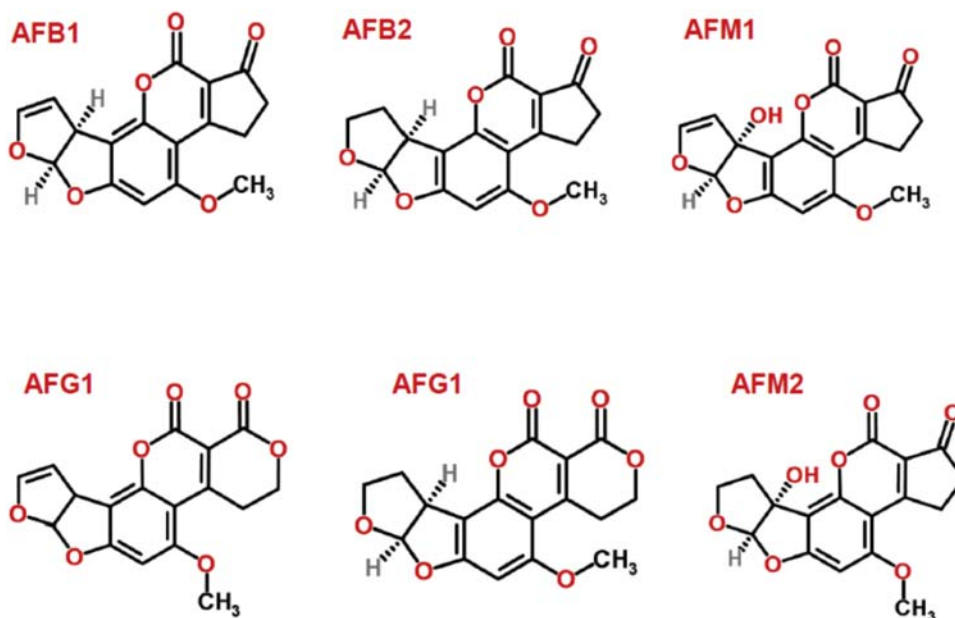


Figure 1 Chemical structures of aflatoxins ([www.chemspider.com](http://www.chemspider.com)).

moisture content of food or feed. In this respect water content in the commodities is specified by the water vapour in between the food kernels (i.e. grains or nuts). This differs from the moisture content of the kernels, as moisture content is water which is bound/included in the kernels itself. Molds grow by utilising intergranular water vapour, the concentration of water which is in equilibrium between free water within the kernels and the water vapour in the actual environment of the kernels. This water concentration is described either in terms of the relative humidity (%) or water activity ( $a_w$ ) at a certain temperature. The latter describes the ratio of the vapour pressure of water in the kernel to that of pure water at the same temperature. The relative humidity is equivalent to the water activity expressed as a percentage, calculated by  $a_w \times 100\%$ . For a given moisture content, different commodities vary by water activity and thus can express different mold type and growth rate. Water activities which are necessary for mold growth range from 0.77 to 0.90, with a tendency for mold growth increasing with temperature. The water activity for growth of *A. flavus* mold ranges from 0.78 to 0.84. Aflatoxin production starts above  $a_w = 0.84$ . The higher the water activity the larger the production of aflatoxins. *A. flavus* grows in the temperature range of 10–43 °C. The optimal growth rate occurs around 30 °C. Aflatoxins are produced at the temperature range 15–37 °C. Aflatoxin production between 20–30 °C is greater than at higher or lower temperatures. The effect of water activity and temperature on mold growth of *A. parasiticus* is similar to that of *A. flavus*. Mold growth of *A. parasiticus* requires an  $a_w$  of 0.84, whereas aflatoxin production starts at an  $a_w$  of 0.87. The effect of temperature on the growth of *A. parasiticus* and the production of aflatoxins is optimal at approximately 28–30 °C.

Aflatoxin contamination can occur pre-harvest when the crop plant is growing or post-harvest during processing, packaging, distribution and storage of commodities. Generally, all crops and cereals that are improperly stored under favourable temperature and humidity conditions for a prolonged time can be subject to mold growth and aflatoxin contamination. Maize is considered to be the crop relatively susceptible to aflatoxins contamination, while rice is the least. In practice this means, for example, that corn can be safely stored for 1 year at a moisture level of 15% and a temperature of 15 °C. However, when stored at 30 °C, the same corn will be significantly affected by molds within 3 months. The presence of molds does not guarantee that harmful aflatoxin levels are present, but indicates a significant risk of contamination by aflatoxins. Corn can also be affected by *A. flavus* in the field, when conidia from soil-inhabiting organisms are carried by air into the silks of the corn plant, where under suitable environmental conditions infections of corn ears can occur. Other commodities, like peanuts, are prone to infection at high temperatures and low-moisture levels, commonly known as drought stress. Irrigation supplies water and lowers soil temperature as well, which allows for normal plant development and greatly reduces aflatoxin risk. Water is also the best insect control available in that it makes the peanut plant much less susceptible to pests such as cornstalk borer, burrower bugs, corn earworm and spider mites. In pistachios another phenomenon favours aflatoxin contamination, as the hulls can split prior to maturity of the nuts, creating an open portal of entry for fungi and also may involve damage by insect larvae.

## Adverse Health Effects

Aflatoxins have carcinogenic, teratogenic, hepatotoxic, mutagenic and immunosuppressive effects, with the liver being the target organ. Aflatoxins are associated with both acute toxicity as well as chronic carcinogenicity in humans and animals. The International Agency of Research on Cancer (IARC, 2012) classified aflatoxin B<sub>1</sub> as a Group 1 carcinogen, with high risks for hepatocellular carcinoma in individuals exposed to aflatoxins. Acute toxicosis, usually rare in developed countries, can occur in developing countries, whereas chronic carcinogenicity is a global problem. The LD<sub>50</sub> values range between 0.5 and 10 mg/kg body weight in different animal species. In humans, acute aflatoxicosis is characterized by vomiting, abdominal pain, pulmonary and cerebral edema, coma, convulsions and even death. In animals, symptoms of gastrointestinal dysfunction, reduced reproduction, reduced feed conversion and efficiency, lowered milk and egg production and anemia have been reported. The toxic effects of AFB<sub>1</sub> are principally due to the binding of bioactivated AFB<sub>1</sub>-8,9-epoxide to cellular macromolecules, particularly mitochondrial and nuclear nucleic acids and nucleoproteins, resulting in cytotoxic effects. The double bond in the furan ring of AFB<sub>1</sub> and AFG<sub>1</sub> can be oxidised and form a reactive 8,9-exoepoxide that readily reacts with DNA and other nucleophiles. In DNA, the AFB<sub>1</sub>-N7-guanine is formed, which may subsequently lead to transversion mutations. Co-exposure to hepatitis viruses, in particular hepatitis B has a strong impact on the carcinogenic risk to aflatoxins. In epidemiological studies, there is interaction with hepatitis B infection, and subjects positive for hepatitis B surface antigen (HBsAg) show at least a multiplicative risk when present together with aflatoxin exposure (FAO/WHO, 2017). After entering the body, aflatoxins are metabolized by the liver to a reactive epoxide intermediate or hydroxylated to become the less harmful aflatoxin M<sub>1</sub>, which will be excreted in the urine. Aflatoxin M<sub>1</sub> as such is less harmful when compared to aflatoxin B<sub>1</sub>. Therefore IARC classified it in Group 2B, possibly carcinogenic to humans. Human exposure to aflatoxin M<sub>1</sub> occurs primarily via consumption of milk and milk products from animals that have consumed contaminated feed (Anonymous, 2004). It has also been found in human breast milk.

Outbreaks of aflatoxicosis directly affecting humans are rare. The first one, reported in India, led to the death of 100 people in 1974. In April 2004, one of the largest aflatoxicosis outbreaks occurred in rural Kenya, resulting in 317 casualties and 125 deaths (Lewis et al., 2005). Aflatoxin-contaminated homegrown maize was the source of the outbreak. The extent of market maize contamination revealed that 55% of maize products had aflatoxin levels greater than the Kenyan regulatory limit of 20 µg/kg, 35% had levels above 100 µg/kg and 7% had levels higher than 1000 µg/kg. Another incident occurred in Serbia, after the country had been affected by severe drought during 2012, causing an increased prevalence and high levels of aflatoxin contamination in maize for feed, reported to have a mean contamination level of 36.6 µg/kg, with 53.5% of the samples above 10 µg kg<sup>-1</sup> (Torović, 2015).

Due to this contamination, in 2013 and 2014 aflatoxin M<sub>1</sub> was determined in 80 samples of milk and 21 samples of infant formulae on the Serbian market. All milk samples collected in 2013 showed AFM<sub>1</sub> contamination in the range 0.02–0.32 µg/kg, with a mean level of 0.13 µg/kg, 75% of the samples exceeding the EU maximum limit for AFM<sub>1</sub> in milk of 0.05 µg/L. In 2014, AFM<sub>1</sub> was found in 83%, 70%, 80% and 58% of the samples collected in April, July, September and December, respectively, where only 5% of the samples taken in July exceeded the limit.

## Legislation

Due to the hazardous effects of aflatoxins almost every national and international public health and governmental authorities such as the US Food and Drug Administration (FDA), World Health Organization (WHO), Food Agriculture Organization (FAO) and the European Food Safety Authority (EFSA CONTAM Panel et al., 2018), have compiled risk assessment documents regarding contamination in food and feed. To achieve as harmonized limits as possible in the world, since 1956 the Joint FAO/WHO Expert Committee on Food Additives (JECFA), an international expert scientific committee that is administered jointly by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) was initiated. They publish risk assessments which are applied by the Codex Committee on Contaminants in Food (CCCF) to set maximum limits. The Codex maximum limit (ML) for a contaminant in a food or feed commodity is the maximum concentration of that substance recommended by the Codex Alimentarius Commission to be legally permitted in that commodity. As many contaminants occur naturally it would be impossible to impose a zero limit on these substances. To protect human health Codex works to keep these levels as low as possible based on sound scientific evidence. In case of contaminants which are considered to be genotoxic carcinogens or in cases where current exposure of the population or of vulnerable groups in the population is close to or exceeds the tolerable intake, maximum limits should be set at a level which is as low as reasonably achievable (ALARA). Such approaches ensure that food business operators apply measures to prevent and reduce the contamination as far as possible in order to protect human and animal health. It is furthermore appropriate for the protection of infants and young children, a vulnerable group, to establish the lowest possible maximum limits, which are achievable through a strict selection of raw materials used for manufacturing of baby food. These considerations resulted in adopting strict regulatory maximum limits for aflatoxin B<sub>1</sub> in most countries in the world on the presence of aflatoxins in food and feed from as low as 0.1 µg/kg in baby food to 8 µg/kg for almonds, pistachios and apricot kernels, intended for direct human consumption or use as an ingredient in foodstuffs. For total aflatoxins, the limits for the sum of aflatoxin B<sub>1</sub>, aflatoxin B<sub>2</sub>, aflatoxin G<sub>1</sub> and aflatoxin G<sub>2</sub> range from 4 to 20 µg/kg. For feed the limits depend on the target animal and vary between 5 and 20 µg/kg for aflatoxin B<sub>1</sub> in feed with a moisture content of 12%. The lowest value, for lactating animals, will result in milk which fulfills the EU limit of 0.050 µg/kg for aflatoxin M<sub>1</sub>, as the metabolism in the cow has a conversion factor of 100. Codex set the limit for aflatoxin M<sub>1</sub> at 0.50 µg/kg, so 10 times higher.

## Sampling

The most crucial step in the analysis of aflatoxins in food is the sampling procedure, which greatly contributes to the reliability of the results and the final decision on compliance or non-compliance for an entire lot or batch. This is due to the fact that mold growth originates only from just those spots in a field or storage where the moisture and temperature are optimal for aflatoxin production. Therefore the contamination is very heterogeneous. Except for liquid commodities such as milk or processed food, i.e. peanut butter, sampling methods which are applied for residue analysis or for checks on nutritional composition of food are not suitable for aflatoxin analyses, as contamination is not homogeneously distributed in grains, nuts, spices or dried fruits. Basic sampling procedures are described in detail in European Commission Regulation (EC) No. 401/2006 and in the standard ISO 24333: 2009 of the International Standardization Organisation. A typical sampling schedule for a 25 tonnes truck load of cereal grains requires taking 100 incremental samples of 100 g according to the EC sampling plan and 20 incremental samples of 500 g according to the ISO schedule, in both cases revealing an aggregate sample of 10 kg to be delivered in the laboratory. In both cases a laboratory sample has to be taken from the homogenized 10 kg. In case of dispute an arbitrary subsample best be taken of the homogenized laboratory sample, which is another challenge, as the subsample for analysis has to be representative for the laboratory sample. Any procedure which does not take the heterogeneity of the contamination into account will lead towards a questionable outcome of the analysis.

## Analysis

Chromatography is the most commonly used technique for aflatoxin analysis in food and feed. The earliest chromatographic method is thin layer chromatography (TLC), which is a rapid screening method for aflatoxins by visual assessment or instrumental densitometry. However, current trends in aflatoxin analysis in food are focused on application of robust, fast, easy to use and cheap technologies that are able to detect and quantify aflatoxins with a high sensitivity and selectivity in a single test. To meet those needs, many chromatographic methods such as HPLC coupled with fluorescence (FLD) or mass spectrometry (MS) detectors have been developed. Rapid tests are more commonly applied when quick screening is required in the field or in industrial processing procedures.

Enzyme-linked immunosorbent assay (ELISA) provides easy testing, with many validated kits commercially available for detection and quantification of aflatoxins. If necessary positive ELISA results could be confirmed by a suitable chromatographic method, especially when used in a matrix not specified by the manufacturer of the assay. For any test has to be kept in mind how representative is the single drop of the obtained extract, as applied in the test, for taking a decision on a complete field, silo, truck or ship load.

## Prevention

Aflatoxins are chemically and thermally stable during food processing, including cooking, boiling, baking, frying, roasting and pasteurization. Once these compounds are present in food or feed it is very difficult to eliminate them. Several efforts have been made to find a chemical decontamination process, without very much success. Only for feed the use of ammonia has gained some industrial application in Brazil, Mexico and some States in the USA (i.e. in Texas). Another option is to inactivate aflatoxins by means of binding the compounds to adsorbing agents, which are excreted by the animal. The critical issue there is whether the bound aflatoxin is not released by the binding agent, for example in the acidic stomach content. After a long period of scientific investigations about inactivation agents (binders) the European Commission concluded that bentonite has the ability to reduce Aflatoxin B<sub>1</sub> contamination in pig, poultry and ruminant feed (EC No 1060/2013). Chemical decontamination of food is forbidden anywhere in the world. For food only sorting out infected kernels is a safe option to reduce aflatoxins. Optical sorting technology is available to reject discoloured, diseased and damaged seeds, nuts, grains, rice, pulses, fruits and spices ([www.buhlergroup.com/optical-sorting](http://www.buhlergroup.com/optical-sorting)).

All together the best way to avoid contamination is prevention. Therefore a number of control strategies for aflatoxin contamination in crops are investigated: controlling preharvest drought stress, breeding programs for heat and pest resistant varieties and assessing potential biocontrol agents. Due to the uncontrollable weather conditions, cultivation of a crop usually encounters a period of drought and stress during growing, flowering and kernel development. The weather conditions are the major factors for increased aflatoxin contamination. Irrigation will reduce drought stress and lower the soil temperature as well. Other factors that have to be taken care of by farmers are plant nutrition (fertilisers), manage plant diseases (fungicides), pest control (pesticides), weed reduction (herbicides) and avoid excessive plant density to achieve better ventilation in the field. An additional option is to apply crop rotation. Growing sugar beet after harvesting peanut will reduce the amount of *Aspergillus species* in the soil, thus giving a better starting point for the next peanut crop. Involving corn in this circulation process is not an option, as corn is easily affected by mold, which will affect any next crop, even after extensive tillage. The Codex Alimentarius Website (<http://www.fao.org/fao-who-codexalimentarius/codex-texts/codes-of-practice/en/>) gives codes of practice for prevention and reduction of aflatoxin contamination in peanuts (CAC/RCP 55-2004), in tree nuts (CAC/RCP 59-2005), dried figs (CAC/RCP 65-2008) and for reduction of aflatoxin B<sub>1</sub> in raw materials and supplemental feeding stuffs for milk producing animals (CAC/RCP 45-1997).

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## Further Reading

Internet: [https://ec.europa.eu/food/safety/chemical\\_safety/contaminants\\_en](https://ec.europa.eu/food/safety/chemical_safety/contaminants_en); this site gives access to legislation, EFSA publications, contamination levels registered in the rapid alert system for food and feed, guidance documents; <https://www.mytoolbox.eu/> and <http://www.mycokkey.eu/>, these websites give information about EU projects with international participants regarding prevention and research.



## Pesticide MRLs and Impact on Global Trade

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### Glossary

**Acceptable Daily Intake (ADI)** The ADI of a chemical is the daily intake which, during an entire lifetime, appears to be without appreciable risk to the health of the consumer on the basis of all the known facts at the time of the evaluation. It is expressed in milligrams of the chemical per kilogram of body weight.

**Acute Reference Dose (ARfD)** The ARfD of a chemical is an estimate of the amount of a substance in food and/or drinking-water, normally expressed on a body-weight basis, which can be ingested in a period of 24 hours or less without appreciable health risk to the consumer on the basis of all known facts at the time of the evaluation.

**Maximum Residue Level (MRL)** The MRL means the upper legal level of a concentration for a pesticide residue in or on food or feed set in accordance with the Regulation (EC) No 396/2005, based on good agricultural practice and the lowest consumer exposure necessary to protect vulnerable consumers (Article 3(2)(d) of Regulation (EC) No 396/2005). The terms 'maximum residue level' and 'maximum residue limit' are often used interchangeably.

**Codex MRLs (CXL)** The CXL means an MRL set by the Codex Alimentarius Commission (Article 3(2)(e) of Regulation (EC) No 396/2005).

**No Observable Adverse Effect Level (NOAEL)** The highest exposure level at which no adverse effects can be identified in tests.

**Pesticide** Pesticide means any substance intended for preventing, destroying, attracting, repelling, or controlling any pest including unwanted species of plants or animals during the production, storage, transport, distribution and processing of food, agricultural commodities or animal feeds, or which may be administered to animals for the control of ectoparasites. The term includes substances intended for use as a plant-growth regulator, defoliant, desiccant, fruit thinning agent, or sprouting inhibitor and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport. The term normally excludes fertilizers, plant and animal nutrients, food additives and animal drugs.

**Note:** Within the European Union the term 'pesticide' is solely used for substances used in PPPs.

### Introduction

Plant protection products (PPPs) are formulations containing one or more active substances and in most cases co-formulants in a solid, liquid or gaseous stage to protect plants or plant products. The application of PPPs on crops in agriculture, horticulture or on plant products during storage can result in measurable levels of pesticide residues in or on harvested products, but also in products of animal origin and processed products. These pesticide residues must not entail any risks for human and animal health and the environment.

The protection of consumers and the trade of the products were from the beginning the two main aspects in MRL setting. MRL setting started about 50 years ago on national level (in Germany: in 1966 for products of plant origin, in 1973 for products of animal origin) and international level (in the European Union (EU): in 1976 for fruits and vegetables with Council Directive 76/895/EEC, followed by 86/362/EEC, 86/363/EEC and 90/642/EEC). Since EU directives needed to be transposed at that time into national legislation, each Member State devised its own laws causing deviations that may hinder free trade within the EU.

As a result of numerous serious food safety incidents during the 1990s (BSE, residues and contaminants (dioxin), etc.), the European Commission launched a White Paper on Food Safety on 12th January 2000 (EC, 2000). In order to achieve the highest standards of food safety in the EU, the White Paper on Food Safety served as a start for a new legal basis for appropriate food and animal feed production and food safety control. It sets out a number of measures which "will enable food safety to be organized in a more coordinated and integrated manner"; these include:

- the establishment of an independent European Food Authority with responsibility for "independent scientific advice on all aspects relating to food safety, operation of rapid alert systems, communication and dialogue with consumers on food safety and health issues as well as networking with national agencies and scientific bodies"
- a new legal framework covering all sectors of the food production and distribution chain, including animal feed production, primary production, food processing, storage, transport and retail sale (integrated approach "from farm to table", also referred to as "from farm to fork", "from stable to table")
- a harmonization of national control systems
- a dialogue with consumers and information of emerging food safety concerns and risks



Therefore, in 2002, the European Parliament and the Council adopted [Regulation \(EC\) No 178/2002](#) laying down the general principles and requirements of food law and setting up the European Food Safety Authority (EFSA) as an independent agency responsible for scientific advice and support. In 2009, the [Regulation \(EC\) No 1107/2009](#) concerning the placing of plant protection products on the market was adopted. Concerning MRLs in or on food and feed of plant and animal origin, the [Regulation \(EC\) No 396/2005](#) was already implemented in 2005. This Regulation “directly concerns public health and is relevant to the functioning of the internal market” (recital 2 to [Regulation \(EC\) No 396/2005](#)).

## Regulation and Control of Pesticides in the European Union

A PPP cannot be placed on the market without prior authorization. In the EU, the placing of the PPPs on the market is based on a two-step procedure. The first step consists of the approval of the active substance, while in the second step the authorization of the PPP containing the approved active substance may be granted. Both procedures are principally regulated by the framework [Regulation \(EC\) No 1107/2009](#). The approval of active substances takes place at EU level, where EFSA prepares the final risk assessment of active substances and Member States vote on the approval. Only after the active substance is approved, the use of PPPs can be evaluated and authorized at Member States level. The period of authorization of a PPP is based on the period of approval of the active substances contained therein as it is limited (max. 10 years for first approval according to Article 5 of [Regulation \(EC\) No 1107/2009](#)). Three years before expiry of the approval, a producer of the active substance may submit an application for renewal of approval (Article 15 of [Regulation \(EC\) No 1107/2009](#)). Similarly, if an authorization holder wishes to continue to place its PPP on the market after the expiry date of its authorization, an application for renewal is required.

[Regulation \(EC\) No 1107/2009](#) describes requirements and procedures for approval of active substance and for the authorization of PPPs with involvement of applicant, Member States, EFSA, European Parliament and Council. As required by [Regulation \(EC\) No 1107/2009](#) (Article 4), an active substance can only be approved for use in PPPs when it has been found to be both effective for pest control and safe for human, animals and the environment. The data requirements for active substances and for PPPs are defined by Regulation (EC) No 283/2013 and by Regulation (EC) No 284/2013, respectively. In addition, further Guidelines and Guidance Documents provided by the European Commission, the EFSA and the Organization for Economic Co-operation and Development (OECD) may apply. The OECD Guidelines for the testing of chemicals provide about 150 of the most relevant internationally agreed testing methods to identify and characterize potential hazards of chemicals ([OECD, n.d. a](#)). Additional OECD Guidance Documents may help in interpretation. Guidelines and Guidance Documents used to fulfill data requirements are described in two Commission Communications attached to Regulations (EC) No 283/2013 and No 284/2013 (Commission Communication, 2013/C 95/01 and 2013/C 95/02). For harmonization of MRL calculations, the OECD has developed an OECD MRL calculator which has been adopted at international level and, therefore, on which MRL proposals should be based ([OECD, n.d. b](#)).

With the application for approval of active substance for placing PPPs on EU market, a complete list of tests and studies in accordance with Article 8 of [Regulation \(EC\) No 1107/2009](#) should be submitted by the applicant. The Member State (i.e. Rapporteur Member State (RMS)) submits a draft assessment report to the EFSA for risk assessment and expert consultation after which EFSA issues a conclusion. Based on EFSA’s independent risk assessments, the European Commission and Member States through the Standing Committee on Plants, Animals, Food and Feed (SCoPAFF) approve pesticides. Approved active substances are included into Regulation (EU) No 540/2011, the “positive” list of active substances approved for use in PPPs. Member States may authorize the use of PPPs containing active substances from this list. However, a PPP cannot be authorized without a fixed MRL. The establishment of a MRL at a level compliant with the envisaged use is one of the main requirements laid down in Article 29(1)(i) of [Regulation \(EC\) No 1107/2009](#).

A MRL for pesticides residue is a legally established maximum concentration of a particular active substance expected to be found in or on a particular food and/or feed commodity after use of the PPP in accordance with the label instructions (Good Agricultural Practice (GAP)). MRLs are set according to the principle “as low as reasonably achievable” (ALARA), following the main principle of using pesticides as much as necessary and as little as possible.

Since 1 September 2008, setting or amending of MRLs is regulated in the EU by [Regulation \(EC\) No 396/2005](#) replacing four Council Directives on MRLs which had to be transposed into national legislation before (Directives 76/895/EEC, 86/362/EEC, 86/363/EEC and 90/642/EEC). The main purpose of [Regulation \(EC\) No 396/2005](#) is to ensure a high level of consumer protection and to establish harmonized provisions relating to maximum levels of pesticide residues in or on food and feed of plant and animal origin. Additional provisions on import tolerances and Codex MRLs (CXLs) are necessary to facilitate international trade. As defined in Annex I to [Regulation \(EC\) No 396/2005](#), MRLs are set for raw agricultural commodities. To all active substances not mentioned in Annexes II, III, IV or V to the Regulation (and thus not approved in the EU), a default MRL of 0.01 mg/kg applies. This value represents a limit of quantification (LOQ) as residues have to be set as close to zero as possible.

[Regulation \(EC\) No 396/2005](#) lays down the rules and the procedure for setting MRLs with clear roles of the Member States, the EFSA and the European Commission. For setting appropriate MRLs, data in accordance with [Regulation \(EC\) No 396/2005](#) shall be submitted (toxicological studies, residue trials, and analytical methods), i. e. the same data requirements apply as set out in Regulations (EC) No 283/2013 and 284/2013 including the accompanying guidelines and guidance documents (s. above). The objective of residue trials, which have to be conducted in accordance with the proposed GAP, is to determine the maximum amount of pesticide residues in food and feed commodities at the time of consumption by humans and animals. Toxicological evaluation is

carried out on the basis of animal testing in order to obtain inter alia toxicological limits. The acceptable daily intake (ADI) and acute reference dose (ARfD) are calculated by dividing the no observed adverse effect level (NOAEL) - the highest dose without harmful effects - by an uncertainty factor of at least 100 to compensate for potential differences between animals and humans and for differences between individuals. The potential chronic (long-term) and acute (short-term) dietary exposure of consumers to pesticide residues are estimated using a calculation model of EFSA, the Pesticide Residue Intake Model (PRiMo). This model implements internationally agreed risk assessment methodologies published by the WHO (WHO, 1997) and contains national food consumption data provided by Member States and in addition WHO cluster diets (EFSA, 2018a). For establishment of MRLs, level of residues determined in trials, data on toxicology and food consumption are to be taken into account. Only if the amount of residues is acceptable in regard to health, i. e. if the estimated long-term and short-term exposure of consumers to residues remains below the ADI and the ARfD thresholds, and the residues can be determined by commonly available methods, a MRL can be set and afterwards an authorization for use of a PPP can be issued. The RMS evaluates the data submitted by the applicant and prepares an evaluation report concerning the necessary MRL. The EFSA prepares the final risk assessment and issues a reasoned opinion regarding the consumer safety of the proposed MRL. Provided that EFSA's risk assessment does not identify any unacceptable risks to consumers, the European Commission, through the SCoPAFF, finalizes the procedure by setting an EU-harmonized MRL and publishing a regulation on MRLs setting, amending the Regulation (EC) No 396/2005. After that, the PPP can be authorized at national level.

It is important to emphasize that MRLs are trading standards and not necessarily toxicological safety levels as they are usually set much lower than the levels that would pose a risk to consumers. Therefore, exceeding the MRL does not necessarily imply an adverse effect on human health. However, products/commodities with residues above the respective MRL must not be placed on the market as they do not comply with legal requirements. Exceedings of MRLs can occur for different reasons such as incorrect use of a PPP, use of authorized PPPs on unauthorized crops or contamination during storage and transport. Competent authorities in the Member States are responsible to enforce the safe use of PPPs.

In accordance with Regulation (EC) No 396/2005 (Article 26ff. to be changed in future by Regulation (EU) 2017/625 (Article 155)), official controls have to be carried out in each Member State, to ensure that food placed on the market is in compliance with afore-mentioned regulations. In each Member State, two control programs are in place for residues: an EU-coordinated control program with a number of samples analyzed each year by each Member States being proportional to their populations, and a national control program. The EU-coordinated control programs represent randomized controls and define pesticides and food products to be monitored by all Member States. In contrast, the national control programs are mainly risk-based as Member States define certain products which are expected to contain pesticide residues in concentrations exceeding MRLs, or products that may pose risks for consumer safety. The monitoring results of both control programs have to be submitted to the EFSA and the European Commission. The EFSA has to prepare an annual report on pesticide residues, analyzing the data in view of the MRL compliance of food products available on the EU market and the exposure of consumers to pesticide residues. The main purpose of the data analysis is to give the European Commission, acting as risk manager, the necessary information to decide on risk management policy issues.

Official Controls according to Article 68 of Regulation (EC) No 1107/2009 are aimed to monitor production, packaging, labeling, storage, transport, marketing, formulation, parallel trade and use of plant protection products. Member States have to report the results to the EU.

A potential risk to consumer health from food or feed is given when the exceeding of the MRL results in an exhaustion of the ARfD. In this case, the affected Member State circulates the information via the EU Rapid Alert System for Food and Feed (RASFF) – an online database designed to ensure the swift exchange of information between its members (28 EU Member States, EFSA, Commission, Norway, Liechtenstein, Iceland and Switzerland). Created in 1979, RASFF database provides a round-the-clock service to ensure that urgent notifications are shared quickly and allows the members to check whether they are affected and if a need for action is necessary. Food safety risks may be averted by taking the required measures such as giving information to the public and withdrawing the products from the market (EFSA, 2018b).

## Regulation and Control of Pesticides in International Trade

At the global level, MRLs are established by the Codex Alimentarius Commission (CAC). The CAC was founded by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). The statutes of the CAC, as stated in Article 1 (a) "protecting the health of the consumers and ensuring fair practices in the food trade" (CAC, 2016), were adopted in 1961 by the 11th Session of the FAO Conference and in 1963 by the 16th Session of the World Health Assembly. The Codex Alimentarius (Latin, meaning Food Code) is a collection of standards, codes of practice, guidelines, and other recommendations relating to foods, food production, and food safety. The CAC is responsible for providing safe and healthy food by setting internationally valid food standards. The founding of the World Trade Organization (WTO) and especially the implementation of the WTO "Agreement on the Application of Sanitary and Phytosanitary Measures" (SPS Agreement), increased the importance of the CAC organization's standards (FAO/WHO, 2018a).

The CAC is supported by an Executive Committee and several subsidiary bodies, referred to as Codex Committees, which prepare draft standards for submission to the Commission. Four different types of Committees exist, horizontal or General Subject Committees (10 Committees), vertical or Commodity Committees (6 Committees), ad hoc Intergovernmental Task Forces (one

Task Force) and Coordinating Committees (6 Committees), through which regions or groups of countries coordinate food standards activities in the region, including the development of regional standards (FAO/WHO, 2018b). The Codex Committee on Pesticide Residues (CCPR) is one of the general Committees. The work is always organized stepwise starting with a proposal and ending up with the adoption of the standard by the CAC. A few general Committees are supported by scientific bodies preparing evaluations. One of scientific bodies is the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group on Pesticide Residues (JMPR) giving advice to the CCPR (FAO, 2018).

### SPS-Agreement

The WTO was established in 1995 after nearly 10 years of trade negotiations (Uruguay Round). The WTO agreements cover goods, services and intellectual properties. One of these agreements is the General Agreement on Tariffs and Trade (GATT) (for goods): The agreement incorporates several Annexes. One of these Annexes covers health regulations for farm products (SPS Agreement). The SPS Agreement by itself does not set standards for food safety, animal and plant health, it mentions bodies responsible for setting these standards. The CAC is one of these standard setting bodies. The standards provided by these bodies are implemented as international standards. Through the SPS Agreement, harmonization is induced by encouraging members to introduce measures meeting these standards. Possible requirements may be the inspection of products that products must come from disease free areas or need to undergo certain processing steps or treatments before reaching the consumer. The use of certain additives or the setting of MRLs are further possible measures. The sovereignty of Member States is not supposed to be reduced by these measures and established measures cannot disadvantage other members. The international standards provide an indispensable level of protection from which deviations are only possible under special conditions. National modifications to the standards need to be scientifically justified by an adequate risk assessment. Additionally, these changes need to be notified to the other members in order to assess compliance with the standards, possible effects on trade and as general information to the other members for acknowledgement. The adoption of sanitary and phytosanitary measures has increased over the past years. This increase is not necessarily positive. Some countries use these measures to establish trade barriers to protect their own producers of agricultural products from foreign competition (WTO, 2018a).

The enforcement of standards is overseen by the responsible national authorities of each individual country (FAO, 2003). Diverse approaches and interpretations can lead to different conclusions resulting in negotiations and a final ruling by the WTO (WTO, 2018b).

### Influence of MRLs on Trade

According to the SPS Agreement, MRLs are a valid standard to globally provide safe and agricultural products. International MRLs (also referred to as CXL) are adopted by the CAC being agreed upon in the CCPR. CXLs are annually aligned, unless concerns are raised. Concerns can be discussed in council meetings prior, at CCPR meetings, shortly before or in between the CAC meetings. They may be addressed as observations documented in the protocol of the CAC meeting or in concern forms as described in the CCPR manual. Usually, the discussions result in a common position presented at the CAC meeting by the EU acting as sole legally entitled force.

A standardized procedure to set MRLs is only available in some countries and regions. The calculation models to determine MRLs still vary in different countries. The EU is one of the members establishing robust MRLs. Setting of MRLs is regulated by Regulation (EC) No 396/2005 which is valid in all Member States and therefore, MRLs are harmonized throughout the EU (Farnsworth, 2018). Countries without the possibility to set a MRL for a specific pesticide-commodity combination can adopt an existing CXL to facilitate trade. More important for those countries is the possibility to produce food of plant and animal origin that can be exported to other countries (Foletti and Shingal, 2014).

The removal of national requirements and differences through harmonization of the MRLs has simplified the exchange of agricultural goods within the EU. For the same reasons, market access for importers from outside the EU has become easier than before. Homogeneity has positively affected agri-trade within the EU but also with non-EU states (BMEL, 2016). Recent figures support the assumption that in general, a harmonization of standards has increased agri-trade. Intra-EU trade has grown by 72% from 2005 to 2016 to reach a value of 350 billion EUR. Trade with Non-EU Members has relatively increased even more. The total trade value has grown from 57.6 billion EUR in 2005 to 129.1 billion in 2015 - a total increase of 124%. Globally the value of trade in food exceeded the mark of 1.12 trillion US\$ in 2017 (EU, 2017).

Nevertheless, fair trade is still an illusion. Not all countries adopt CXLs. Different interpretations of study results may lead to different toxicological endpoints and/or different residue definitions that may hinder acceptance of a CXL.

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## Relevant Websites

- <http://www.fao.org/agriculture/crops/thematic-sitemap/theme/pests/jmpr/en/> – The Joint FAO/WHO Meeting on Pesticide Residues (JMPR).
- <http://www.who.int/en/> – WHO.
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- <http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN> – EU Pesticides database.
- <http://www.fao.org/fao-who-codexalimentarius/en/> – Codex Alimentarius.
- <http://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/pestres/en/> – Codex Pesticides Residues in Food Online Database.

## Pesticides: An Update on Mass Spectrometry Approaches

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### List of Definitions

APCI Atmospheric pressure chemical ionization  
APPI Atmospheric pressure photoionization  
CAC Column adsorption chromatography  
CDB Compound database  
CDFA California Drug and Food Administration  
CI Chemical ionization  
DC Direct current  
DDA Data dependent acquisition  
DIA Data independent acquisition  
dsPE Dispersive solid phase extraction  
ECD Electron-capture detector  
EICD Electrolytic conductivity detector  
EI Electron impact ionization  
EMR Enhanced matrix removal-lipid  
ESI Electrospray ionization  
FDA U.S. Food and Drug Administration  
FID Flame ionization detector  
FLD Fluorescence detector  
FT Fourier transformation  
FWHM Full width at half maximum  
FPD-P Flame photometric in phosphorus mode  
FPD-S Flame photometric detector in sulfur mode  
GC-MS Gas chromatography-mass spectrometry  
German DFG Deutsche Forschungsgemeinschaft (German Research Foundation)  
GPC Gel permeation chromatography  
HRMS High resolution mass spectrometry  
IT-MS Ion trap mass spectrometry  
IUPAC International Union of Pure and Applied Chemistry  
K-D Kuderna-Danish evaporator  
LC High performance liquid chromatography  
LC-MS Liquid chromatography-mass spectrometry  
LODs Limits of detection  
LOQs Limits of quantitation  
MRM Multiple reaction monitoring  
MS/MS Tandem mass spectrometry  
MS<sup>E</sup> Alternate low-energy and high-energy MS/MS experiment  
NFA National Food Administration of Sweden  
NIST National Institute of Standards and Technology  
NPD Nitrogen phosphorus detector  
Orbitrap Orbital trap mass analyzer  
PAM Pesticides analytical manual  
PCD Post column derivatization  
PRM Parallel reaction monitoring  
PSA Primary-secondary amine linked to silica particles  
Q-HRMS Quadrupole high resolution mass spectrometry



QMF Quadrupole mass filter  
QqIT Quadrupole-ion trap  
Q-Orbitrap Quadrupole orbital trap mass analyzer  
QqQ-MS/MS Triple quadrupole tandem mass spectrometry  
Q-TOF-MS Quadrupole time-of-flight mass analyzer  
QuEChERS Quick, Effective, Cheap, Easy. Rugged and Safe  
QuPPE Quick polar pesticide  
RF Radio frequency  
RSD Relative standard deviation  
SIM Single ion monitoring  
SIM Single ion monitoring  
SNR Signal-to-noise ratio  
SPE Solid phase extraction  
SRM Selected reaction monitoring  
TOF-MS Time-of-flight mass analyzer  
UHPLC Ultrahigh performance liquid chromatography  
vDIA Variable DIA  
XSD Halogen selective detector

### **Pesticide Identification by Chromatography-Mass Spectrometry**

Since the 1940s, synthetic organic pesticides have been used to control a variety of pests to protect crops and agricultural commodities. Government agencies such as the U.S. Environmental Protection Agency and the Pest Management Regulatory Agency of Health Canada are involved in the regulation of pesticides by establishing pesticide tolerances in foods. The [U.S. Food and Drug Administration \(2012\)](#), the [European Food Safety Authority \(2017\)](#), and the organizations that provide guidance on international food standards, such as [CODEX Alimentarius \(2017\)](#), have adopted identification criteria for pesticides based on chromatography-mass spectrometry procedures. Guidance criteria using mass spectrometry require retention times based on a chromatographic separation technique, the presence of the precursor and/or diagnostic ions based on the fragmentation of the precursor, and ratios between prominent characteristic ions ([Lehotay et al., 2008](#)). These diagnostic fragment or product ions are generated by in-source fragmentation when using selected ion monitoring (SIM) on a single quadrupole mass analyzer, or by collision induced dissociation when using selected or multiple reaction monitoring (SRM, MRM) on a tandem (i.e., triple quadrupole or quadrupole-ion trap) mass analyzer. GC-MS or LC-MS equipped with a triple quadrupole mass analyzer operating in SRM or MRM mode (GC-QqQ-MS/MS or LC-QqQ-MS/MS) is the preferred platform for pesticide analysis because of its capability to quantitate as well as identify pesticides based on retention time of the precursor mass and the selective mass fragmentation of the precursor into product ions. Identification of the pesticide is crucial for regulatory, compliance, and enforcement purposes to ensure that a pesticide is unambiguously present in the complex food matrix above a level of concern.

Before mass spectrometry became the dominant detector, confirmation of pesticides using non-selective detectors based on element selection (presence of heteroatoms such as halogens, nitrogen, and phosphorus) or spectrophotometric detection (e.g., UV, fluorescence) was achieved by chromatographic retention time from different capillary chromatography columns, analysis performed on different detector systems, or use of another orthogonal method. Identification by chromatography-MS eliminates the need for confirmatory or secondary analysis since one injection provides retention time, precursor and product ion masses, and ion ratios provide unequivocal evidence that the pesticide is present in the food sample. The retention time and the resulting unique fragmentation pattern from a mass spectrum can be used to differentiate a pesticide (or any other analyte) from other molecular components in the food extract.

### **The Influence of Mass Spectrometry on Sample Preparation for Pesticide Analysis**

Mass spectrometry has made an important impact for the multiresidue pesticide methods that are currently being used for analysis. Prior to the use of mass spectrometry, pesticide methods used large sample sizes, large amounts of organic solvents, and usually required expensive clean-up to minimize the influence of co-eluting matrix components that may interfere in the detection of the pesticide by a less selective detection system ([Anastassiades et al., 2003](#)). One of the first multiresidue pesticide procedures was developed by [Jones and Riddick \(1952\)](#) when they extracted organochlorine pesticides from animal and plant



tissue and dairy products using acetonitrile as an organic extraction solvent and detected the pesticides from the extracts by colorimetry. Paul Mills at the U.S. Food and Drug Administration (FDA) applied the procedure to extract and isolate pesticides from fruits and vegetables and detected the pesticides by thin-layer chromatography (Mills, 1959) and later, gas chromatography-microcoulometric detection (Mills et al., 1963). Typically, these procedures involve extracting the food sample with acetonitrile, partitioning with petroleum ether and a sodium chloride saturated solution, cleaning up on a florisil column with an eluting solvent, and removing the excess solvent using steam and a Kundera-Danish concentrator. Over the years, pesticide sample preparation methods were modified as new pesticides, such as organophosphorus pesticides, pesticides containing heteroatoms such as nitrogen, sulfur, and oxygen, and pesticides with different chemical and physical properties, were added to the multiresidue procedures. Acetone, ethyl acetate, and methanol have been used instead of acetonitrile as alternative extraction solvents. In the 1970s, FDA replaced acetonitrile with acetone as the extraction solvent, followed by a dichloromethane/petroleum ether partitioning and various extract cleanup strategies using adsorption column chromatography (florisil) or solid-phase extraction cartridges (using modified silica and later polymer-based particles crosslinked with octadecyl C18-linked ligands) allowing pesticide detection using gas chromatography equipped with element selective (nitrogen-phosphorus, flame photometric, electron capture, electroconductivity) (Luke et al., 1975, 1981). Ethyl acetate was another extraction solvent which was popularly used for gel permeation chromatography clean-up procedures to remove lipids and high molecular weight matrix components from the food extracts. These different procedures were modified over the years as GC-MS and LC-MS replaced GC and HPLC.

Anastassiades et al. (2003) evaluated the pros and cons of current sample preparation procedures for pesticides. They optimized certain steps which formed the basis of the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) procedure. This procedure streamlined acetonitrile-based extraction processes by using smaller sample sizes (i.e., from 50–100 g to 10–15 g), less solvent (50–100 mL to 10–15 mL), and simplified the extraction and cleanup steps so that each step is performed manually with 50 mL tubes and a centrifuge. Extraction and partition of pesticides from food into acetonitrile are enhanced by the addition of salts, initially anhydrous magnesium sulfate and sodium chloride. The preparation is simplified by using a clean-up step transferring the acetonitrile extract into a second centrifuge tube containing either one or more sorbents such as primary-secondary amine- (PSA) and octadecyl (C18)-linked silica or graphitized carbon that can remove matrix interfering components. The QuEChERS procedure is flexible and modular as many steps of the procedure can be modified, such as sample size, solvent volume, solvent choice, sorbent choice, and adopted for either GC-MS or LC-MS analysis. No longer are laboratories required to use large sample sizes or solvent volumes except for very unusual circumstances. This trend is being accelerated by the increasing sensitivity, simplicity and flexibility of GC-MS and LC-MS.

Many analytical and laboratory-based companies sell QuEChERS consumable products commercially that offer different variations of QuEChERS to allow the tailoring of the sample procedure to target specific pesticides and different food matrices. New sorbents such as carbon nanotubes (Abdulrauf and Tan, 2016; Qin et al., 2015), zirconia (Rajski et al., 2013), and polymers (Han et al., 2016) have been created to remove pigments, fats, lipids, and other matrix components that may interfere in the analysis of pesticides (López-Blanco et al., 2016; Parrilla Vázquez et al., 2016). As acetonitrile extraction procedures became optimized with QuEChERS, acetone- and ethyl acetate-based extraction procedures also underwent their own simplification and modification. Laboratories using acetone or ethyl acetate-based extraction procedures have shown that these methods demonstrate equivalency with QuEChERS over a large number of pesticides and range of food matrices, as well as with sample preparation time and consumable use (Lehotay et al., 2005; Pihlström et al., 2007; Uclés et al., 2014, 2017a,b; Lozano et al., 2016). The introduction of QuEChERS and other efficient sample preparation products and procedures have improved the preparation and analysis of pesticides and other non-polar analytes of importance in food and environmental analyses. Currently, QuEChERS and generic “dilute-and-shoot” procedures are the preferred approaches to analyze a wide variety of pesticides, chemical residues, and chemical contaminants in food for analysis by GC-MS and LC-MS (Romero-González et al., 2016). Table 1 lists some sample preparation procedures for pesticide analysis they have been simplified and amenable to both GC-MS and LC-MS analysis using QuEChERS or modified or simplified versions of previously developed methods using the commonly used extraction solvents acetonitrile, acetone, and ethyl acetate.

### From GC to GC-FS-MS to GC-MS/SIM to GC-QqQ-MS/MS

During the 1980s, GC-MS was used to compliment established gas chromatography instruments equipped with element selective detection such as electron capture (ECD), nitrogen-phosphorus (NPD), flame photometric in phosphorus and sulfur mode (FPD-P, FPD-S), electrolytic conductivity (ELCD), and halogen selective (XSD) detectors that were selective for specific heteroatoms present in pesticides such as chlorine, bromine, nitrogen, phosphorus, and sulfur. As the technology matured and improved, costs became affordable. Eventually GC-MS transitioned into the sole and dominant instrument for analyzing pesticide residues. During the same time, the replacement of packed glass columns with capillary columns became commercially available in the 1970s and 1980s offering improved separations and chromatographic resolution. Improvements in chromatography and mass spectrometry, as well as instrument availability and cost, accelerated its use in pesticide analysis.

The commercialization of the quadrupole mass spectrometer in the 1970s led to the use of mass spectrometry as the major detector for pesticide analysis used today. The quadrupole mass filter (QMF) was relatively simple and inexpensive to manufacture over other mass spectrometers, such as sector and time-of-flight instruments (Sparkman et al., 2011). The quadrupole mass

**Table 1** LC-MS/MS and GC-MS/MS procedures using a single multiresidue pesticide procedure for fresh produce

Sample preparation procedure				LC-MS/MS				GC-MS/MS				Reference
Procedure	Extraction solvent	Partition solvent	Clean-up	LC column (cm × mm × μm)	Mobile phase	LC run time	No of pesticides by LC-MS/MS	GC column (m × mm × μm)	Injection mode	GC run time	No of pesticides by GC-MS/MS	
Mini-Luke	Acetone	Petroleum ether/ Dichloromethane	None	C18 (10 × 2.1 × 1.7)	MeOH/H <sub>2</sub> O/AF	10 min	109	VF-5MS (30 × 0.25 × 0.25)	PTV/LVI	21.34	71	Dutch mini-Luke method ( <a href="#">EURL-FV, 2014-M12</a> )
SweEt	Ethyl acetate	None	None	C18 (15 × 2.1 × 1.7)	MeOH/H <sub>2</sub> O/AF	16 min	240	VF-5MS (30 × 0.25 × 0.25) with Guard Column	Not described	Not described	147	Swedish EtOAc method ( <a href="#">EURL-FV, 2010-M4</a> )
QuEChERS	Acetonitrile	None	dSPE (PSA)	C18 (10 × 2.1 × 3)	MeOH/H <sub>2</sub> O/AF/ formic acid	18 min	75	HP-5MS (30 × 0.25 × 0.25)	Splitless	41.9	66	QuEChERS EN method ( <a href="#">EURL-FV, 2010-M1</a> )
ACN Extraction	Acetonitrile	None	SPE (GCB/ PSA)	C18 (15 × 2.1 × 3)	ACN/H <sub>2</sub> O	38 min	63	DB-1701 (30 × 0.25 × 0.25)	Splitless	38	383	<a href="#">Pang et al. (2006)</a>

spectrometer consists of four parallel rods aligned to form a circular or hyperbolic cross section. It is a scanning mass analyzer that uses the stability of ion trajectories in oscillating electric fields to select and separate ions according to their mass-to-charge ratios ( $m/z$ ). A positive and negative direct current (DC) potential and an alternating radiofrequency (RF) potential is superimposed on each rod pair. This RF voltage causes the ions to spiral as they traverse through the quadrupole toward the detector. The DC and RF potentials are adjusted to allow ions of a given  $m/z$  ratio to remain stable within the rods and pass through to the detector, while others are ejected. To acquire a mass spectrum, the DC and RF potentials must be ramped in a constant ratio across a specified range (Mellon, 2003; Niessen and Correa, 2017).

Quadrupole mass spectrometers typically operate in either full scan (FS) or selected ion monitoring (SIM) modes. In GC-full scan-MS mode (GC-FS-MS), charged ions are monitored over a large  $m/z$  range giving a complete spectrum of electron impact (EI) mass fragments formed in the source. In SIM mode (GC-MS/SIM), a finite number of specific ions are selected and measured. The advantage of operating in full scan mode is that identification of the pesticide of interest can be determined by comparing the mass spectrum obtained from the sample to a reference mass spectrum of the pesticide. Full scan analysis includes comprehensive MS libraries of many chemical compounds and software algorithms to match spectra and deconvolute spectra by removing interfering peaks and subtracting the background (Ueno et al., 2008; Ragnar Norli et al., 2010). There are full scan MS databases available that contain thousands of reference compounds such as the one developed by the National Institute of Standards and Technology (NIST). Used in conjunction with software tools and algorithms, experimental MS spectra for an unknown can be rapidly screened and matched to an MS spectrum for a reference compound. One of the first demonstrations of GC-MS for pesticide analysis was performed by Stan (1989, 2000), whose work demonstrated that identification of pesticides using GC-FS-MS and GC-MS/SIM could be achieved by probability-based matching using the NIST database. Major disadvantages of the GC-FS-MS approach are that full scan screening procedures lack the higher sensitivity of SIM due to scanning over a full  $m/z$  range in the presence of the sample matrix and matching criteria between experimental and reference spectra could be complicated limiting confident identification. Additionally, GC-FS-MS is not effective to detect pesticides at the default tolerances, with limits of quantitation greater than the 0.01 ppm level.

A more popular approach is to use GC-MS/SIM for the analysis of pesticides because of the increased sensitivity to measure at the parts-per-billion (ppb) level. The current identification criteria for GC-MS/SIM is better defined due to the limited mass information collected. In SIM, a focused scan range permits only the ions that are characteristic of the pesticide to be selected, increasing the dwell time and thereby the sensitivity of the targeted analyte. Identification using GC-MS/SIM is achieved by providing typically three or four characteristic ions of the pesticide analyte with ion ratios in an acceptable range, typically  $\pm 30\%$  relative or  $\pm 10\%$  absolute. These criteria ranges have been established by governing agencies such as European Food Safety Agency (2017) and the U.S. Food and Drug Administration (2012). One of the first comprehensive works that included a validated sample preparation method and utilized GC-MS/SIM for instrumental analysis of pesticides was performed by Fillion et al. (1995) of Health Canada. The sample preparation procedure involved an acetonitrile salt-out extraction with sodium chloride of the pesticides from the food matrix, followed by clean-up of the acetonitrile extracts using solid-phase extraction using tandem primary-secondary amine-linked silica (PSA) and graphitized carbon black sorbents column cartridges. The analysis was performed using GC-MS/SIM using two injections to cover 199 pesticides, each run requiring approximately 65 mins. Identification was achieved by the presence of quantitation and qualification ions at defined retention times and ion ratio tolerances. This work represented one of the first multiresidue methods for pesticide analysis solely utilizing mass spectrometry detection for both quantitation and identification. The method developed by Fillion et al. (1995, 2000) is the basis for procedures used by other organizations and laboratories (Japan Department of Food Safety Ministry of Health, Labour and Welfare; Pang et al., 2006, 2015). Other stand-alone GC-MS/SIM procedures soon followed based on varying extraction solvents and clean-up procedures (Mercer and Hurlbut, 2004; Ueno et al., 2004; Mercer, 2005). Many of these procedures are still being used today.

The quadrupole ion trap (QIT) mass analyzer was commercially available in the 1980s and evaluated for pesticide analysis. In a three-dimensional (3D) ion trap, RF and DC potentials are applied to oscillate ions at a secular frequency and selectively eject ions into the detector (Lacorte et al., 2015; Niessen and Correa, 2017). This analyzer employs the same principles as the quadrupole analyzer mentioned above, as it uses a RF only electric field for the storage and scanning of ions by  $m/z$  ratios. One of the benefits of the ion trap is its capability of performing tandem (MS/MS) and MS<sup>n</sup> experiments. One of the earlier applications of the ion trap was performed by Cairns et al. (1993) who analyzed fresh produce extracts prepared by the Luke Method in both FS and SIM mode for over 245 pesticides. Schachterle et al. (1994) demonstrated collision-induced dissociation steps by GC-QIT-MS/MS to study malathion in an orange extract. Hirahara et al. (2006) evaluated 200 pesticides in various crops at 0.01 mg/kg, determined recoveries in the range 50%–150% for 194 pesticides, and found that identification could be better achieved by GC-MS/MS rather than GC-MS/SIM.

The use of gas chromatography-tandem mass spectrometry (GC-MS/MS) has shown to improve the selectivity to the analytes, reduce the background, and increase the sensitivity. Multiresidue pesticide analysis by ion trap MS/MS tends to be slower than triple quadrupoles for selected reaction monitoring (SRM) acquisition, requiring more time to complete a SRM event per scan cycle due to the necessity of ion storage. GC-QqQ-MS/MS using a triple quadrupole platform operating in SRM or MRM mode proved to be much more efficient for multi-residue methods. As the name suggests, the original triple quadrupole mass analyzer consisted of “three” quadrupoles arranged in series after the source, the first of which selects the precursor ion, a second which functions as a collision cell using an energetic, inert gas such as nitrogen or argon, and a third quadrupole to filter selected fragment or product ions for detection (Yost and Enke, 1979). Modern triple quadrupole instruments consist of a dedicated collision cell instead of a second quadrupole to provide better collision efficiencies, improved electronics to increase scanning speeds, and faster computers

that can process data acquisition. GC-QqQ-MS/MS provides improved selectivity and sensitivity for target pesticides by selecting specific precursor-to-product ion transitions that reduce contributions from matrix background and other co-eluting and isobaric analytes.

GC-QqQ-MS/MS has replaced GC-MS/SIM due to compound selectivity resulting in less interference of co-eluting chemical components from a complex food matrix and superior signal-to-noise allowing for lower limits of detection and quantitation. Garrido-Frenich et al. (2005) demonstrated the use of GC-QqQ-MS/MS for the screening, identification, and quantitative analysis of 130 pesticides. Samples were prepared using a simple method procedure by extracting/homogenizing the food sample with ethyl acetate, filtering through anhydrous sodium sulfate, and solvent exchanging to cyclohexane. A 11.6 min GC temperature program using one MS/MS transition was used for screening step. Any presumptive pesticide positive would require a second GC-MS/MS analysis consisting of two or three MS/MS transitions for confirmation and quantitation. Current GC-QqQ-MS procedures can achieve screening, identification, and quantification in one determinative step. A review on the role of GC-QqQ-MS/MS in pesticide residue analysis in food and environment matrices by Hernández et al. (2013) provides several examples and references that reveal improvements in methods performance in comparison to traditional GC and GC-MS approaches. GC-QqQ-MS/MS has been used to analyze a large number of pesticides in fresh produce (Okiihashi et al., 2007; Pihlström et al., 2007; Wong et al., 2010; Hakme et al., 2018; Lozano et al., 2016; Lee et al., 2017; Khan et al., 2018), dried botanical supplements (Hayward et al., 2013), spices (Ahammed Shabeer et al., 2018), and tea (Hayward et al., 2015; Pang et al., 2015; Chang et al., 2016).

Studies by Portolés et al. (2010, 2012a,b) and Cherta et al. (2013) evaluated the use of chemical ionization (APCI) instead of the traditional electron impact ionization (EI) to generate intact molecular ions in GC-QqQ-MS/MS. EI (70 eV) tends to be excessive which may result in extensive fragmentation of the pesticides that will compromise selectivity, sensitivity, and identification (Portolés et al., 2012a,b). Existing chemical ionization (CI) methods in both positive and negative (PCI, NCI) modes for GC-MS analysis involve introducing an energetic gas such as ammonia, methane or isobutane to induce fragmentation have been previously investigated for pesticides (Biros et al., 1972; Dougherty et al., 1972). However, CI is not frequently used in multi-residue pesticide procedures because it is not as universal as EI and requires different modes (positive and negative CI) and multiple injections to cover a wide range of pesticides (Alder et al., 2006). APCI, a softer form of ionization used in LC-MS, was coupled a GC-QqQ-MS instrument to evaluate a wide range of pesticides in food samples and compared to results generated by GC-EI-QqQ-MS/MS (Portolés et al., 2012a,b). Of the pesticides studied, the molecular ion could not be generated using EI but the APCI source generated  $M^+$  and/or  $[M+H]^+$  ions. The use of GC-API-QqQ-MS/MS allowed for more selective and sensitive transitions in SRM mode using  $[M+H]^+$  as a precursor ion and could further expand the use of GC-MS applications to pesticide analysis.

### Liquid Chromatography-Triple Quadrupole Tandem Mass Chromatography (LC-QqQ-MS/MS)

Liquid chromatography (LC) was primarily used for the analysis of carbamate insecticides that involved liquid chromatographic separation, followed by post-column derivatization, and the fluorogenic derivatives were analyzed by fluorescence detection (Moye and Scherer, 1977). The pesticides that are used today have replaced their more toxic predecessors and are designed to be more species specific, less persistent, and are designed to degrade to less toxic by-products in the environment. Most of these pesticides also tend to be thermally-labile and not amenable to the elevated temperature conditions used in GC and GC-MS analysis. Additionally, many current target pesticides do not possess significantly unique chromophores that can be spectrophotometrically detected. While the majority of pesticide laboratories transitioned directly to LC-QqQ-MS/MS, single quadrupole and ion trap mass analyzers were also explored. Single quadrupole mass analyzers were limited in identification of pesticides in complex matrices. The major disadvantage of the early LC-QIT-MS/MS systems was that quantitation could be challenging due to the limited dynamic range especially in the presence of sample matrix (Soler et al., 2005). Despite limited selectivity, one advantage of LC-MS applications was that the intact molecular adduct in the form of  $[M+H]^+$ ,  $[M+NH_4]^+$ ,  $[M-H]^-$ , could be detected unlike in GC-MS with electron impact (EI) ionization. LC-MS typically uses electrospray ionization (ESI) in both positive and negative modes to generate the formation of precursor ions  $[M+H]^+$ ,  $[M+NH_4]^+$ , and  $[M-H]^-$ , based on the atomic composition and properties of the pesticide. However, other related ionization sources such as atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) have been used in limited studies (Takino et al., 2004; Yoshioka et al., 2004).

The work of Klein and Alder (2003) was one of the first to successfully demonstrate the use of LC-QqQ-MS/MS for the determination of 108 pesticide analytes, of which 98 and 10 pesticides were analyzed by positive and negative electrospray ionization mode, respectively. The sample procedure utilized a methanol extraction, clean-up of the aqueous methanol extract using a diatomaceous earth (ChemElut) column, elution of the pesticides from the column with dichloromethane, and evaporation and filtration of the extract into a vial for LC-QqQ-MS/MS analysis. Recoveries at the 10 µg/kg (ppb) level in a variety of produce matrices were in the 70%–120% range with a relative standard deviation  $\leq 25\%$ . This seminal paper also provides criteria to optimize the triple quadrupole analyzer by establishing the optimal collision energies to generate the most intense precursor-to-product transition ions, assessing the magnitude of matrix effects, and demonstrating the applicability of LC-MS/MS for multiclass pesticide analysis. In a 2006 review, Alder et al. (2006) evaluated the selection of pesticides or classes of pesticides that would optimally be determined either by GC-MS (or GC-MS/MS) or LC-MS/MS. Although the authors realized no one technique is capable of analyzing the more than 860 active substances used in pesticide formulations, an evaluation of approximately 500 pesticides (e.g., organophosphorus, carbamate, organochlorine, sulfonylurea, triazole, triazine, urea, pyrethroid, aryloxyphenoxy propionate, aryloxy alkanolic acids, and other non-classified pesticides) revealed 135 and 47 of those that could not be detected by GC-MS and LC-MS,

respectively. However, 33 of the 47 pesticides that were not detected by LC-MS/MS were organochlorine pesticides, many of which are legacy compounds and are no longer being registered for use as pesticides. Many of the pesticides that are not amenable to GC-MS analysis tend to be thermally unstable and are at risk of decomposing due to the heated conditions of the GC inlet and column oven. For all compounds that are analyzed by both GC-MS and LC-MS, better performance of LC-MS/MS was typically observed and attributed to larger injection volumes and sample capacity in LC-MS instruments.

Since the work of Klein and Alder (2003), LC-QqQ-MS/MS or hybrid quadrupole-ion trap (LC-QqIT-MS/MS) tandem mass spectrometry has been the primary procedure used by many laboratories to perform multiresidue analysis for pesticides in a variety of food matrices. Due to the declined use of chlorinated pesticides and the increased use of thermally-labile pesticides, LC-MS has overtaken GC-MS as the primary instrument for pesticide analysis. Although QuEChERS is probably the most popular method used to prepare the food matrices for extracts, other procedures that had origins from GC and GC-MS procedures have been modified for LC-MS analysis (EURL-FV(2010-M1), EURL-FV (2010-M4), EURL-FV (2014-M12), Pang et al., 2006, Payá et al., 2007).

### Future Status of MS: High Resolution MS (GC-HRMS, LC-HRMS)

Although tandem mass spectrometry in GC-QqQ-MS/MS and LC-QqQ-MS/MS provides nearly unambiguous identification of pesticides in foods, its shortcomings did not go unrecognized as laboratories began to evaluate high resolution mass spectrometry with applications for full scanning analysis as possible complements or replacements to triple quadrupole platforms. Chromatography coupled to tandem mass spectrometry is a targeted and optimized procedure for quantitation and identification in multiresidue pesticide analysis but it requires *a priori* knowledge of the pesticide such as retention time, collision energies, and product ions. Chromatography-HRMS shows potential as a screening procedure and/or an alternative quantitative procedure to LC-QqQ-MS/MS with comparative sensitive to quadrupole mass analyzers. Full scan HRMS workflows do not require retention time and compound-specific MS parameters, except in data processing, allowing data to be re-evaluated for retrospective analysis. The high resolving power that allows accurate mass assignments is a main advantage of these mass analyzers. The IUPAC definition of resolution is defined as  $m/\Delta m$ , where  $m$  is the mass of an individual ion of interest and  $\Delta m$  is the width of the individual peak (Niessen and Falck, 2015). Resolution allows for increased confidence of identification of the pesticide analytes by improving the quantitative mass accuracy, and therefore, assists in providing structural assignments and fragmentation of product ions. Another important term associated with HRMS is resolving power, which determines the separation required between two Gaussian peaks at their apices,  $m_1$  and  $m_2$ , expressed as the peak width in mass units (Niessen and Falck, 2015). Mass resolving power is defined as  $m_1/(m_1 - m_2)$  in relation to mass,  $m_1$ . High resolution mass spectrometry can distinguish ions with the same nominal mass but with different elemental compositions (isobaric ions), measure the exact monoisotopic mass and the isotopic mass distribution (due to the isotopic contributions of the atoms, i.e.,  $^{12}\text{C}$ ,  $^{13}\text{C}$ , and  $^{14}\text{C}$ ), determine elemental composition, and provide structural elucidation of the chemical of interest.

HRMS mass analyzers provide mass accuracy as co-eluting compounds can be discriminated and measured to the 0.0001 amu and can therefore provide more definitive information for structure assignments of precursor and product ions. Early research on TOF and QTOF mass analyzers and the commercial availability of the benchtop Orbitrap in the last decade accelerated interest in the use of HRMS as a complement to LC-QqQ-MS/MS or as a standalone platform to screen for less frequent pesticides, pesticide metabolites, degradation products, and other co-residues and contaminants in the same food matrix. Development of qualitative screening methods can streamline the laboratory approaches to identify unwanted and undesirable chemicals in food products by minimizing the quality control measurements that are associated for quantitative analysis (Mainero Rocca et al., 2017). The design between the two mass analyzers, time-of-flight and orbitrap, are based on two entirely different physical principles. Further detailed descriptions are provided by Radionova et al. (2016), Boesl (2017) for the TOF, and Perry et al. (2008), Zubarev and Makarov (2013) for the Orbitrap, respectively, and the review by Kaufmann and Teale (2017) provides a detailed description of the advantages and disadvantages of both high resolution mass analyzers. Both GC- and LC-HRMS procedures for multiresidue pesticides procedures for fresh produce using TOF and Orbitrap platforms, have been thoroughly investigated and evaluated as indicated in Table 2. Most of the sample preparation procedures used were based on QuEChERS and the number of pesticides increased when a hybrid instrument (Q-TOF, QOrbitrap) was used for the analysis.

### GC-TOF-MS and GC-Q-TOF-MS Pesticide Analysis

Time-of-flight (TOF) mass analyzers determine the  $m/z$  of ions based on the time ions travel and separate from each other in a field-free region (flight or drift tube) under the influence of an electric field as they reach the detector (Lacorte et al., 2015). TOF instruments are commonly manufactured with a reflectron, which is an ion optical devices that “reflects” or changes the direction of the ions which increases their path length and brings differing ion kinetic energies into phase before the ions enter the detector, allowing for increased separation and increasing resolution. The advantages of the TOF mass analyzer are its high scan and data acquisition rates which can acquire spectra at a faster rate (10–50 spectra/s) than other mass analyzers and generate high resolution spectra containing accurate mass measurements. Unit-resolution TOF also trades resolution for high acquisition rates (500 spectra/s) and gives linearity of four orders of magnitude (Hernández et al., 2011). The high acquisition rate is advantageous for characterizing narrow and sharp peak widths that are generated in capillary, high-speed, and comprehensive two-dimensional GC (GC×GC). A



TOF mass spectrometer provides sufficient acquisition rates capable of producing sufficient data points to define chromatographic peak shapes. GC×GC-TOF MS has been used for pesticide analysis in produce (Zrostlíková et al., 2003; Banerjee et al., 2008; Lehotay et al., 2011).

Early work on high resolution GC-TOF was evaluated for its sensitivity in full scan acquisition mode, resolving power, and accurate mass measurements (Leandro et al., 2007; Hernández et al., 2011). While these features are desirable, early TOF analyzers had limited dynamic range and resolution (~7000 full width at half mass, FWHM). Cajka and Hajšlova (2004), Cajka et al. (2008) were one of the first groups to analyze pesticides using GC-TOF-MS and found that most of the pesticides could be detected below 10 µg/kg and demonstrating performance characteristics, such as mass accuracy and pesticide detectability, exceeding those performed by other conventional mass analyzers. Cervera et al. (2012) evaluated a GC-TOF-MS for 55 pesticides for targeted and non-targeted analysis of pesticides in fruits and vegetables. QuEChERS extracts of various food matrices were analyzed in full scan mode and the most abundant ion was used as the quantitation ion while the other diagnostic ions were used for confirmation. The recoveries were between 70%–120% and precision < 20% but only half the pesticides could be validated at the 0.01 µg/kg level. Hayward and Wong (2009) compared the performances of GC-TOF-MS (resolving power of ~6000 at  $m/z$  200 and mass accuracy of 5 ppm) and GC-MS-SIM methods of 170 pesticides in ginseng root extracts and found that there were no significant differences in the concentrations or geometric limits of detection (LODs) measured for pesticides in commercial ginseng roots using either of the two techniques.

Increased resolution and the addition of a quadrupole improved the instrument performance for GC-QTOF-MS analysis. Portolés et al. (2010) and Zhang et al. (2014) developed methods to screen and identify more than 100 pesticide residues in fresh produce using GC-QTOF-MS/MS. Portolés et al. (2010) expanded their work from QqQ to QTOF using an atmospheric pressure ionization (API) source rather than the highly fragmented EI ionization source to generate molecular ions while in the Zhang et al. (2014) study, a screening workflow for screening and analyzing pesticides was developed that included a retention time index, accurate mass full scan MS database, and MS/MS identification. A database or library is a collection of reference retention times and exact masses of ions from MS and MS/MS scans for each pesticide that can be used to search, retrieve, and match the experimental data. This workflow involved full scan screening to search for 165 pesticides by chemical formula match and MS/MS identification of the product ion using accurate mass measurement. Over 80% of pesticides at 5 ng/mL or lower concentrations could be confirmed in each matrix using at least two representative ions with their response ratios from the MS spectra. In Portolés et al. (2010), a list of 100 GC-amenable pesticides including organochlorine, organophosphorus and organonitrogen compounds, were evaluated using the API source. The addition of water as a modifier was tested to promote the generation of protonated molecules and revealed that screening could be easily performed with this source by investigating the presence of the protonated molecule ion  $[M+H]^+$ . The developed procedure was applied to pesticide screening in different food samples and has allowed the presence of several pesticides to be confirmed such as chlorpyrifos ethyl, deltamethrin and endosulfan sulfate. The results shown by both research groups revealed the promising use and application of GC-API-QTOF-MS, high resolution MS/MS experiments, and mass spectra libraries/database to better and rapidly identify detected pesticide residues in complicated food matrices.

### GC-Orbitrap MS and GC-QOrbitrap-MS for Pesticide Analysis

Recently, GC-EI-QOrbitrap MS and GC-EI-Orbitrap MS became commercially available a decade after the deployment of LC-Orbitrap systems (Peterson et al., 2010, 2014). Mol et al. (2016) first evaluated the GC-EI-QOrbitrap MS for pesticide residue analysis in fruits and vegetables prepared by QuEChERS. The instrument performed in full scan mode with a resolving power of 60,000 and achieved a mass accuracy within  $\pm 2$  ppm over a wide concentration range. Sufficient LOD/Qs  $\leq 0.5$  pg in produce matrices, a wide concentration range (up to 5 orders of magnitude), acceptable recoveries 70%–120% and repeatability (RSD  $\leq 10\%$ ), and good matching against NIST reference library were observed. Tienstra and Mol (2018) further extended the study to effectively analyze pesticides and polychlorinated biphenyls in cereals and feed ingredients. Lozano et al. (2018) evaluated the GC-EI-QOrbitrap for the identification and quantitation in multiresidue pesticide analysis of baby foods in full scan mode. Their results were similar to those produced by Mol et al. (2016) and identification was determined by retention time and the presence of two diagnostic product ions with a mass accuracy  $\leq 5$  ppm and signal-to-noise ratio of the peaks  $\geq 3$ . GC-EI Orbitrap MS was determined to be suitable for GC-MS applications and an alternative to GC-QQQ-MS/MS. However, since EI is the only ionization source available for the QOrbitrap, the excessive fragmentation from EI limits its potential and MS/MS capabilities.

### LC-TOF-MS and LC-QTOF-MS

One of the first LC-HRMS procedures for pesticide analysis was performed by Ferrer and Thurman (Ferrer et al., 2005, 2006; Thurman et al., 2006) who developed a multiresidue method utilizing LC-TOF-MS for the analysis of several classes of pesticides in water and vegetable samples. In-source fragmentation was used to generate one and two fragment ions for 96 and 49, respectively, of the 101 pesticides studied. Quantitation of water and produce samples was determined using matrix-matched calibration curves with correlation coefficients  $\geq 0.99$  or higher and the average instrument limits of detection (LODs) in the presence of the matrix was  $\sim 3$  µg/kg. Pesticides with higher LODs tend to be halogenated consisting of Br, Cl, and F (e.g., bromoxynil, captan,



chlorpyrifos-methyl, fluoroacetamide, fluoroxypry, spiromefesin, teflubenzuron, and trifluralin). The strength of the study pertains to the determination of high mass accuracies of the product ions which can be used to deduce the structure of the fragment ions, and how this information can be useful for identification. The approach by Ferrer et al. was also used by other groups to screen pesticides in fruit and vegetable commodities as listed in [Table 2](#).

As in the case of GC-MS, the addition of a quadrupole in a hybrid QTOF mass analyzer can significantly improve screening applications. An example of a screening and identification procedure using LC-QTOF-MS was developed by [Pang et al. \(2018\)](#) to evaluate 485 pesticides in fruits and vegetables. An accurate mass database based on retention times and accurate masses of the precursor using LC-TOF-MS in full scan mode and an MS/MS spectral library based on the product ions using reference standards was developed by LC-QTOF-MS/MS. The screening process was established and mass deviation, retention time assignments, and ionization was optimized improving the accuracy of the screening procedure and reducing the rates of false-positive and false-negative results. The screening procedures were applied to over 12,500 samples of fruit and vegetable samples throughout the country over a four-year survey.

### LC-Orbitrap-MS and LC-QOrbitrap-MS

The Orbitrap is composed of a spindle-shaped central electrode and a barrel-shaped outer electrode ([Perry et al., 2008](#); [Eliuk and Makarov, 2015](#)). From the ion source, ions traverse through a series of optical lens and stages of differential pumping to achieve a low vacuum environment and are transferred into a curved RF-only trapping quadrupole known as the C-trap ([Bateman et al., 2009](#)). Ions are injected from the C-trap through additional stages of differential pumping into the Orbitrap, where a mass spectrum is acquired by image current detection. The injected ions orbit the central electrode and oscillate along the horizontal axis at frequencies related inversely to the square root of  $m/z$ . A Fourier transformation (FT) translates this frequency into  $m/z$  values and their amplitudes into intensities. The longer the transient signal is recorded, the higher is the resolution of the mass spectrum obtained. The FT of a transient signal yields results in a highly-resolved, accurate-mass Orbitrap mass spectrum. The QOrbitrap consists of a quadrupole mass filter followed by a high-energy collision dissociation (HCD) cell used to induce fragmentation of selected precursor ions prior to mass analysis by the Orbitrap.

When the LC-Orbitrap MS became commercially available approximately 10 years ago, several groups began its evaluation of its feasibility for pesticide analysis ([Alder et al., 2011](#); [Hayward et al., 2011](#); [Mol et al., 2012](#); [Rajski et al., 2014](#)). [Alder et al. \(2011\)](#) assessed the sensitivity to be sufficient to screen for pesticides prepared from a QuEChERS procedure in full scan mode. [Mol et al. \(2012\)](#) investigated the use of the LC-full scan HRMS and all-ion fragmentation (AIF) for qualitative screening for a test set of 130 pesticides spiked in commodities at 10, 50 and 200  $\mu\text{g/kg}$ , prepared using QuEChERS without cleanup, using a database of 556 pesticides in 21 fruit and vegetable commodities. Detection was based on the extraction of the exact mass at  $\pm 5$  ppm and  $\pm 30$  s of the major precursor and diagnostic ions. [Rajski et al. \(2017\)](#) confirmed the work of [Mol et al. \(2012\)](#) by evaluating QuEChERS extracts of plant foods and determined that a resolution of 35,000 and 70,000 was sufficient for the analysis of pesticides in fruits and vegetables.

Although the hybrid ion trap-orbitrap mass spectrometer had been available for some time, the commercialization of the QOrbitrap mass analyzer was of tremendous interest for pesticide analysis and was studied by several groups ([Jia et al., 2014](#); [Wang et al., 2014](#)). One of the first comprehensive studies utilizing LC-QOrbitrap MS was performed by [Wang et al. \(2014\)](#). Pesticides were extracted from samples using the QuEChERS procedure. LC-ESI-Q-Orbitrap MS in full MS scan mode, and LC-ESI QOrbitrap Full MS/dd-MS/MS (i.e., data-dependent scan mode) obtained product ion spectra for identification. LC-ESI-QOrbitrap MS quantification was achieved using matrix-matched standard calibration curves along with the use of isotopically-labeled standards or chemical analogues as internal standards to achieve optimal method accuracy. The method performance characteristics include overall recovery, intermediate precision, and measurement uncertainty evaluated according to a nested experimental design. For the 10 matrices studied, 94% of the pesticides in fruits and 91% in vegetables had recoveries between 81%–110%, 99% of the pesticides in fruits and 99% of the pesticides in vegetables had an intermediate precision of  $\leq 20\%$ , and 97.8% of the pesticides in fruits and 96% of the pesticides in vegetables demonstrated measurement uncertainty of  $\leq 50\%$ . Overall, the LC-ESI-QOrbitrap MS demonstrated acceptable performance for the quantification of pesticide residues in fruits and vegetables. The LC-ESI-QOrbitrap Full MS/dd-MS/MS along with library matching shows great potential for identification and is being investigated further for routine practice.

### Pesticide Screening Using LC-HRMS

Much of the early work on pesticides using LC-HRMS tends to focus on single stage mass analyzers, consisting of either a TOF or Orbitrap. Although the single stage Orbitrap system consists of a multipole collision cell adjacent to the Orbitrap, known as an HCD cell to induce fragmentation, the system was limited to all-ion fragmentation (AIF) experiments. It was the introduction of hybrid instruments, particularly a QTOF or QOrbitrap mass analyzer that provided opportunities to perform targeted and non-targeted MS/MS data acquisition experiments such as data-dependent acquisition (DDA), and data-independent acquisition (DIA) ([Zhu et al., 2014](#); [Wang et al., 2016](#); [Renaud et al., 2017](#); [Rajski et al., 2017](#); [Rauniyar et al., 2017](#)). DDA (or dd-MS/MS discussed in the preceding section) involves an inclusion list of retention times and precursor ions and a narrow quadrupole isolation window of 1–4 Da. An MS/MS scan is triggered when a targeted precursor is detected above a defined intensity threshold. The result is a very selective MS/MS spectrum of the analyte with minimal interference from the matrix co-extractives ([Wang et al., 2016](#); [Rajski et al.,](#)

**Table 2** LC and GC methods coupled to high resolution mass spectrometry (HRMS)

GC-HRMS											
Commodity	Sample preparation	MS analyzer	Acquisition	Resolution	Pesticides	Column type Ionization (m × mm × μm)		Injection	Run time	Sensitivity	Reference
Fresh produce	ASE (EtOAc)/GPC clean-up	Quad-TOF	Full Scan and MS/MS	8500 (FWHM)	100	APCI	HP-5MS (15 × 0.25 × 0.25)	Splitless	45	> 10 μg/kg	Portolés et al. (2010)
Fresh produce	QuEChERS	TOF	Full Scan	6700 (FWHM)	55	EI	HP-5MS (30 × 0.25 × 0.25)	Splitless	45	> 10 μg/kg	Cervera et al. (2012)
Fresh produce	ACN Extraction/SPE Cleanup	Quad-TOF	Full Scan and MS/MS	13,500 (FWHM)	187	EI	DB-35MS (30 × 0.25 × 0.25)	Splitless	36.3	> 5 μg/kg	Zhang et al. (2012)
Fresh produce	QuEChERS	Quad-TOF	Full Scan and MS/MS	18,000 (FWHM)	132	APCI	DP-5MS (30 × 0.25 × 0.25)	Pulsed splitless	45	> 10 μg/kg	Portolés et al. (2014)
Fresh produce	ACN Extraction/SPE Cleanup	Quad-TOF	Full Scan and MS/MS	12,500 (FWHM)	165	EI	DB-35MS (30 × 0.25 × 0.25)	Splitless	36.3	> 5 ng/mL	Zhang et al. (2014)
Fresh produce	EtOAc Extraction	Quad-TOF	Full Scan and MS/MS	12,000 (FWHM)	71	NCI	HP-5MS UI (15 × 0.25 × 0.25)	Splitless	40.5	> 1 μg/kg	Besil et al. (2015)
Fresh produce	ACN Extraction/SPE Cleanup	Quad-TOF	Full Scan	10,000 (FWHM)	210	EI	VF-1701 MS (30 × 0.25 × 0.25)	Splitless	38	< 5 μg/kg	Cao et al. (2015)
Fresh produce	QuEChERS	Quad-Orbitrap	Full Scan	60,000 (FWHM)	64	EI	TG-OCP I (30 × 0.25 × 0.25)	Splitless	30.2	< 5 μg/kg	Mol et al. (2016)
Fresh produce	QuEChERS	Quad-Orbitrap	Full Scan	60,000 (FWHM)	210	EI	TG-5SiIMS (30 × 0.25 × 0.25)	Splitless	33.6	< 10 μg/kg	Uclés et al. (2017a,b)
Cereals and Feed	QuEChERS	Quad-Orbitrap	Full Scan/SIM	60,000 (FWHM)	86	EI	TG-OCP I (30 × 0.25 × 0.25)	PTV	27.5	< 10 μg/kg	Tienstra and Mol (2018)
Infant Food- Fruit and Vegetables	QuEChERS	Quad-Orbitrap	Full Scan	60,000 (FWHM)	15	EI	TG-5SiIMS (30 × 0.25 × 0.25)	Splitless	35.6	1 μg/kg	Lozano et al. (2018)
Fresh produce	ACN Extraction/SPE Cleanup	Quad-TOF	Full Scan and MS/MS	14,000 (FWHM)	439	EI	VF-1701 MS (30 × 0.25 × 0.25)	Splitless	38	< 10 μg/kg	Li et al. (2018)
LC-HRMS											
Fresh produce	QuEChERS	TOF	Full Scan	Not provided	101	ESI	C8 (15 cm × 4.6 mm × 5 μm)	On-column	45	0.04–120 μg/kg	Ferrer et al. (2007)
Fresh produce	MeOH:Water Extraction	Quad-TOF	Full Scan and MS/MS	10,000	11	ESI	C18 (5 cm × 2.1 mm × 1.7 μm)	On-column	26.1	0.47–12.5	Grimalt et al. (2010)

Fresh produce	QuEChERS	Quad-TOF	Full Scan and MS/MS	15,000	138	ESI	C18 (10 cm × 2.1 mm × 1.7 μm)	On-column	14	10 μg/kg	<a href="#">Wang and Leung (2009)</a>
Fresh produce	QuEChERS	TOF	Full Scan	10,000 and 20,000	300	ESI	C18 (5 cm × 4.6 mm × 1.8 μm)	On-column	15	> 10 μg/kg	<a href="#">Mezcua et al. (2011)</a>
Fresh produce	QuEChERS	Orbitrap	Full Scan	50,000	240	ESI	C18 (10 cm × 2.1 mm × 1.8 μm)	On-column	15	> 1 μg/kg	<a href="#">Kaufmann et al. (2012)</a>
Fresh produce	QuEChERS	Orbitrap	Full Scan	50,000	556	ESI	C18 (10 cm × 3 mm × 3 μm)	On-column	28.5	> 10 μg/kg	<a href="#">Mol et al. (2012)</a>
Fresh produce	QuEChERS	TOF	Full Scan	Not provided	949	ESI	C18 (5 cm × 4.6 mm × 1.8 μm)	On-column	41	Not provided	<a href="#">Polgár et al. (2012)</a>
Fresh produce	QuEChERS	Quad-Orbitrap	Full Scan/DDA	70,000/17,500	166	ESI	C18 (10 cm × 2.1 mm × 1.9 μm)	On-column	14	10 μg/kg	<a href="#">Wang et al. (2012)</a>
Fresh produce	QuEChERS	LIT-Orbitrap	Full Scan/DDA (MS/MS, MS3)	7500	54	ESI	C18 (5 cm × 2.1 mm × 1.7 μm)	On-column	15	> 0.1 ng/mL	<a href="#">Farré et al. (2014)</a>
Baby food	QuEChERS	Quad-Orbitrap	Full Scan/DDA	70,000/17,500	258	ESI	C18 (10 cm × 2.1 mm × 2.6 μm)	On-column	15	> 10 μg/kg	<a href="#">Jia et al. (2014)</a>
Fresh produce	QuEChERS	Quad-TOF	Full Scan/AIF	26,500	199	ESI	C18 (10 cm × 2.1 mm × 1.7 μm)	On-column	17	10 μg/kg	<a href="#">López et al. (2014)</a>
Fresh produce	QuEChERS	Quad-Orbitrap	Full Scan/DDA	70,000/17,500	451	ESI	C18 (10 cm × 2.1 mm × 1.9 μm)	On-column	14	10 μg/kg	<a href="#">Wang et al. (2014)</a>
Baby food	ACN Extraction	Orbitrap	Full Scan/AIF	25,000/10,000	260	ESI	C18 (10 cm × 2.1 mm × 1.7 μm)	On-column	14	0.5–50 μg/kg	<a href="#">Gomez-Perez et al. (2014)</a>
Fresh produce	QuEChERS	Quad-Orbitrap	Full Scan/vDIA	70,000/35,000	184	ESI	C18 (10 cm × 3 mm × 3 μm)	On-column	20	> 10 μg/kg	<a href="#">Zomer and Mol (2015)</a>
Fresh produce	QuEChERS	Quad-TOF	Full Scan	40,000	161	ESI	C18 (15 cm × 2.1 mm × 2.2 μm)	On-column	65	> 0.0005 ng/mL	<a href="#">Amelin et al. (2016)</a>
Fresh produce	QuEChERS	Quad-TOF	Full Scan	Not provided	152	ESI	C18 (10 cm × 2.1 mm × 1.8 μm)	On-column	15	10–40 μg/kg	<a href="#">Munaretto et al. (2016)</a>
Fresh produce	QuEChERS	Quad-TOF	Full Scan/AIF	15,000–20,000	342	ESI	C18 (5 cm × 2.1 mm × 1.8 μm)	On-column	19	> 1 μg/kg	<a href="#">Pérez-Ortega et al. (2016)</a>
Fresh produce	QuEChERS	Quad-TOF	Full Scan/DDA	Not provided	546	ESI	C18 (10 cm × 2.1 mm × 1.7 μm)	On-column	18	> 0.3 μg/kg	<a href="#">Zhu et al. (2017)</a>
Fresh produce	QuEChERS	Quad-Orbitrap	Full Scan/vDIA	70,000/35,000	166	ESI	C18 (15 cm × 2.1 mm × 2.6 μm)	On-column	17	> 10 μg/kg	<a href="#">Rajski et al. (2017)</a>
Fresh produce	QuEChERS	Quad-Orbitrap	Full Scan/AIF	70,000/17,500	64	ESI	C18 (15 cm × 75 μm × 3 μm)	On-column	47	> 10 μg/kg	<a href="#">Moreno-González et al. (2017)</a>
Fresh produce	QuEChERS	Quad-Orbitrap	Full Scan/vDIA	70,000/17,500	448	ESI	C18 (10 cm × 2.1 mm × 1.9 μm)	On-column	14	10 μg/kg	<a href="#">Wang et al. (2017)</a>
Fresh produce	ACN Extraction/SPE Cleanup	Quad-TOF	Full Scan/AIF	Not provided	485	ESI	C18 (10 cm × 2.1 mm × 3.5 μm)	On-column	27	> 10 μg/kg	<a href="#">Pang et al. (2018)</a>

2017). For a non-targeted procedure, DIA is often used where the entire mass range is divided into  $m/z$  isolation segments by the quadrupole. The quadrupole isolation window is opened to larger mass segments i.e., 25–100 Da and each mass window is subjected to fragmentation one segment at a time. AIF is the least selective mode of fragmentation because there is no quadrupole pre-selection involved as all ions undergo fragmentation, while DDA is the most selective and specific. DIA lies in between the other two modes, where the degree of selectivity is based on the width of the mass isolation windows.

The first demonstration of DIA was performed by Zomer and Mol (2015) using a non-targeted approach of data acquisition with fragmentation. Their non-targeted approach used a combination of a full-scan acquisition event followed with variable DIA (vDIA) MS/MS for screening applications. vDIA involves smaller isolation windows of 100  $m/z$  width for the mass range 100–500  $m/z$  and larger isolation windows of 500  $m/z$  for the range of 500–1000  $m/z$ .

Other laboratories (Rajski et al., 2017; Goon et al., 2018; Wang et al., 2017) have also used vDIA acquisition for pesticide screening. Wang et al. (2017) developed a target screening of 448 pesticide residues in fruits and vegetables using ultrahigh-performance liquid chromatography coupled with electrospray ionization quadrupole-Orbitrap high-resolution mass spectrometry (UHPLC-ESI-QOrbitrap) and a compound database (CDB). Their work, for the first time, described a procedure for the development of a CDB and its applications for target screening using LC/ESI QOrbitrap Full MS/dd-MS/MS and Full MS/DIA. They optimized screening applications by addressing in-spectrum mass correction, retention time alignment, and response threshold adjustment when building the CDB, which are critical parameters to minimize false negative/positive rates for routine screening. The validated target screening method is capable of screening at least 94% and 99% of 448 pesticides at 10 and 100  $\mu\text{g/kg}$ , respectively, in fruits and vegetables without having to evaluate every compound manually during data processing, significantly reducing the workload in routine practice. The advantage of DIA analysis is that the instrumental setup is very simple, no specific retention times and masses are required *a priori*, and the only scans required are mass ranges provided in the mass isolations windows. This makes DIA very manageable compared to DDA and provides better specificity than AIF.

## Conclusions

Current instruments are more sensitive and improved than their predecessors but there are existing problems regarding matrix effects with GC-MS and LC-MS. Ion suppression is a problem for LC-MS because matrix components tend to compete with the pesticide for charge in the ion source. Matrix enhancement in GC-MS is a common observation because the enhanced analyte signal is a result of the co-extractives in the matrix (as opposed to a calibration standard prepared in solvent) protecting the analytes of interest in the heated regions (i.e., liner) of the gas chromatograph. These matrix effects on GC-MS and LC-MS systems require the use of matrix-matched calibration standards or the method of standard addition for quantitation. In addition to the instrument itself, improvements in sample preparation has also evolved and this will continue.

GC-MS was the primary instrument for the analysis of pesticides, but because many of the pesticides that were compatible for GC and GC-MS analysis are no longer in use and are being replaced by less toxic and persistent pesticides that also tend to be thermally-labile, they are more suited for LC-MS analysis. Although there are advances in GC-MS instruments, such as APCI interfaces for providing less fragmentation compared to electron impact ionization and other mass analyzer platforms such as high speed TOF for multidimensional gas chromatography (GC $\times$ GC-TOF MS), high resolution TOF, QTOF, Orbitrap, and QOrbitrap mass analyzers, the number of pesticides primarily used for GC-MS analyzers is becoming smaller compared to the increasing number of pesticides analyzed by LC-MS procedures.

The use of GC-QqQ-MS/MS and LC-QqQ-MS/MS tend to be the current standard of analyzing pesticides because both identification and quantitation can be achieved in a single injection. These techniques are sensitive enough to detect most of the pesticides at the default tolerances or assigned limit of quantitation. Criteria for the identification of pesticides have been well studied and established and are based on MS/MS results. Sample preparation procedures for multipesticide analysis have evolved and have become streamlined and less complexed due to the introduction and continual use of mass spectrometry. Recent interest in chromatographic methods coupled to HRMS platforms forecasts a future where these instruments may play an important role in screening. Screening tends to require less validation than quantitative methods but requires the use of robust software tools, compound databases, and mass spectral libraries that can search for more pesticides as well as other residues and chemical contaminants, toxins, and important chemical analytes. The use of compound databases and/or libraries can reduce the need to have available pesticide standards that are needed for targeted GC-MS/SIM, GC-MS/MS or LC-MS/MS methods.

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## Pesticides: Evaluation Process in the EU

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### Glossary

The Glossary below is mainly taken from Regulation EC (No) 1107/2009 and from the European Commissions' webpage ([https://ec.europa.eu/food/plant/pesticides\\_en](https://ec.europa.eu/food/plant/pesticides_en))

**Authorisation of a plant protection product** An administrative act by which the competent authority of a Member State authorises the placing on the market of a plant protection product in its territory.

**Competent authority** Any authority or authorities of a Member State responsible for carrying out the tasks established under this Regulation.

**Environment** Waters (including ground, surface, transitional, coastal and marine), sediment, soil, air, land, wild species of fauna and flora, and any interrelationship between them, and any relationship with other living organisms.

**Good Agricultural Practise (GAP)** The nationally recommended, authorised or registered safe use of plant protection products under actual conditions at any stage of production, storage, transport, distribution and processing of food and feed.

**Harmful organisms** Any species, strain or biotype belonging to the animal kingdom or plant kingdom or pathogenic agent injurious to plants or plant products.

**Maximum residue level (MRL)** Highest level of a pesticide residue that is legally tolerated in or on food or feed when pesticides are applied correctly (Good Agricultural Practice).

**Metabolite** Any metabolite or a degradation product of an active substance, safener or synergist, formed either in organisms or in the environment. A metabolite is deemed relevant if there is a reason to assume that it has intrinsic properties comparable to the parent substance in terms of its biological target activity, or that it poses a higher or comparable risk to organisms than the parent substance or that it has certain toxicological properties that are considered unacceptable. Such a metabolite is relevant for the overall approval decision or for the definition of risk mitigation measures.

**Micro-organisms** Any microbiological entity, including lower fungi and viruses, cellular or non-cellular, capable of replication or of transferring genetic material.

**Pesticide** Something that prevents, destroys, or controls a harmful organism ("pest") or disease, or protects plants or plant products during production, storage and transport. The term includes, amongst others: herbicides, fungicides, insecticides, acaricides, nematocides, molluscicides, rodenticides, growth regulators, repellents, rodenticides and biocides. They are primarily used in the agricultural sector but also in forestry, horticulture, amenity areas and in home gardens.

**Plants** Live plants and live parts of plants, including fresh fruit, vegetables and seeds.

**Plant products** Products of plant origin in an unprocessed state or having undergone only simple preparation, such as milling, drying or pressing, but excluding plants.

**Plant protection products** "Pesticides" that protect crops or desirable or useful plants.

**Producer** A person who manufactures plant protection products, active substances, safeners, synergists, co-formulants or adjuvants on his own, or who contracts this manufacturing to another party, or a person designated by the manufacturer.

**Residues** One or more substances present in or on plants or plant products, edible animal products, drinking water or elsewhere in the environment and resulting from the use of a plant protection product, including their metabolites, breakdown or reaction products.

**Substances** Chemical elements and their compounds, as they occur naturally or by manufacture, including any impurity inevitably resulting from the manufacturing process.

**Tests and studies** Investigations or experiments whose purpose is to determine the properties and behaviour of an active substance or of plant protection products, predict exposure to active substances and/or their relevant metabolites, determine safe levels of exposure and establish conditions for the safe use of plant protection products.

**Vulnerable groups** Persons needing specific consideration when assessing the acute and chronic health effects of plant protection products. These include pregnant and nursing women, the unborn, infants and children, the elderly and workers and residents subject to high pesticide exposure over the long term.

**Zone** A group of Member States as defined in Annex I Regulation (EC) No 1107/2009; Zone A – North (Denmark, Estonia, Latvia, Lithuania, Finland, Sweden); Zone B – Centre (Belgium, Czech Republic, Germany, Ireland, Luxembourg, Hungary, Netherlands, Austria, Poland, Romania, Slovenia, Slovakia, United Kingdom); Zone C- South (Bulgaria, Greece, Spain, France, Italy, Cyprus, Malta, Portugal).

## Legal Basis of the Evaluation of Active Substances of Plant Protection Products in the EU

The term “pesticides” is commonly used broader than “plant protection products” (PPP) and covers also products such as biocides. In this article the terms “pesticides” and “PPP” are used as synonyms, and do not refer to biocides.

In the EU the evaluation of a new active substance, amendment or renewal of approval of an active substance, that can be used in plant protection products follows a comprehensive process with different responsibilities for risk assessors in the European Union Member States (MS) and the European Food Safety Authority (EFSA), and risk managers in the European Commission (EC) and EU MS. The respective legislative process and procedure is outlined in the “General Food Law”, Regulation EC (No) 178/2002 (EU, 2002) and specific pesticide Regulations such as Regulation (EC) No 1107/2009 (EU, 2009) related to new active substances, and in the Commission Implementing Regulation EU 844/2012 (EU, 2012a) for the renewal of approval of active substances. Regulation EU 686/2012 (EU, 2012b) provides information on the evaluation of active substances whose approval will expire between 1 January 2013 and 31 December 2018. Commission Implementing Decision 2016/C 357/05 (EU, 2016a) and Regulation EU 2016/183 (EU, 2016b) allocates MS for the evaluation of active substances which approval will expire between 1 January 2019 and 31 December 2021.

Special provisions for approval apply for basic substances, which are, generally speaking “*active substances, not predominantly used as plant protection products such as beer, fructose or sodium chloride, but which may be of value for plant protection and for which the economic interest of applying for approval may be limited*” as outlined in Art. 23 of Regulations (EC) No 1107/2009 (EU, 2009).

## The Process of the Evaluation

The process starts with a data package provided by an applicant such as a producer who wishes to receive authorisation for a new active substance or renewal of approval of an active substance, with the submission of an application to a MS, called the rapporteur MS (RMS). The RMS carries out an admissibility check of the application. If the application is admissible, the RMS notifies the applicant, the other MS, the EC and EFSA. The RMS prepares a draft assessment report (DAR) for new active substances, or - for renewal of approval - a renewal assessment report (RAR). During this process, the RMS can request additional studies or information to the applicant. The DAR/RAR provides an assessment if the active substance can be expected to meet the approval criteria. This report is submitted to the EC and EFSA. It should be noted that there is a common structure of the DAR agreed by the EU. For basic substances the dossier is sent directly to the EC, which sends a mandate to EFSA. EFSA carries out an independent scientific review to ensure consistency in the evaluation. This process is also called “peer review process” and comprises the following steps:

1. Commenting: EFSA makes the summary dossier and the DAR/RAR available to the public, all MS, EC, ECHA, and the applicant, and invites all to submit comments on the DAR/RAR during a 60 day period. EFSA also comments on the DAR/RAR. The RMS collates the comments in a table.
2. Consideration of the comments: EFSA invites the RMS to address the comments and the applicant has the possibility to react. In addition, EFSA can ask the applicant to submit additional information to MS, EC, and EFSA during a maximum period of 90 days. The need for additional information is identified between EFSA, EC and RMS.
3. Expert meeting: If needed, EFSA organises expert consultation meetings in the different disciplines, e.g. toxicology, residues, ecotoxicology with MS experts, including the RMS. The outcome of the meeting is summarized in a report.
4. Drafting of the EFSA conclusions: EFSA has to adopt within 120 days - or 150 days if there is an expert consultation meeting - after the commenting period a conclusion whether the active substance can be expected to meet the approval criteria. The MS receive the final draft conclusion for written consultation before the adoption by EFSA. The EFSA conclusion is the conclusions of the peer-reviewed assessment, identifies data gaps, issues which could not be finalised and critical areas of concern. It may include options for risk mitigation measures.

To ensure the same level of protection for humans, animals and the environment, the approval or non-approval is done at EU level. On the basis of the EFSA conclusion the EC prepares a proposal for approval or non-approval of an active substance. A committee comprising MS representatives, called the Standing Committee on Plants, Animals, Food and Feed (PAFF), votes on the EC proposal. The final legislative decision whether or not to approve the active substance is published in the EU Official Journal. This information is also available in the EU pesticide database of active substances. This database is kept regularly updated and contains also information on EU pesticides maximum residue levels (MRLs) (link provided under Chapter “relevant websites”). A new active substance is usually approved for 10 years, while an application for renewal of approval can be granted for up to 15 years. The whole process can vary greatly, depending on the complexity of the dossier, and may take between 2.5 and 3.5 years.

## The Application

The application consists of a summary and a dossier, and provides information on i) the intended use of the active substance, which must be representative, this means used on a widely grown crop, in each geographical area (zone), ii) the amount to be applied to protect the crop, as well as iii) the pesticide residue level remaining on the crop after such application (EU, 2009). In addition, the dossier contains the respective scientific information and the results of tests and studies, in line with the regulatory data requirements (see Chapter 1.3). In addition, the dossier must contain scientific peer-reviewed open literature related to the active substance

and its relevant metabolites providing information on possible side-effects on human and animal health, the environment and non-target species.

A similar procedure needs to be followed when the authorisation of a PPP is asked by an applicant to a MS (EU, 2009). Under EU law, MS are responsible for the evaluation and authorisation of the use of an active substance-based formulation only if the pesticide active substance is on the EU list, although MS have the possibility for exceptional authorisations of products containing non-approved substances. Applications are assessed and approved by the national authority where they are submitted. For pesticide authorisation, the EU is split into three zones: North, South and Centre where countries with similar agricultural, plant health and environmental conditions are grouped together. When a pesticide is authorised for use in one MS, its use could be also allowed in other MS of the same zone. This process is called mutual recognition.

### Data Requirements for Active Substances

For new or renewal of approvals of active substances which expired on 1 January 2016 or later, the data requirements are outlined in Commission Regulation (EU) No 283/2013 (EC, 2013a), commonly referred to as “new data requirements”. A high-level overview of the data requirements, meaning what type of information needs to be provided in the dossier by the applicant is outlined below, according to the Annex to Commission Regulation (EU) No 283/2013.

- Identity of the active substance
- Physical and chemical properties of the active substance
- Further information on the active substance (e.g. use, function, mode of action)
- Analytical methods
- Toxicological and metabolism studies
- Residues in or on treated products, food and feed
- Fate and behaviour in the environment
- Ecotoxicological studies
- Literature data
- Classification and labelling

Furthermore, specific guidance documents and test methods based on international or European standards for the areas mentioned above are available to assist applicants and risk assessors. They are summarized in the EU notice (EC, 2013c), or - if developed after 2013 - available on the Website of the Directorate General SANTE (link provided under Chapter “relevant websites”). Guidance documents can be developed by EFSA, are published on the Authority’s Website, and may be adopted by MS representatives in PAFF, e.g. the EC guidance on the assessment of exposure of operators, workers, residents and bystanders in risk assessment for plant protection products (EFSA, 2014; EC, 2017). As long as an EFSA guidance document is not adopted by PAFF, e.g. guidance on risk assessment on bees (EFSA, 2013), or guidance on the establishment of the residue definition for dietary risk assessment (EFSA, 2016), it is not formally applicable.

For the data requirements for active substances which approval expired before 1 January 2016, transitional measures apply as stated under Article 3 (active substances) and 4 (plant protection products) of Commission Regulation (EU) No 283/2013 (EC, 2013a). Within this context, reference to Commission Regulation (EU) No 544/2011 (EU, 2011a) for active substances and Commission Regulation (EU) No 545/2011 (EU, 2011c) for plant protection products, the so called “old data requirements” is made. The EC guidance document on the interpretation of transitional measures for the data requirement for active substances and plant protection products according to Regulation (EC) No 283/2013 and Regulation (EC) No 284/2013 provides further details (EC, 2015).

The above is relevant for active substances, however it should be noted that the “new” data requirements for plant protection products are available in Regulation (EU) No 284/2013 (EU, 2013b) and the specific guidance documents and test methods are summarized in an EU notice (EC, 2013d). The “old data requirements” for plant protection products are outlined in Commission Regulation (EU) No 545/2011 and in the EC guidance document on the interpretation of transitional measures for the data requirements for active substances and plant protection products according to Regulation (EC) No 283/2013 and Regulation (EC) No 284/2013 (EC, 2015). In addition, MS have to perform the assessment in line with the “Uniform Principles for the Evaluation and the Authorisation of Plant Protection Products” adopted by Commission Regulation (EU) No 546/2011 (EU, 2011b).

### Risk Assessment of Active Substances

The objective of the risk assessment of an active substance is to evaluate the safety of an active substance and its residues, this means assessing if the active substance has any harmful effect on human and animal health, including those on vulnerable groups or any unacceptable effects on the environment. This is done by concluding whether the active substance can be expected to meet the approval criteria, which are outlined in Article 4 of Regulation (EC) No 1107/2009 (EU, 2009). This is a stepwise approach by first establishing if the active substance or renewal of approval of an active substance fulfils first the approval criteria related to genotoxicity, carcinogenicity, reproductive toxicity and fate and behaviour as outlined under 3.6.2 to 3.6.4 and 3.7 of Annex II of Regulation (EC) No 1107/2009 (EU, 2009). If these criteria are satisfied the assessment shall continue to establish whether the other approval criteria set out in points 2 and 3 of Annex II are satisfied. Point 3.6.5 outlines the criteria related to endocrine disrupting properties. It



should be noted that on 12 December 2017 the measures concerning specific scientific criteria for the determination of endocrine disrupting properties proposed by the EC were agreed with a qualified majority by PAFF, and subsequently adopted by the EC. These measures will be published in the EU Official Journal in 2018.

The EFSA conclusion is the conclusion on the peer-reviewed assessment (EFSA, 2016).

Appendix A of the EFSA conclusion contains a “list of end points” for an active substance and the representative formulation, listing the agreed end points of studies submitted in support of the application.

## **Legal Basis of Pesticides Residues in Food and Feed**

Pesticides or plant protection products - when applied to plants or plant products - might leave traces, which are called pesticides residues. However, as outlined in Article 4, 2a, and 2b of Regulation (EC) No 1107/2009 (EU, 2009), residues of plant protection products “shall not have any harmful effects on human health, including that on vulnerable groups, or animal health, taking into account known cumulative and synergistic effects where the scientific methods accepted by the Authority [EFSA] to assess such effects are available, or on groundwater” and “they shall not have any unacceptable effect on the environment”.

Regulation (EC) No 396/2005 establishes the rules governing the setting and the review of pesticide maximum residue levels (MRLs) at European level (EU, 2005). In the EU the highest legally permitted amount of pesticides on food or feed when applied correctly, meaning according to the “Good Agricultural Practice”, is called the maximum residue level (MRL). The amounts of residues in food must be as low as possible, in view to protect consumers and vulnerable groups such as children and the unborn. If there is no specific MRL set, a default value of 0.01 mg/kg is applicable. In the EU, MRLs apply to products of plant and animal origin and parts thereof and listed in Annex I of Regulation (EC) No 396/2005 to be used as fresh, processed and/or composite food or feed in or on which pesticide residues may be present. Annex I comprises MRLs on around 315 fresh and processed products of different pesticides (app. 1100) which are used in the EU and Third countries, and which are recorded in the database of pesticides EU MRLs, published on the Website of the Directorate General SANTE (link provided under Chapter “relevant websites”) in the EU pesticides database, and are regularly updated. EU MRLs are also applicable to food imported into the EU.

## **The Process of Setting MRLs**

The MRL setting is a comprehensive process with different responsibilities for risk assessors in the European Union Member States (MS) and the European Food Safety Authority (EFSA), and for risk managers in the European Commission (EC) and the MS. The respective legislative process is outlined in specific Regulations, such as Regulation (EC) No 1107/2009 and in particular in Regulation (EC) No 396/2005 (EU, 2005). If the intended use of the pesticide requires an amendment of the existing MRL set out in Regulation EC 396/2005, the details are outlined under Article 6 to 11 of Regulation (EC) No 396/2005. The process to modify the MRL starts with an application which needs to be submitted by the applicant to a MS, called evaluating MS (EMS). The EMS prepares an initial risk assessment and an “evaluation report (ER)”. This report is reviewed by EFSA. EFSA issues the outcome in a “reasoned opinion”. MS and EC decide whether to accept the proposed MRL, and if so, the MRL is listed in Annex II (new MRL) or Annex III (modified MRL) of Regulation (EC) No 396/2005. Following this, the MS can authorise the pesticide. A MRL application may also be submitted by importers in case the legal limits need to be revised for food produced in third countries that is intended for the EU market.

In addition, EFSA is asked to carry out a review of all existing MRLs 12 months after the inclusion or non-inclusion of an active substance in Annex I to Directive 91/414/EEC. Under Directive 91/414/EEC, only a few representative uses were evaluated, whereas MRLs set out in Regulation (EC) No 396/2005 (EU, 2005) have to accommodate all uses authorised within the EU, and uses authorised in third countries that have a significant impact on international trade. EFSA developed the Pesticide Residues Overview File (PROFile) which is an inventory of all pesticide residue data relevant to the risk assessment and MRL setting for a given active substance. The PROFile provides data on the nature and magnitude of residues in primary crops; processed commodities; rotational crops; livestock commodities; and the analytical methods for enforcement of the proposed MRLs. The process is slightly different to the one outlined above on modification of MRLs. The RMS submits to EFSA the PROFile and the ER, and during a completeness check period, MS can submit additional information. Based on this overall information, EFSA prepares a draft reasoned opinion, circulates the reasoned opinion for written comments to MS. EFSA considers these comments during the finalisation of the reasoned opinion. In 2016, the EC published a guidance document on the MRL setting procedure aiming to providing clarity on the various steps involved in the procedure, on the timelines and on specific circumstances related to the MRL setting process (EU, 2016c). Since spring 2017 this procedure was slightly revised to gain efficiency. The first review using this new procedure is related to the review of existing MRLs for glyphosate, which will be published by EFSA beginning 2018.

## **Data Requirements for Pesticide Residues**

The date of submission of the application to the EMS determines which data requirements and guidance documents are applicable. This could be either the “new data requirements” outlined in Commission Regulation (EU) No 283/2013 (EU, 2013a) or the “old data requirements” outlined in Commission Regulation (EU) No 544/2011 (EU, 2011a) (see also Chapter 1.3). The data requirements which are applicable in the transitional period of “new” and “old” data requirement are provided in the EC guidance document on



the interpretation of transitional measures for the data requirements for active substances and plant protection products according to Regulation (EC) No 283/2013 and Regulation (EC) No 284/2013 (EC, 2015), respectively. In addition, the assessment is performed in accordance with the legal provisions of the Uniform Principles for the Evaluation and the Authorisation of Plant Protection Products (EU, 2011b).

To assist applicants and risk assessors, specific technical guidelines are available and are outlined under Chapter 1.3 related to the “new” data requirements. The guidance documents related to “old data requirements” established in Regulation (EU) No 544/2011 (EC, 2011a) are published on the Website of the Directorate General SANTE (link is provided under “relevant websites”) and refer for example to metabolism and distribution in plants (EU, 1997a), general recommendations for the design, preparation and realisation of residue trials (EU, 1997b), testing of plant protection products in rotational crops (EU, 1997c), processing studies (EU, 1997d), metabolism and distribution in animals (EU, 1997e), storage stability studies (EU, 1997f), and MRL plant calculator EU-OECD (OECD, 2011).

### The Risk Assessment of Pesticide Residues

EFSA carries out a risk assessment based on the toxicological reference values (acute reference dose and/or acceptable daily intake) for the active substance, and the expected exposure of consumer groups including e.g. children and vegetarians, based on the residues expected in food. EFSA uses the Pesticide Residue Intake Model, called PRIMo, which includes national food consumption data and unit weights provided by MS, to estimate the chronic (long-term) and acute (short-term) dietary consumer exposure to pesticide residues. A new version (rev.3) of PRIMo was released at the beginning of 2018. The risk assessment, including the MRL proposals, is presented in “reasoned opinions” and may be approved by the EC and MS. The reasoned opinions need to provide information whether the analytical method for routine monitoring is appropriate, the anticipated LOD for the pesticide/crop combination, and an assessment of the risk for exceeding the acceptable daily intake or acute reference dose in light of the modification of the MRL, the contribution of the residues in the product for which the MRL was requested to the overall intake. The most critical information is provided in a list of end points annexed to the reasoned opinion. It provides information on the studies assessed by EFSA in the framework of the EU pesticides peer review, including the end points of studies submitted in support of the current MRL application.

### Monitoring of Plant Protection Products

Inspection services in MS verify if the residues in the food comply with the legal limits, i.e. the EU harmonised MRLs as laid down in Regulation (EC) 396/2005. According to Article 31 of Regulation (EC) No 396/2005, MS are requested to share the results of the official controls and other relevant information with the EC, EFSA and the other MS. Specialised laboratories test more than 700 pesticides in around 370 food products/food groups and more than 20 million individual data are reported to EFSA. This data is summarized on an annual basis in the EU pesticide monitoring report, as laid down in Article 32 of Regulation (EC) No 396/2005. EFSA also carries out a dietary exposure assessment. EU risk managers, the EC and MS use this data to e.g. carry out a review of existing MRLs, or enhance future monitoring programmes. The latest results from the 2015 EU report on pesticide residues in food demonstrated that 97% of 84,000 samples were compliant with the legal limits, the respective MRLs (EFSA, 2017).

### Urgent Scientific Advice

Under specific circumstances, the EC can also ask urgent scientific advice to EFSA in case there are questions related to the potential health risk due to the presence of e.g. elevated levels of an active substance in food. For example, this was the case in 2009 where the presence of nicotine in wild mushrooms of up to 0.5 mg/kg was identified. EFSA carried out a risk assessment by estimating the short and long-term dietary exposure to nicotine compared to the derived health based guidance values, the acute reference dose, and the acceptable daily intake. Based on the scientific advice by EFSA, risk managers were able to take measures to allow the placing on the markets of dried mushrooms from the 2008 season, provided that they are safe for EU consumers (EFSA, 2009).

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<sup>a</sup>The positions and opinions presented in this article are those of the author alone and do not necessarily represent the views/any official position or scientific work of EFSA. For the views or scientific outputs of EFSA, please consult its Website under <http://www.efsa.europa.eu>.

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- <http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN> – EC, EU Pesticides Database (information on active substances and pesticides EU MRLs).
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- <https://www.efsa.europa.eu/en/applications/pesticides> – EFSA pesticides application section.
- <http://www.efsa.europa.eu/en/applications/pesticides/regulationsandguidance> – EFSA guidance documents pesticides.
- EFSA guidance documents MRLs.
- [http://www.efsa.europa.eu/en/interactive\\_pages/pesticides\\_authorisation/PesticidesAuthorisation#activesubstances](http://www.efsa.europa.eu/en/interactive_pages/pesticides_authorisation/PesticidesAuthorisation#activesubstances) – EFSA. How Europe ensures pesticides are safe. Interactive info graphic.
- <http://eur-lex.europa.eu/homepage.html> – European Union Official Journal.
- <http://www.oecd.org> – OECD (Organisation for Economic Co-operation and Development).

# Polycyclic Aromatic Hydrocarbons in Food and Feed

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## General

Depending of the area of work and the closely-linked number of markers used to estimate the polycyclic aromatic hydrocarbons (PAHs) content in different matrices, many people tend to limit their thoughts to one to 16 compounds. In fact, PAHs constitute a whole class of substances of fused aromatic rings, which are composed of carbon and hydrogen atoms only. PAHs are ubiquitous, semi-volatile substances. They originate from natural and anthropogenic sources. Natural sources for PAH emissions could be forest, bush-, and prairie fires, or volcano eruptions (Manzetti, 2013). Anthropogenic sources comprise domestic and industrial combustion processes, aluminium and coke production, crude oil spills, as well as exhausts of all kinds of combustion engines (Achten and Hofmann, 2009; Djinojic-Stojanovic et al., 2013; Mastral and Callen, 2000). High molecular weight PAHs may be formed during catalytic hydrocracking of petroleum or may be found amongst others in coal tar pitches, carbon black, or Diesel particulate matter (Fetzer, 2007; Alegbeleye et al., 2017). Rubber debris of wheels of cars and trucks containing carbon black are another source for the release of PAHs into the environment. The European Environment Agency (EEA) reported for the period of 1990 to 2011 a decrease of PAH emissions in EU by 58%. The total release of PAHs in 27 EU Member States was in 2011 still almost 1100 t, which however is an underestimation due to the limitations in the number of PAHs covered and gaps in reporting (Schindlbacher et al., 2013).

The formation reactions of PAHs depend on the type of fuel and reaction conditions (Richter and Howard, 2000). Mechanisms governing the formation of aromatic compounds in differently fuelled flames were reviewed elsewhere (Richter and Howard, 2000). The pattern of PAHs and derived indices are often used to conclude on their origin (Stogiannidis and Laane, 2015).

The higher the molecular mass of PAHs becomes, the lower is their water solubility and the higher their octanol/water partition coefficient, indicating increasing unpolarity. The simplest PAH is naphthalene, containing two fused aromatic rings. The International Union for Pure and Applied Chemistry (IUPAC) developed a harmonised system for denominating PAHs (International Union, 1998; Ehrenhauser, 2015). However, it did not find full acceptance, which is reflected in scientific literature by the preferred use of common names for denoting PAHs rather than employing IUPAC terminology. An overview of IUPAC and alternative names was presented by the US National Institute of Standards and Technology (NIST), which compiled the structures, names, CAS numbers and some physical data of 660 PAHs (Sander and Wise, 1997). This report evolved to a free accessible online database (NIST PAH Database, 2015).

Large PAHs are of less importance in the food area. The largest compounds that were monitored so far in a wider range of food are dibenzopyrenes, which consist of six fused benzene rings. However, their content in food is usually very low. More abundant are in food PAHs with lower molecular weight.


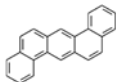
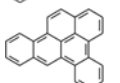
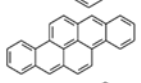
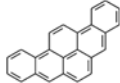
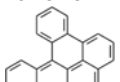


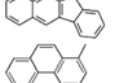




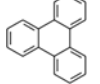
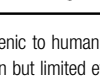
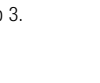
In addition to the terrestrial research on PAHs, it shall be mentioned that PAHs are common and abundant compounds in space (Boersma et al., 2013).

## Toxicology of PAHs

Despite not knowing the compounds, first observations of the carcinogenicity of PAHs were made end of the 18th century by Sir Percival Pott, who linked scrotal cancer of young chimney sweepers to the exposure to soot (Earl, 1819). Later it was proven by experiment that PAHs contained in soot were responsible for the cancer. The acute toxicity of PAHs is low. However, metabolic activation to form di-epoxides, radical cations, or o-quinones may lead to the formation of stable DNA adducts, which might initiate both mutations and cancer (Xue and Warshawsky, 2005; Ewa and Danuta, 2017). The metabolism and toxicology of PAHs, and their risk to human health were assessed and reviewed by several authors and international organizations, such as the Scientific Committee on Food (SCF), the FAO/WHO Joint Experts Panel on Food Additives (JECFA), the International Agency for Research on Cancer (IARC), and the European Food Safety Authority (EFSA) (European Community, 2002; Xue and Warshawsky, 2005; International Agency, 2010, 2012; Joint FAO/WHO Expert Committee, 2006; Culp et al., 1998; Rota et al., 2014; Koganti et al., 2001; European Food Safety Authority, 2008). The IARC assessed the carcinogenicity of 60 PAHs and several occupations involving coal derived PAHs. It concluded that different occupational exposures, such as during coal gasification, are carcinogenic to humans (International Agency, 2010). Additionally it identified benzo[a]pyrene as Group 1 carcinogen (carcinogenic to humans), and some other 16 PAHs as Group 2A (3 substances) respectively Group 2B (13 substances) carcinogens, which indicate probably, and respectively, possibly carcinogenic to humans (International Agency, 2010). The majority of evaluated PAHs were not classifiable as to their carcinogenicity to humans, which was frequently caused by the lack of adequate data. Details on composition of sets of PAHs assessed by the different expert panels and the outcome of the assessment are provided in Table 1.

**Table 1** Assessment of PAHs by different expert panels and composition of different priority lists

Common name	IUPAC name	Abbr.	Structure	CAS	MW g/mol	SCF	JECFA	IARC <sup>a</sup> group	16 US EPA PAHs	EU 15 + 1 priority PAHs	4 EU marker PAHs
Acenaphthene	=	AC		83-32-9	154.208	i	nr	3	x		
Acenaphthylene	=	ACL		208-96-8	152.192	i	nr	na	x		
Anthanthrene	Dibenzo[def,mno]chrysene	ATR		191-26-4	276.331	i	nr	3			
Anthracene	=	AN		120-12-7	178.229	nG	nG	3	x		
Benzo[a]anthracene	Tetraphene	BaA		56-55-3	228.288	GC	GC	2B	x	x	x
Benzo[a]fluorene	11H-Benzo[a]fluorene	BaFL		238-84-6	216.277	i	nG	3			
Benzo[b]fluorene	11H-Benzo[b]fluorene	BbFL		243-17-4	216.277	i	nr	3			
Benzo[c]fluorene	7H-Benzo[c]fluorene	BcFL		205-12-9	216.277	na	na	3		x	
Benzo[b]fluoranthene	Benzo[e]acephenanthrylene	BbFA		205-99-2	252.309	GC	GC	2B	x	x	x
Benzo[ghi]fluoranthene	=	BghiFA		203-12-3	226.272	i	nr	3			
Benzo[j]fluoranthene	=	BjFA		205-82-3	252.309	GC	GC	2B		x	
Benzo[k]fluoranthene	=	BkFA		207-08-9	252.309	GC	GC	2B	x	x	
Benzo[ghi]perylene	=	BghiP		191-24-2	276.331	G	G	3	x	x	
Benzo[c]phenanthrene	=	BcPH		195-19-7	228.288	i	nr	2B			
Benzo[a]pyrene	benzo[pqr]tetraphene	BaP		50-32-8	252.309	GC	GC	1	x	x	x
Benzo[e]pyrene	=	BeP		192-91-2	252.309	i	nr	3			
Chrysene	=	CHR		218-01-9	228.288	GC	GC	2B	x	x	x
Coronene	=	COR		191-07-1	300.352	i	nr	3			

Cyclopenta[ <i>cd</i> ]pyrene	=	CPP		27208-37-3	226.272	<b>GC</b>	<b>G</b>	2A		<b>x</b>
Dibenzo[ <i>a,h</i> ]anthracene	Benzo[ <i>k</i> ]tetraphene	DBahA		53-70-3	278.347	<b>GC</b>	<b>GC</b>	2A	<b>x</b>	<b>x</b>
Dibenzo[ <i>a,e</i> ]pyrene	Naphtho[1,2,3,4- <i>def</i> ]chrysene	DBaeP		192-65-4	302.368	<b>GC</b>	<b>GC</b>	3		<b>x</b>
Dibenzo[ <i>a,h</i> ]pyrene	Dibenzo[ <i>b,def</i> ]chrysene	DBahP		189-64-0	302.368	<b>GC</b>	<b>GC</b>	2B		<b>x</b>
Dibenzo[ <i>a,i</i> ]pyrene	Benzo[ <i>rsf</i> ]pentaphene	DBaiP		189-55-9	302.368	<b>GC</b>	<b>GC</b>	2B		<b>x</b>
Dibenzo[ <i>a,l</i> ]pyrene	Dibenzo[ <i>def,p</i> ]chrysene	DBalP		191-30-0	302.368	<b>GC</b>	<b>GC</b>	2A		<b>x</b>
Fluoranthene	=	FA		206-44-0	202.251	i	nr	3	<b>x</b>	
Fluorene	9H-fluorene	FL		86-73-7	166.219	i	nr	3	<b>x</b>	
Indeno[1,2,3- <i>cd</i> ]pyrene	=	ICP		193-39-5	276.331	<b>GC</b>	<b>GC</b>	2B	<b>x</b>	<b>x</b>
1-Methylphenanthrene	=	1 MP		932-69-9	192.256	i	nr	3		
5-Methylchrysene	=	5 MC		3697-24-3	242.314	<b>GC</b>	<b>GC</b>	2B		<b>x</b>
Naphthalene	=	NA		91-20-3	128.171	i	<b>nG</b>	2B	<b>x</b>	
Perylene	=	PE		198-55-0	252.309	i	nr	3		
Phenanthrene	=	PHE		85-01-8	178.229	i	nr	3	<b>x</b>	
Pyrene	=	PY		129-00-0	202.251	<b>nG</b>	<b>nG</b>	3	<b>x</b>	
Triphenylene	=	TRI		217-59-4	228.288	x	nr	3		

IARC classification: group 1 = carcinogenic to humans, group 2A = probably carcinogenic to human, group 2B = possibly carcinogenic to humans, group 3 = not classifiable as to carcinogenicity to humans.

G: genotoxic, nG: not genotoxic; GC: genotoxic and carcinogenic; i: included in evaluation but limited evidence or not conclusive data; na: not assessed; nr: included in evaluation, but not rated; x: included in list; = : identical with common name.

<sup>a</sup>IARC evaluation comprised additional, not listed PAHs, which were classified into group 3.

## Exposure to PAHs

For humans, different routes of exposure to PAHs were identified and extensively studied. They comprise inhalation, ingestion and skin contact. More than 500 native, alkylated, and partially hydrated PAHs were described in main stream cigarette smoke (Rodgman and Perfetti, 2006). Inhalation of combustion products and uptake via food are the primary routes of exposures to PAHs for non-smokers. The relative contribution of one or the other route cannot be generalized, as the exposure depends both of the place of residence and of eating habits. Environmental exposure to PAHs is higher in large cities and highly industrialized areas than in rural areas (Zhang and Chen, 2017; Cachada et al., 2016; Lambert et al., 2012). Also the uptake of PAHs by migration from plastic and rubber materials of consumer products through skin contact cannot be neglected (Bokkers et al., 2016). The potential leaching of PAHs from toys into the saliva of infants led to the setting of restrictive measures (European Union, 2013).

However, both JECFA and EFSA concluded that the major route of exposure of humans to PAHs is food (Joint FAO/WHO Expert Committee, 2006; European Food Safety Authority, 2008). EFSA calculated a median dietary exposure to BaP and the 4 EU marker PAHs (PAH4, see Table 1) for the mean European dietary consumer of 3.9 ng/kg body weight per day (bw/d) and 19.5 ng/kg bw/d respectively, resulting in margins of exposure (MOEs) of 17900 and 17500 respectively (European Food Safety Authority, 2008). As both MOEs were significantly above the threshold value of 10000, EFSA concluded a low concern for consumer health. The MOEs for high dietary consumers were close or even below the MOE threshold, raising potential concern for consumer health. Australia concluded for the local population that the exposure to PAHs from food is of low health concern (MOEs > 10000) and does not justify legal action. Similar was the outcome of the second French total diet study, which revealed for French consumers a low health risk from the dietary exposure to PAHs (Santonicola et al., 2017a). However, this study was performed after the EU implemented stringent maximum levels (Yu et al., 2011). Scientific literature contains besides these favourable findings also reports on the average dietary exposure of large populations to BaP amounting to about twice the median European level (Cai et al., 2012).

Exposure to PAHs, respectively to their metabolites, occurs already prenatal and continues postnatal via intake from the air and human breast milk (Perera et al., 2005; Urbancova et al., 2017; Pulkrabova et al., 2016; Santonicola et al., 2017a; Yu et al., 2011; Loutfy et al., 2017).

PAHs are not equally distributed in the diet. Certain food products bear higher risk of contamination than others. Details on the potential PAH content of food commodities such as edible oils, meat and fish, vegetables, spices and herbs, food supplements, and other relevant food products are presented further down.

## Monitoring and Maximum Levels

The interpretation of data on the PAH content of different matrices such as soil, water, air, and food is complicated by the disagreement of the composition of the sets of PAHs studied in the different areas (Wenzl et al., 2006). One has to understand that each combination of PAHs was established with a certain aim, and none of them can be used to fully picture the exposure to PAHs. However, the kind and number of monitored PAHs is in some areas more of historical relevance than founded on current requirements (Keith, 2015; Andersson and Achten, 2015). The continued monitoring of the so called 16 EPA PAHs in food, a set of 16 PAHs that was prioritized end of the 1970s by the US Environmental Protection Agency (EPA) may be mentioned in this context, as only part of them were identified as of major concern for human health (Joint FAO/WHO Expert Committee, 2006; Keith, 2015). Toxicological evaluations by JECFA and the SCF led in the EU to the establishment of a list of 16 priority PAHs, which were classified carcinogens (European Union, 2005; European Community, 2002; Joint FAO/WHO Expert Committee, 2006). For reasons of distinction from the 16 EPA PAHs, they are frequently denominated as 15+1 EU priority PAHs. An overview of the composition of the priority lists is given in Table 1.

Differences exist also in the evaluation and interpretation of analytical data. Some countries, such as EU member states favour evaluation of exposure based on individual substances, or combinations thereof (European Union, 2011). Among these countries, differences exist in the number of marker substances used to represent the overall exposure to PAHs, which is indicated by differences in the number of regulated PAHs (Table 2). Studies revealed that BaP alone is not a good indicator for exposure to PAHs as it was absent in many samples containing other PAHs. A set of four PAHs, consisting of BaP, BaA, BbFA, and CHR, was found more representative for the overall PAH content of food (European Food Safety Authority, 2008). Consequently, maximum levels (MLs) were set in the EU for the sum of these four PAHs in addition to the MLs for BaP alone (Table 2) (European Union, 2011, 2015a, 2015b).

Other countries such as Australia, Canada and the USA use toxic equivalency factors (TEFs) to estimate the potential threat brought by PAHs contained in certain food product to human health (Food Standards, 2004). The latter system works in analogy to the one established for chlorinated dibenzo-p-dioxines and dibenzofurans. However, expert panels contested the suitability of TEFs in the area of PAHs in food due to the lack of oral toxicity data of individual PAHs, and differences in the mode of action (Joint FAO/WHO Expert Committee, 2006; European Food Safety Authority, 2008). Canada is the only of the three before mentioned countries that set maximum levels based on toxic equivalents (TEQ), particularly for olive pomace oil. Toxic equivalents represent the concentration of a certain PAH in a food product corrected with the corresponding TEF.



**Table 2** Maximum levels for PAHs in food in EU and other jurisdictions

			Maximum levels [ $\mu\text{g/kg}$ ]	
Foodstuffs			BaP	Sum of BaP, BaA, BbFA and CHR
European Union	6.1.1	Oils and fats (excluding cocoa butter and coconut oil) intended for direct human consumption or use as an ingredient in food	2.0	10.0
	6.1.2	Cocoa beans and derived products	5.0 $\mu\text{g/kg}$ fat	30.0 $\mu\text{g/kg}$ fat as from 1.4.2015
	6.1.3	Coconut oil intended for direct human consumption or use as an ingredient in food	2.0	20.0
	6.1.4	Smoked meat and smoked meat products	2.0 as from 1.9.2014	12.0 as from 1.9.2014
	6.1.5	Muscle meat of smoked fish and smoked fishery products, excluding fishery products listed in points 6.1.6 and 6.1.7. The maximum level for smoked crustaceans applies to muscle meat from appendages and abdomen. In case of smoked crabs and crab-like crustaceans it applies to muscle meat from appendages.	2.0 as from 1.9.2014	12.0 as from 1.9.2014
	6.1.6	Smoked sprats and canned smoked sprats (25) (47) ( <i>Sprattus sprattus</i> ); Smoked Baltic herring $\leq 14$ cm length and canned smoked Baltic herring $\leq 14$ cm length (25) (47) ( <i>Clupea harengus membras</i> ); Katsuobushi (dried bonito, <i>Katsuwonus pelamis</i> ); bivalve molluscs (fresh, chilled or frozen) (26); heat treated meat and heat treated meat products (46) sold to the final consumer	5.0	30.0
	6.1.7	Bivalve molluscs (smoked)	6.0	35.0
	6.1.8	Processed cereal-based foods and baby foods for infants and young children	1.0	1.0
	6.1.9	Infant formulae and follow-on formulae, including infant milk and follow-on milk	1.0	1.0
	6.1.10	Dietary foods for special medical purposes intended specifically for infants	1.0	1.0
	6.1.11	Cocoa fibre and products derived from cocoa fibre, intended for use as an ingredient in food	3.0	15.0
	6.1.12	Banana chips	2.0	20.0
	6.1.13	Food supplements containing botanicals and their preparations	10.0	50.0
		Food supplements containing propolis, royal jelly, spirulina or their preparations		
	6.1.14	Dried herbs	10.0	50.0
	6.1.15	Dried spices with the exception of cardamom and smoked <i>Capsicum</i> spp.	10.0	50.0
Peoples Republic of China		Paddy, wheat	5.0	
		Smoked or baked meats	5.0	
		Smoked or baked aquatic products	5.0	
		Fats and oils, and fat emulsions	10.0	
Russian Federation		Vegetable oils – all types, vegetable oil fractions	2	
South Korea		Edible oil	2.0	
		Sukjihwang & gunjihwang	5.0	
Canada		Olive pomace oil	3 $\mu\text{g/kg}$ TEQ <sup>a</sup>	
Hong Kong		Edible oils [also applies to edible fats]	10 $\mu\text{g/kg}$ <sup>b</sup>	
Brazil		Olive oil	2.0 $\mu\text{g/kg}$	

<sup>a</sup>PAHs expressed as toxic equivalents (TEQ) of BaP.<sup>b</sup>Action level.

### Analytical Methods to Determine PAHs in Food and Feed

Analytical methods for the determination of PAHs were developed already long before the publication of the US EPA's priority list. However, their number increased rapidly after this milestone was set, focusing further mainly on the 16 prioritised PAHs. The number of published analytical methods is enormous. A search in Scopus(R), using the terms "PAH" and "analytical method"

resulted in more than 570 hits. A detailed discussion of these publications would go far beyond the length and scope of this manuscript. Therefore, only the overall concepts of analytical methods for the determination of PAHs in food are presented, omitting a systematic discussion of their pros and cons. The latter information can be found in several review papers, which focus on different matrices and analytical techniques (Moret and Conte, 2000; Plaza-Bolaños et al., 2010; Purcaro et al., 2013; Wise et al., 2015; Bansal et al., 2017; Buczyńska et al., 2013; Wenzl et al., 2006).

The development of analytical methods for the determination of PAHs in food has to respect the particular properties of the targeted analytes and of the matrix. Robustness of the method and potential for automatization are as well important aspects.

It is necessary to separate PAHs from the food matrix prior to measurement by either gas chromatography or liquid chromatography. A difference in performance of one or the other with regard to accuracy of results was not observed (Sykes et al., 2013). Both separation techniques may be combined for PAH analysis with different detection systems. Single quadrupole gas chromatography mass spectrometry (GC-MS) and high performance liquid chromatography with fluorescence detection (HPLC-FD) were for decades the methods of choice. Few laboratories applied gas chromatography high resolution magnetic sector mass spectrometry for the determination of PAHs in food (Simon et al., 2008; Ziegenhals et al., 2008a). The advancement of mass spectrometry combined with increased affordability of instruments led in the last decade to the broad application of tandem mass spectrometers combined with both gas chromatography (GC-MS/MS) and ultra-performance liquid chromatography (UPLC-MS/MS) (Veyrand et al., 2007). Gas chromatography time of flight mass spectrometry (GC-TOF) and GC-quadrupole TOF (GC-QTOF) mass spectrometers are employed for PAH analysis only in special cases, as the detection systems of TOF mass spectrometers are not yet as sensitive as those of (tandem-) quadrupole mass spectrometers (Drabova et al., 2012). However, modern GC-MS instruments are sufficiently sensitive for the implementation of current legislation on PAHs contents of food. A further improvement of the detectability of PAHs is therefore considered of rather low priority.

Measurements by GC electron ionisation mass spectrometry suffer from the high stability of the condensed aromatic ring systems, resulting in poor analyte fragmentation. The low informative value of EI mass spectra requires good chromatographic separation of isobaric species, as co-elution could lead to wrong peak assignment and consequently wrong conclusions. Despite improvements in GC column technology, it is not yet possible to baseline separate all critical pairs, such as TRI and CHR, within one run (Gómez-Ruiz and Wenzl, 2009; Bordajandi et al., 2008).

Whereas EI is the preferred ionisation method for all gas chromatographic mass spectrometric measurements, dopant assisted atmospheric pressure photo ionisation (APPI) is applied in place of electro spray ionisation (ESI) in the measurement of PAHs by LC-MS/MS (Itoh et al., 2006; Hollosi and Wenzl, 2011). However, at present PAHs are rarely analysed by LC-MS/MS in routine, which is probably due to the need of higher investment in instrumentation, limited applicability of the APPI source, and risk for quick deterioration of instrument performance due to matrix deposition on the lamp. Experiences from 12 proficiency tests on the determination of the 15+1 EU priority PAHs, respectively the 4 EU marker PAHs, in various food showed that HPLC-FLD is suitable for the determination of almost all PAHs in most of the studied matrices. CPP cannot be detected by FD due to the absence of fluorescence. Also complex matrices such as cocoa products and black pepper posed problems to laboratories applying HPLC-FD.

As in all chemical analyses, sample preparation and measurement must be dovetailed and optimised for obtaining high quality results. In PAH analysis, the lipophilicity and volatility of PAHs are directly affecting the design of analytical methods. The high volatility of two- and three ring PAHs might lead to losses during sample preparation, whereas the low volatility of six-ring and larger PAHs challenges the measurement by gas chromatography. Consequently, internal standardisation with, ideally, carbon 13 labelled PAH analogues is a prerequisite for achieving a high level of accuracy. Many food products containing potentially PAHs are rich in fat content. Thus for the analysis of these products it is necessary to separate PAHs from the fat fraction, as the latter interferes usually with chromatographic analysis. The initial separation from the matrix occurs in an extraction, respectively partitioning step. Solid food is extracted with an unpolar to mid-polar organic solvent or solvent mixture such as petrol ether, *n*-hexane, or a mix of *n*-hexane and acetone. The extraction step alone is usually not selective enough to provide extracts that can be injected into the measurement instrument. One or more clean-up steps are commonly integrated in the analytical procedure. Open column normal phase chromatography on silica, gel permeation chromatography, and/or solid phase extraction are traditionally applied clean-up techniques (Ziegenhals et al., 2008a; Veyrand et al., 2007). The downside of these techniques is the consumption of sometimes large amounts of organic solvents. Thus, more environmentally friendly methods were developed, which however might have a narrow scope, or whose method performance characteristics were sometimes inferior to those of traditional methods (Purcaro et al., 2007; Bansal et al., 2017).

Miniaturization was seen as a possibility of reducing the consumption of organic solvents, which however might put the representativeness of the analysed sample at stake. Attempts were also made to adapt the QuEChERS method, which is widely used for the determination of pesticides in fruits and vegetables, for PAH analysis (Anastassiades et al., 2003). The developed methods were applied for different fatty and non-fatty food items (Portolés et al., 2017; Urban and Lesueur, 2017).

Standardized analytical methods are available from different sources (Table 3). They are based on either HPLC-FD or GC-MS analysis and are applicable starting from edible oils up to a broad range of food. In addition to the international standards, analytical methods were validated by collaborative trial for the determination of PAH in certain food commodities (Simon et al., 2010; Mastovska et al., 2015).

Scientific literature did not yet report on non-chromatographic rapid analysis methods for the determination of PAHs in food in routine.

**Table 3** Standardised analytical methods for the determination of PAHs in food

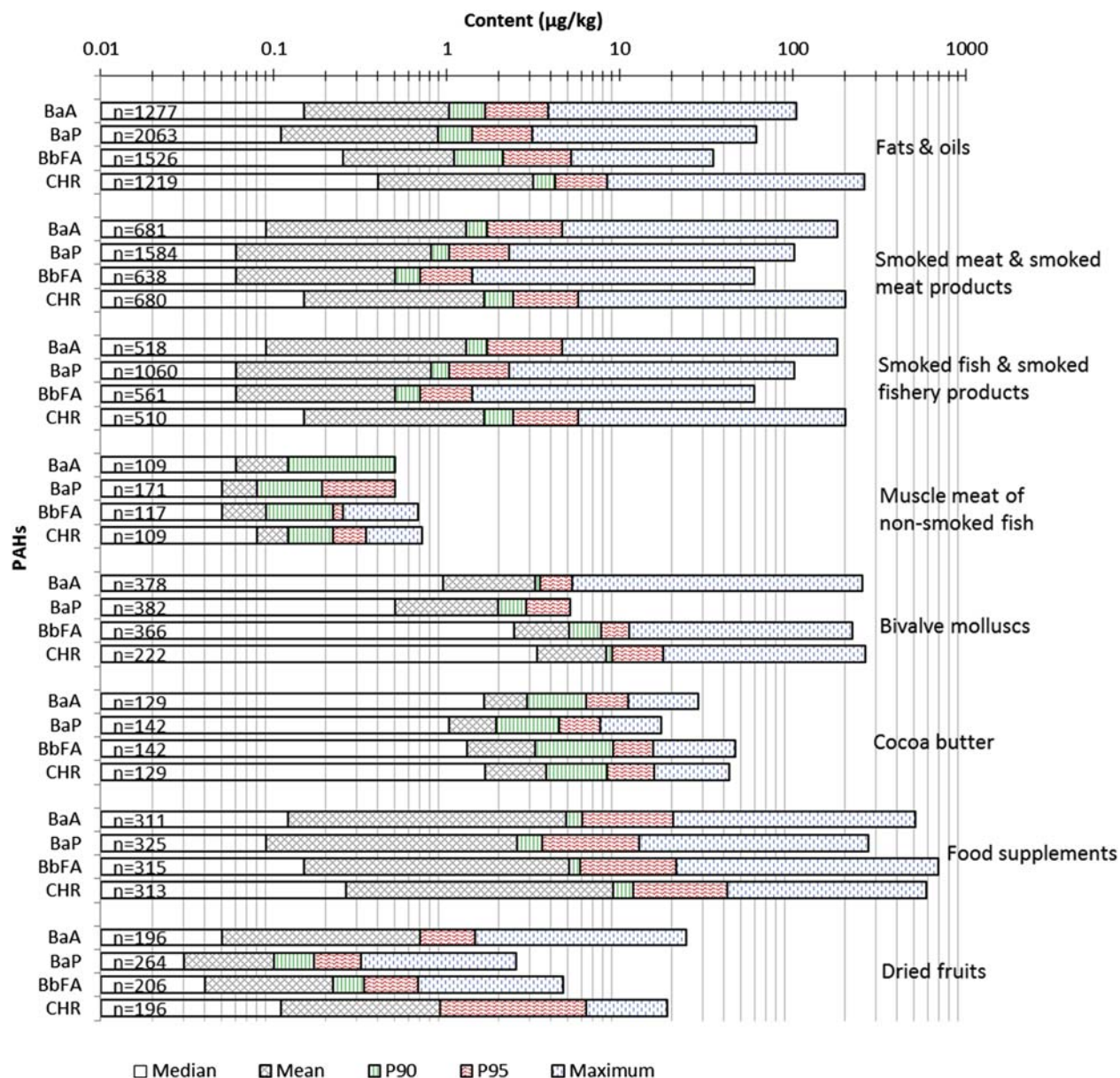
	<i>Title</i>	<i>Analytical technique</i>	<i>Scope</i>	<i>PAHs covered</i>	<i>Working range</i>
ISO 15753:2016 <sup>a</sup>	Animal and vegetable fats and oils — Determination of polycyclic aromatic hydrocarbons	HPLC-FD	Animal and vegetable fats and oils Excluded: palm oil, olive pomace oil, and fat from milk and milk products	NA, AC, ACL, FL, AN, PHE, FL, CHR, BaA, PY, BaP, BbFA, BkFA, BghiP, DBahA, ICP	0.2–1.0 µg/kg
ISO 22959:2009	Animal and vegetable fats and oils – Determination of polycyclic aromatic hydrocarbons by on-line donor–acceptor complex chromatography and HPLC with fluorescence detection	HPLC-FD	Determination of polycyclic aromatic hydrocarbons (PAHs) in edible fats and oils.	AN, PHE, FA, PY, CHR, BaA, BeP, BaP, PE, BghiP, ANT, DBahA, COR, ICP, BaFA, BbFA, BkFA	0.1 µg/kg – 3.5 µg/kg
ISO 15302:2017	Animal and vegetable fats and oils – Determination of benzo[a]pyrene – Reverse-phase high performance liquid chromatography method	HPLC-FD	Determination of benzo[a]pyrene in crude or refined edible oils and fats in the range 0.1 µg/kg to 50 µg/kg Excluded: fat from milk and milk products	BaP	0.1 µg/kg
AOAC 973.30 <sup>b</sup>	Polycyclic Aromatic Hydrocarbons and Benzo[a]pyrene in food (1974)	Thin layer chromatography followed by UV and Fluorescence Spectrophotometry	Food	PAH	
EN 16619:2015	Food analysis - Determination of benzo[a]pyrene, benz[a]anthracene, chrysene and benzo[b]fluoranthene in foodstuffs by gas chromatography mass spectrometry (GC–MS)	GC–MS	Determination of 4 of the 16 EU priority polycyclic aromatic hydrocarbons (PAHs) .... in extruded wheat flour, smoked fish, dry infant formula, sausage meat, freeze-dried mussels, edible oil and wheat flour	BaA, BbFA, BaP, CHR	0,5 µg/kg to 11,9 µg/kg
CEN/TS 16621:2014	Food analysis - Determination of benzo[a]pyrene, benz[a]anthracene, chrysene and benzo[b]fluoranthene in foodstuffs by high performance liquid chromatography with fluorescence detection (HPLC-FD)	HPLC-FD	Determination of benzo[a]pyrene (BaP) plus benz[a]anthracene (BaA), benzo[b]fluoranthene (BbF) and chrysene (CHR) in several food matrices. in-house validated via the analysis of spiked samples of edible olive oil, fresh mussels, smoked fish, smoked meat products, processed cereal-based foods for young children, infant formulae, chocolate and food supplements (isoflavones)	15 + 1 EU priority PAHs	0.25 µg/kg respectively 1.0 µg/kg to 4.95 µg/kg respectively 23,53 µg/kg

<sup>a</sup>specifies a) General method b) Specific method for coconut oil and vegetable oils with short-chain fatty acids.<sup>b</sup>outdated methodology.

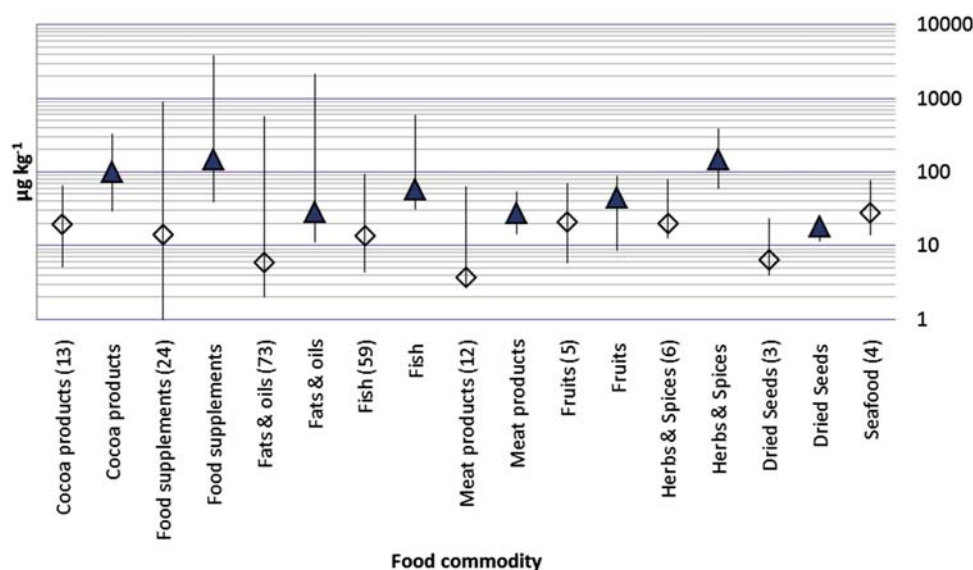
## Occurrence of PAHs in Food

### Fats and Oils

Environmental contamination as well as production processes such as drying, grilling, roasting, and smoking can affect the contents of PAHs in food significantly. The lipophilicity of PAHs makes especial fatty food matrices prone to PAH contamination. No wonder that edible fats and oils belong to the most relevant food matrices in this respect and that maximum levels were set in different jurisdictions primarily for this food category. Environmental contamination is considered the main source for contamination with PAHs of virgin edible oils, presuming state of the art harvest and production processes are used (Rodríguez-Acuña et al., 2008). Failing to meet current standards, by e.g. exposing olives during harvest to exhausts of harvesting machines, or ventilating storage tanks with unfiltered ambient air might result in substantial increase of PAH contents. Application of special clays during the refining of crude fats and oils serve to reduce PAH content levels of highly contaminated batches (Hua et al., 2016). The collection of PAH occurrence data by EFSA revealed that 90% of the about 2000 fats and oil samples complied with the at that time applicable maximum level for BaP of 2.0 µg/kg (Fig. 1) (European Food Safety Authority, 2007). However, the compilation of notifications entered from January 2010 until March 2018 into the EU Rapid



**Figure 1** PAH contents compiled by EFSA for different food categories (European Food Safety Authority, 2007). n: indicates the number of samples used for the calculation of statistical descriptors.



**Figure 2** Levels of PAH contents of notification to the Rapid Alert System for Food and Feed over the period 2010 to 2018 (European Union RASFF). ◇: indicates BaP; △: indicates the sum of contents of BaA, BaP, BbFA, and CHR. Number within brackets indicates the number of notifications entered in RASFF for the particular food category, independent of the number/kind of PAHs concerned.

Alert System for Food and Feed (RASFF) reveals that elevated PAH levels can still be found in products intended for the market (Fig. 2) (European Union RASFF). The situation on other markets is similar to that in EU (Molle et al., 2017; Shi et al., 2016a; Tfouni et al., 2014; Jiang et al., 2015).

Contact of cocoa beans with exhausts of drying installations during artificial drying might cause increased PAH levels in cocoa products, such as cocoa butter and consequently in chocolate, and cocoa powder (Misnawi, 2012; Raters and Matissek, 2014; Ziegenhals et al., 2009).

### Meat, Fish, and Products Thereof, and Seafood

Smoking of meat and fish is one of the oldest methods used for conserving these otherwise fast deteriorating foods. However, smoked meat and smoked fish, as well as products thereof can contain high levels of PAHs. EFSA reported mean levels for BaP in smoked fish and smoked meat in the 1 µg/kg range, whereas maximum levels were two orders of magnitude higher, and consistent with maximum levels reported to the RASFF for smoked fish. High PAH contents of smoked food are usually linked to poor smoking practices such as direct contact of food items to hot smoke from glow, to the intensity of smoking, and to unfavourable surface to volume ratios of the smoked food (Pöhlmann et al., 2013; Ledesma et al., 2015; Zachara et al., 2017). The latter applies to smoked sprats and small smoked herring, of which several consignments produced within the EU were withdrawn in recent years from the EU market, as they exceeded the applicable maximum levels for PAHs for this type of food. There are reports on high PAH contamination levels of intensely smoked small fish also from other regions of the world (Essumang et al., 2012). However, elevated PAH contents of smoked meat and smoked fish are not the general rule as observed for commercial smoked meat products analysed in Germany (Jira, 2009; Zachara et al., 2017). Codex Alimentarius acknowledged the shortcomings in manufacturing practices, and potential lack of awareness of contamination of food with PAHs during smoking and issued a code of practice for reducing the contamination of food with PAHs during smoking and direct drying processes (Codex Alimentarius, 2009).

The levels of PAHs in non-smoked fish are much lower than in smoked fish (Drabova et al., 2013).

Pollution of the sea by e.g. environmental deposition of PAHs, by leakages in petroleum production, and after ship accidents may cause contamination of seafood with PAHs. EFSA reported for bivalve molluscs a mean BaP content of about 2 µg/kg, whereas the mean contents of BaA, BbFA, and CHR were higher. Bivalve molluscs are of special concern in this respect, as they bioaccumulate PAHs (Hellou et al., 2005). For this reason they are used for biomonitoring of the PAH contamination of the sea. Accidents such as on the Deepwater Horizon rig in the Gulf of Mexico, or the sinking of the Prestige oil tanker close to the Spanish coast caused consequently the temporary ban of harvesting of bivalve molluscs such as oysters and other seafood. However, studies showed that the initially increased PAH contents of mussels went quickly back to background levels (Óscar et al., 2006; Gohlke et al., 2011).

Smoked products are usually prepared on industrial scale, whereas barbecued meat, another source for exposure to PAHs is mainly prepared at household level, and therefore much more difficult to control. Depending on eating habits, barbecued meat might significantly contribute to the overall exposure to PAHs (Duedahl-Olesen et al., 2015). In this respect parameters such as



the type of barbecue (charcoal, gas, electrical), distance between heat source and meat, type of meat product cooked, the use of barbecuing pans, and exposure time influence the PAH content of the prepared food (Rose et al., 2015; Lee et al., 2016; Viegas et al., 2012; Gorji et al., 2016). EFSA reported a maximum value of 19.9 µg/kg BaP in heat-treated meat, which does not necessarily refer to barbecued meat (Fig. 1). However, a Danish study on different types of commercially barbecued meat revealed for e.g. 26 tested beef burgers an average BaP content of 3.0 µg/kg and for PAH4 7.7 µg/kg, with maximum values of 17.5 µg/kg and 48.0 µg/kg (Duedahl-Olesen et al., 2015).

### Beverages

A number of studies focused on the PAH content of beverages, especially in tea (*Camellia sinensis*) and coffee. PAHs are formed in coffee during roasting of the beans, whereas they are deposited on the tea leaves mainly during drying (Tfouni et al., 2013; Guatemala-Morales et al., 2016). Both food items might contain significant amounts of PAHs in the dry product (Drabova et al., 2012; Tfouni et al., 2013; Shi et al., 2016b; Kayali-Sayadi et al., 1999; Orecchio et al., 2009; Badolato et al., 2006; Houessou et al., 2005; Pincemaille et al., 2014; Guatemala-Morales et al., 2016; Garcia Londoño et al., 2015; Ziegenhals et al., 2008b; Lin et al., 2006). However, the concentrations in the ready-to-drink beverages are low due to the small amount of powder used per serving (Pincemaille et al., 2014; Orecchio et al., 2009; Lin et al., 2006). Herbal teas can also contain high levels of PAHs in the powder, but as for tea and coffee low amounts in the prepared infusion (Ziegenhals et al., 2008b; Thea et al., 2016).

### Herbs Spices and Food Supplements

The contamination of herbs and spices with PAHs is known since long. However, growing attention was put in recent years on the contamination of these food items with PAHs. This led in the EU to the setting of maximum levels for herbs and spices (see Table 2) (European Union, 2015a). PAHs might be deposited on herbal plants from the air, taken up from the soil, or enter the product during processing such as drying. The levels of PAHs in herbs and spices vary a lot. Contents for the sum of four EU marker PAHs were reported from “not detected” up to a few tenths of microgram per kilogram product (Ziegenhals et al., 2007; Rozentale et al., 2018). This agrees very well with levels observed in medicinal herbs (Tripathy et al., 2015; Ishizaki et al., 2011). A recent survey covering 150 samples from the market, comprising six types of dried herbs and spices, did not reveal any exceedance of the EU maximum levels (Rozentale et al., 2018).

Special cases constitute cardamom and smoked herbs and spices, such as smoked *Capsicum* species, smoke pepper, or smoked salt. These food items might contain hundreds of microgram per kilogram PAHs (Ziegenhals et al., 2007). The smoked spices are used to provide a smoky taste to food, without the need to apply smoke flavours. However, cardamom and smoked *Capsicum* species are exempted from the EU legislation, due to the on the one hand low consumption volumes of these commodities, and on the other hand otherwise severe consequences for the supply with them.

Food supplements constitute a large group of different products ranging from minerals to vitamins. PAHs were primarily found in food supplements containing edible oils, in propolis products and herbal food supplements such as spirulina, St. John's wort, or *Ginkgo biloba* products (Zelinkova and Wenzl, 2015; Martena et al., 2011; Danyi et al., 2009; Moret et al., 2010). Some of these products contained BaP at levels of tenths of µg/kg (Moret et al., 2010; Zelinkova and Wenzl, 2015). Also the PAH levels described before for dry powdered tea have to be seen in a different light, if tea powder is used as a food supplement, instead of a hot beverage. The contribution to the daily exposure to PAHs depends of course on the consumed amount of product. Despite the intake of food supplements is usually low, their contribution to the overall exposure could in exceptional cases be relevant (Zelinkova and Wenzl, 2015; Danyi et al., 2009; Moret et al., 2010).

### Cereal Products, Milk, and Infant Food

EFSA concluded in 2008 that cereal products are the major contributor to the exposure of Europeans to PAHs (European Food Safety Authority, 2008). This is not at all caused by a high PAH content of cereal products, which are usually, from the analytical point of view, even at challenging levels, but by the high consumption volumes of cereal products (Ciecierska and Obiedziński, 2013; Rozentale et al., 2017; Kacmaz et al., 2016).

Similarly, to human breast milk, PAHs can also be found in milk of ruminants. Studies on milk and milk powders revealed low levels of PAHs (Girelli et al., 2014; Naccari et al., 2011). Milk powder and vegetable fats are important ingredients of infant formulae. To protect infants from high exposure to PAHs, restrictive maximum levels were set in EU for infant food (see Table 2). Several studies, conducted in different geographical regions, demonstrated that the tested commercial infant formulae would largely comply with EU maximum levels (Petrarca and Godoy, 2018; Iwegbue et al., 2014; Cho and Shin, 2012; Ciecierska and Obiedziński, 2010; Rey-Salgueiro et al., 2009). However, some exceedances were reported for ready-to-eat baby food containing various ingredients such as meat or fish (Santonicola et al., 2017b).

### Vegetables

Vegetables might be contaminated with PAHs via atmospheric deposition or by uptake from the soil (Amato-Lourenco et al., 2017; Tuteja et al., 2011). However, the former is more likely as PAHs with high molecular weights (four rings and above) adsorb strongly



to particulate matter and the organic fraction of soil, and get therefore less likely in contact with roots or tubers. Low molecular weight PAHs (2-ring and 3-ring PAHs) are transported via the air and get easier in contact with leafs and fruits (Amato-Lourenco et al., 2017; Tuteja et al., 2011). The levels of PAHs in fresh fruits and vegetables are normally low, but can in exceptional cases, which might include the cultivation of the plant in a heavy polluted area and favoured uptake of PAHs by the plant/fruit due to e.g. a waxy surface, reach levels in the low microgram per kilogram range (Paris et al., 2018).

## Feed

Due to their ubiquity, animals are exposed to PAHs via inhalation and uptake with the feed (Takagi et al., 2005; González-Gómez et al., 2018). Few studies dealt with PAH contents in feed and the resulting exposure of animals (Ciganek et al., 2002; Ciganek and Neca, 2006; Beauchamp et al., 2002; Berntssen et al., 2010; Easton et al., 2002). For example, the daily intake of the sum of 16 EPA PAHs by lactating cows fed a combination of hay and compound feed, and of pigs fed a feed mixture was estimated to 14160 µg and 164 µg respectively (Ciganek et al., 2002). The high exposure levels of the ruminants were caused by the large ingestion of hay. Concerns regarding the transfer of PAHs from feed to animal products, particularly milk proved to be unsubstantiated (Grova et al., 2000, 2006; Kan et al., 2003). This finding is supported by experiments with radio-labelled PAHs, in which three <sup>14</sup>C-labelled PAHs supplied to a lactating goat were excreted without entering milk in substantial amounts (Grova et al., 2002).

Among 25 popular feed ingredients, Alfalfa and calcium phosphate were identified as potentially significant contributors to the PAH levels of animal feed (Fernández-González et al., 2012). The fibre component was recognised already earlier as a major source of PAHs in feed (Yebra-Pimentel et al., 2012).

The recycling of food waste into aquafeed pellets raised questions regarding the potential contamination of feed with PAHs stemming from processed food. However, the recycled food did not significantly contribute to the PAH content of the pellets.

Salmon from aquaculture contained higher levels of PAHs than wild-caught salmon, which was reasoned by the exposure of farmed salmon to PAHs contained in commercial aquafeed (Easton et al., 2002). The input of PAHs via aquafeed was reflected in the PAH contents of sediments in the direct vicinity of aquaculture cages (Tsapakis et al., 2010). The level of PAHs in fish depends on the composition of the aquafeed. The replacement of fish meal and fish oil in aquafeed to a large extent with vegetable based ingredients, aiming at reducing the levels of persistent organic pollutants (POPs, such as dioxins (PCDD) and polychlorinated biphenyls (PCBs)), caused an increase of PAH levels in farmed salmon (Berntssen et al., 2010). This increase was the consequence of a higher PAH content of the vegetable-based ingredients compared to the fish based ingredients. However, using vegetable ingredients with a low PAH content for the preparation of aquafeed might achieve the desired reduction of POP levels by simultaneously keeping the PAH levels in finished fish low (Nácher-Mestre et al., 2010).

Little is known about the content of PAHs in pet food. One study aimed to identify patterns of bioaccumulation, of amongst others PAHs in cats and dogs, presented also PAH content data of 16 commercial pet foods (Ruiz-Suárez et al., 2015). They contained two to four ring PAHs, but hardly any larger PAHs.

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## Relevant Websites

- Codex Alimentarius, Codes of Practice: <http://www.fao.org/fao-who-codexalimentarius/codex-texts/codes-of-practice/en/>.
- European Commission, Directorate General Health and Food Safety (DG SANTE), Chemical food safety: [https://ec.europa.eu/food/safety/chemical\\_safety/contaminants\\_en](https://ec.europa.eu/food/safety/chemical_safety/contaminants_en).
- European Food Safety Authority (EFSA), Opinion on PAHs in food: <http://www.efsa.europa.eu/en/press/news/080804>.
- European Union Reference Laboratory for PAHs (EURL PAH, until 2017): <https://ec.europa.eu/jrc/en/eurl/pahs>.
- US National Institute for Standardization: NIST Polycyclic Aromatic Hydrocarbon Structure Index: <https://pah.nist.gov/>.
- International Agency for Research on Cancer (IARC), IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: <http://monographs.iarc.fr/>.

### Standard Methods for the Determination of PAHs in Food:

- International Organisation for Standardization (ISO): <https://www.iso.org/store.html>.
- European Committee for Standardization (CEN): <https://www.cen.eu/Pages/default.aspx>.



## Veterinary Drugs: Progress in Multiresidue Technique

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### Veterinary Drugs: A Whole World of Residue Possibilities Out There

Veterinary drugs are pharmacologically active substances that may be administered to animals in order to maintain and/or to restore an optimal health status. In this sense, veterinary formulations are widely used in animal husbandry as therapeutics to treat diseases and infections, in the control of parasitic diseases, and also to help alleviate pain and inflammation in sick or injured animals. In addition to their therapeutic and prophylactic uses, veterinary drugs can also be intended for zootechnical purposes, in the form of products applied to healthy animals for non-pathologic objectives (i.e. non therapeutic claims), for instance estrus synchronization or fertility improvement. These chemicals can also be administered to domestic or wild animals to ease management, including tranquilizers, sedatives and anesthetics. Lastly, certain veterinary drugs have the ability to enhance animal production through various metabolic mechanisms (anabolic hormonal compounds, antimicrobials,  $\beta$ -agonists, thyreostatics, corticosteroids) and as such they have been used extensively as growth promoters in livestock farming for many years. However, this latter use has been banned in most European countries as a measure to protect consumers (EC, 1996a; EC, 2003a,b; EC, 2008), meaning that alternative products must be used.

In Europe, the classification of medicinal products for veterinary use is governed by Directive 2001/82/EC, as amended by Directive 2004/28/EC (EC, 2004). In accordance with Article 1 of Directive 2001/82/EC, as amended, the definition of veterinary medicinal product includes any substance or combination of substances presented as having properties for treating or preventing disease in animals; or any substance or combination of substances which may be used in or administered to animals with a view either to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis. In parallel, medicated feedstuffs are mixtures of veterinary medicinal products and feeds which are ready prepared for marketing and intended to be fed to animals without further processing, because of its curative or preventive properties or other properties. In accordance with Article 67 of Directive 2001/82/EC, as amended, veterinary medicinal products require a veterinary prescription and the prescription is also required for food-producing animals unless an exemption has been granted.

The rational use of veterinary drugs in animal husbandry, along with improvements in management and animal feeding, have greatly contributed to achieving the high rates of productivity that most farms worldwide are showing nowadays. Strictly, veterinary drugs can also be used to treat wild, companion and/or sports animals, but their administration to non-food-producing animals is not important from the point of view of food safety, at least not directly. The use of veterinary drugs in food-producing animals has the potential to generate residues in animal-derived edible products (meat, milk, eggs and honey), and in this sense their irrational administration may pose a health hazard to the consumer (EU, 2010). Many factors can influence the occurrence of residues in animal products, including pharmacokinetic characteristics of the drug, animal metabolism, dose, withdrawal period, etc. To prevent risk, it is necessary to use these drugs rationally, only when they are really indicated, at the right dose and moment, and respecting withdrawal periods established for each edible product. Ideally, all veterinary drugs should only be used in consultation and under the direction of veterinarians as their particular knowledge and training have remarkable significance in preventing unnecessary residues and consequent public health hazards.

In Europe, national monitoring plans should sample feed and animals with the aim of detecting illegal treatments or controlling compliance of the permitted ones with the maximum residue limits (MRLs) for veterinary medicinal products set out in the Annex to Commission Regulation (EC) 37/2010, the maximum levels for pesticides set out in Regulation (EC) 396/2005, or the maximum levels laid down in relevant legislation on contaminants (EC, 2005; EU, 2010). The EU requires that foodstuffs (meat, milk, eggs) obtained from animals treated with veterinary medicines or exposed to chemicals used in animal husbandry must not contain any residue at a level that might represent a hazard to the health of consumers. An evaluation of the residues and the subsequent establishment of MRL are only considered for active substances for which consumer exposure represents a particular concern. Before a veterinary preparation can be authorized in Europe, the active drug must be included as an allowed substance in the first table of the Annex to Commission Regulation (EC) 37/2010. The second table of the same Annex includes a list of substances that must not be used in veterinary medicines for use in food producing animals or in biocidal products for use in animal husbandry. Similar examples take place in other regions and countries, for instance in the USA, where US Food and Drug Administration regulates veterinary drugs through the FDA's Center for Veterinary Medicine (CVM) (<https://www.fda.gov/>). Before a new animal drug may receive FDA approval, the sponsor must establish that the new animal drug is safe (for the animal, persons administering it or otherwise associated to the animal and in terms of environmental impact) and effective. Canada, Japan, China, Argentina, Brazil or Australia follow similar trends, but important differences exist in the authorized substances among countries (Masía et al., 2016).

Roughly, veterinary drugs can be classified according to not only their therapeutic objective, mode of action, chemical properties and/or structure, but also to the existence (or not) of authorization for their administration in food-producing animals in different regions of the world. Based upon the foregoing, and undertaking a food-residue perspective, the following classification has been preferred in this work as presented in Table 1: permitted, banned and borderline drugs in the European Union (EU).



**Table 1** Veterinary drugs classified according to the existence of authorization for using them in food-producing animals in the European Union

Permitted	Antimicrobials (antibiotics, antibacterials, coccidiostats, histomonostats) Corticoids/Glucocorticoids Antiparasitic agents, including carbamates, pyrethroids and other pesticides (against endo and ecto-parasites) Sedatives, tranquilizers, anesthetics $\beta$ -Agonists: clenbuterol Non-steroidal anti-inflammatory drugs (NSAIDs)
Banned	Antimicrobials (as growth promoters) Corticoids/Glucocorticoids (as growth promoters) $\beta$ -Agonists Steroids (androgens, estrogens, gestagens) Stilbenes, stilbene derivatives, and their salts and esters Antithyroid agents Resorcylic acid lactones Recombinant bovine somatotropin Chloramphenicol, chloroform, chlorpromazine, colchicine, dapsone, dimetridazole, metronidazole, nitrofurans (including furazolidone) and ronidazole Synthetic dyes (malachite green)
Borderline	Natural hormones and mycotoxins (zearalenone, $\alpha/\beta$ -zearalenol) Prohormones SARMs SERMs Cascade prescription

### Permitted Drugs

Antimicrobials are, by far, the largest and most varied of all the existing groups of veterinary drugs. These compounds are also the most commonly used in animal husbandry worldwide, mainly due to the constantly shifting production practices, from extensive farming systems to large-scale intensive farming operations that routinely require antimicrobials. Recent research has projected a rise of 67% in antimicrobial consumption by 2030, in comparison to 2010, and nearly double in Brazil, Russia, India, China, and South Africa (Van Boeckel et al., 2015). In the previous article, authors stated that chicken and pigs appear as the most frequent target species, mainly because of the relatively lower use of antimicrobials per animal unit and the lower animal densities characteristic of cattle farms.

Antimicrobials means substances produced either synthetically or naturally, used to act against (kill or inhibit) micro-organisms, including bacteria, viruses or fungi, or of parasites, in particular protozoa (EC, 2003b). The term antimicrobial includes antibiotics, but not vice versa. Antibiotic means antimicrobials/antibacterials produced by (or derived from) a microorganism, but does not include substances that are synthetic or semisynthetic, or those obtained from plants or animals. Coccidiostats and histomonostats are antimicrobials too, in this case intended to kill or inhibit protozoa infections. Notice that residues of antibacterials, being the largest and most widely known and studied class of antimicrobials, are amongst the most important in food analytical chemistry. Antibacterial drugs are administered to animals to treat a disease or infection, and may therefore result in residues in food of animal origin. They can also be used to promote growth as feed additives. In this sense, their use was banned in the EU more than a decade ago (EC, 2003b).

Shortly after antimicrobial drugs were discovered and introduced into human medicine, they were used in veterinary medicine. There are hundreds of antimicrobial drugs, most of which belong to a few major classes ( $\beta$ -lactams, aminoglycosides, quinolones and fluoroquinolones, sulphonamides, tetracyclines, lincosamides, phenicols, nitrofurans, nitroimidazoles, ionophores ...) (Reeves, 2012). However, only some of these drugs are approved for their use in food-producing species, and the presence of their residue in food is therefore also regulated, as in the particular case of Europe (EU, 2010). Yet besides the already mentioned residue concerns, the influence of animal treatments with antibiotics on the phenomenon of antimicrobial resistances is of particular importance. Already in 1945, the doctor who revolutionized medicine with his discovery of penicillin, Sir Alexander Fleming, warned of the potential for these resistances to become a public health problem: "then there is the danger that the ignorant man may easily under-dose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant" (Hudson et al., 2017). Because of the emergence of microbes resistant to antibiotics that are used to treat human and animal infections, the European Commission decided to phase out the marketing and use of antibiotics as growth promoters in animal feed (EC, 2003b). In this context, the WHO list of critically important antimicrobials for human medicine provides a ranking of medically important antimicrobials for risk management of antimicrobial resistance due to non-human use. The current revision (fifth) took place at the seventh meeting of the Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) held in Raleigh, United States of America in 2016 (Aidara-Kane et al., 2018).

Apart from the well-known antimicrobials, many other veterinary drugs have been authorized for administration in animal production. Those include antiparasitic agents, drugs acting on the nervous system (sedatives, tranquilizers,  $\beta$ -agonists –not for growth promotion), corticoids or glucocorticoids (methylprednisolone, dexamethasone, betamethasone, prednisolone –not for growth promotion) and non-steroidal anti-inflammatory drugs (NSAIDs) (EU, 2010). It is obvious that antibacterial drugs have attracted much attention in the field of analytical chemistry, but the monitoring of residues from other veterinary medicines in food should not be neglected. In this sense, multi-residue and multi-class techniques offer a very attractive opportunity from an analytical point of view as the analyst may perform determinations of several groups of drugs in a single analysis.

### Banned Drugs

Some substances are considered to represent a clear hazard to the safety of the consumer when present at any level in food, and they must not be used in veterinary practice in stockfarming. Council Directive 96/23/EC classified all these drugs together in a single group called group A, comprising banned substances according to Directive 96/22/EC, i.e. growth promoters and drugs abused in animal fattening, and compounds with no MRL (EC, 1996b). The group of growth promoters includes substances having a hormonal action (steroid hormones, stilbenes, resorcylic acid lactones), antithyroid agents or thyreostats (thiouracils and mercaptoimidazole analogues) and  $\beta$ -agonists, all capable of enhancing growth rate (EC, 2003a). These compounds are banned in the EU and other countries, but authorized in others like the US. Strictly speaking, thyreostats cannot be considered growth promoters since their effect is not anabolic. In turn, corticosteroids are steroid hormones used in human and veterinary medicine as antipyretic, anti-inflammatory and anti-allergy drugs. They are authorized for therapeutic treatments in food producing animals with their corresponding withdrawing times between treatment and slaughtering. Accordingly, maximum residue limits (MRLs) have been established for authorized corticosteroids in milk, muscle, liver, kidney and fat (EU, 2010). Even though formally they are permitted drugs, they can be also used illegally as growth promoting agents, synergistically combined with other molecules such as  $\beta$ -agonists or anabolic agents. Estrogenic, gestagenic and androgenic compounds (EGAs), as well as thyreostatic, corticosteroids and  $\beta$ -agonists, may be used alone or combined in growth promoting cocktails with low concentrations of several drugs, making even more difficult their detection. In this context, multiresidue approaches should be preferred for monitoring purposes.

The administration of exogenous somatotropin to cattle is considered a hormonal tool to increase profit. Recombinant bovine somatotropin (rbST) can be used to enhance milk production in cows and in other dairy ruminants, and its potential as growth promoter has been also demonstrated. The administration of rbST is permitted in several countries including the United States, Brazil, and Mexico, among others, while it has been banned in others, for instance Canada, Australia, and New Zealand. In 1999, the European Union decided to definitively ban possible use of rbST (EC, 1999). Due to the particular characteristics of this veterinary drug (polypeptide hormone), no multiresidue methods have been developed so far to determine somatotropin and its recombinant variants in food (Regal et al., 2017).

Veterinary drugs with no MRL because a safe residue limit could not be established are chloramphenicol, chloroform, chlorpromazine, colchicine, dapsone, dimetridazole, metronidazole, nitrofurans (including furazolidone) and ronidazole. The identification of these banned substances is important in a large number of matrices and at trace levels because there is a zero-tolerance policy on their presence in food. Lastly, synthetic dyes as malachite green have been used as therapeutic multi-usage drugs to reduce parasitic, microbial, and fungal diseases aquaculture. Dyes with pharmacological activity can be categorized into triarylmethanes, phenothiazines, xanthenes, acridines, and azo compounds. Their use in the EU is not permitted, but they can be still utilised illegally because of relatively low cost, availability and high efficacy.

### Borderline Residues

Borderline drugs can often be some of the most challenging. For example, a coccidiostat for use in pigs or poultry in the EU should be assessed as a feed additive, whereas the same product for use in cattle has to be assessed as a veterinary medicinal product. Also, certain natural compounds such as zearalenone may be found in animal feed due to cereal contamination with fungi. Despite belonging to the group of prohibited compounds (resorcylic acid lactones), its presence can be considered natural and unavoidable. In this particular example, the *in vivo* metabolites of zearalenone are the key to distinguish illegal use of zeranol from consumption of food contaminated with *Fusarium* spp. toxins. Likewise, natural hormones have arisen as a real weak point of residue monitoring plans due to their natural origin. The existence of high variability through animals in terms of natural hormonal levels has been reported. This latest fact makes almost impossible to establish legal thresholds to control any exogenous administration of natural hormones to animals. Innovative *in vivo* methods that are useful include targeted and untargeted profiling methods, metabolic pathways analyses, the omics technologies, and gas chromatography coupled to combustion/isotope ratio mass spectrometry (GC-C/IRMS).

Selective androgen receptor modulators (SARMs) constitute a novel class of androgen-receptor ligands with anabolic properties (~steroids) but with less androgenic side-effects. They have been already banned by World Anti-Doping Agency (WADA) but their potential misuse in animals cannot be discarded (Cesbron et al., 2017). Similarly, the group of anti-estrogenic substances includes selective estrogen receptor modulators (SERMs), but also aromatase inhibitors and anti-estrogens. They are prohibited in human sports doping but they also pose a risk of illegal use in animal husbandry for fattening purposes (Meijer et al., 2017).

An additional and interesting example of borderline situations is the so-called 'Cascade' prescription. The 'Cascade' is an exceptional EU mechanism designed to deal with situations where there is no suitable authorized product for the treatment of an animal. In order to avoid unacceptable suffering in an animal, a veterinary practitioner is enabled to prescribe medicines off-label, within very strict limitations. If a medical product is used outside its authorized uses, the veterinarian must specify an appropriate withdrawal period for the slaughter of treated animals or for the production of milk, eggs or honey. Cascade shall apply provided that pharmacologically active substances included in the medicinal product have a MRL established by Commission Regulation (EU) 37/2010, but not necessarily in the species for which it is intended to be used. Also, the veterinarian responsible for prescribing the medicine must specify an appropriate withdrawal period of not less than 7 days for eggs and milk, and 28 days for meat from poultry and mammals including fat and offal (EC, 2004). A good example is lidocaine hydrochloride (2%), approved in the US for use in cattle as a local anaesthetic but licensed only for horses in the European Union. Nevertheless, LIDO may be used in other species under the provision of the cascade.

## Residue Analysis: Past, Present and Future

Two international conferences, in particular, the EuroResidue (ER) series and the Ghent series (the International Symposium on Hormone and Veterinary Drug Residue Analysis – VDRA), give a recognized platform to discuss all aspects of residues of veterinary drugs in the food production chain (van Ginkel and Bergwerff, 2008). The renowned organizing committees of EuroResidue conferences and Ghent symposia established an agreement to deliberately alternate dates, offering food chemist, veterinarians, analyst and other food experts a great opportunity to meet on a regular basis, reflecting the worldwide trends in residue analysis (<http://www.euroresidue.nl/> and <http://www.vdra.be/>). Thus, an international meeting of experts on veterinary drug residues takes place every two years from 1994 on, alternatively in Egmond aan Zee (ER, The Netherlands) and in Ghent (VDRA, Belgium). In this context, one could consider that the specific matters discussed at each edition of these conferences have covered the state-of-the art and future trends in residue science, technologies and policies, at that time. Therefore, a journey through the posters, oral communications, lectures, special issues, books of abstracts ... resulting from ER and VDRA meetings, since their very beginnings, will clearly depict the history and development of veterinary residue analysis, for those interested in exploring the past, present and future of this analytical field.

It was in the ER of 2008 (van Ginkel and Bergwerff, 2008) that the so-called omic techniques (transcriptomics, proteomics, metabolomics) emerged as feasible and applicable alternatives for food residue analysis, especially in the field of hormonal growth promoters. Also, the stunning capabilities of high resolution mass spectrometers (time-of-flight, Orbitrap, FTICR ...) received great attention and these instruments were suggested as representative options of modern instrumental residue analysis. As highlighted during VDRA 2010, the use of ultra-high performance liquid chromatography (UHPLC) hyphenated with accurate mass spectrometry became much more common, along with omic approaches (De Brabander et al., 2010). In ER 2016, these approaches consolidated their position in the development of modern multiresidue methods, and the interest for methods detecting (multiple) antibiotics (simultaneously) exploded (Bergwerff, 2016). In Table 2, a summary of the multiresidue methods published in the corresponding special issues of EuroResidue VI and VIII is presented.

In this context, conventional residue analysis dealing with classic immunoassays, growth inhibition assays, thin layer chromatography, liquid and gas chromatography, UV detectors, triple quadrupole mass spectrometers ..., are still present, and such classic techniques play an important role in routine residue control programs and should coexist with more innovative techniques, since residue analysis must be affordable for everyone.

## Method Validation

Clear rules on how laboratory analysis had to be carried out and results interpreted were provided in Commission Decision 2002/657/EC, implementing Council Directive 96/23/EC, which established criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories (EC, 2002). Moreover, the Decision introduced a procedure to establish minimum required performance limits (MRPL) for analytical methods employed to detect substances for which no permitted limit (MRL) had been established. For the first time, the concepts of decision limit ( $CC\alpha$ ) and detection capability ( $CC\beta$ ) were introduced, as quality parameters that must be established during the validation of an analytical method. Currently, the evolution in analytical equipment and progress in scientific research, accompanied by recent European regulatory changes, seems to demand an update or revision of the 2002/657/EC. The current validation method described in European Commission Decision 2002/657/EC is designed for quantitative methods. As for qualitative screening methods, the approach provided in the Guideline for the validation of screening methods for residues of veterinary medicines (initial validation and transfer) by Community Reference Laboratory in 2010, is better adapted and detailed.

## Trends in Sample Size and New Matrices

Current trends in residue analysis include the use of small sample amounts, and innovative non-invasive matrices such as saliva, hair and feathers. On the other hand, more classic options such as urine and blood samples, are still of importance for

**Table 2** Summary of the multiresidue methods published in the corresponding Special Issues of EuroResidue VI (2009, *Analytica Chimica Acta*, vol. 637, issues 1–2) and VIII (2017, *Food Additives and Contaminants: Part A*, vol. 34, Issue 4, pages 451–652). Purely methodological, comparative, or pharmacokinetic studies have been excluded

<i>Conference</i>	<i>Authors</i>	<i>Vet drugs and metabolites (group: family)</i>	<i>Matrix</i>	<i>Clean-up/Extraction protocol</i>	<i>Instrument</i>	<i>Claims</i>
EuroResidue VI (2008)	Gamba et al.	7 (Antimicrobials: sulphonamides)	Milk (bov)	LLE + SPE	HPLC-DAD	Multiresidue, quantitative, confirmatory, 2002/657/EC
	Cristofani et al.	8 (Antimicrobials: tetracyclines & epimers)	Muscle (bov)	LE + MCAC + SPE	HPLC-DAD	Quantitative, confirmatory, 2002/657/EC
	Dusi et al.	6 (Estrogenic: resorcylic acid lactones)	Urine (bov, por)	IAC	HPLC-ESI-MS/MS	Quantitative, confirmatory, 2002/657/EC
	Granja et al.	1 (Antimicrobials: aminoglycosides)	Honey	Dissolve + SPE	HPLC-ESI-MS/MS	Quantitative, confirmatory, 2002/657/EC
	Stubbings and Bigwood	20 (Antimicrobials: nitroimidazoles, sulphonamides, fluoroquinolones, quinolones, ionophores, dinitrocarbanilide)	Muscle (chicken)	QuEChERS	HPLC-ESI-MS/MS	Multiresidue/multiclass, screening, 2002/657/EC
	Xia et al.	7 (Antimicrobials: nitroimidazoles & metabolites)	Kidney (por)	LLE + SPE	HPLC-ESI-MS/MS	Multiresidue, quantitative, confirmatory
	Granelli et al.	19 (Antimicrobials: tetracyclines, sulfonamides, quinolones, $\beta$ -lactams, macrolides)	Muscle (por, bov)	LE	HPLC-ESI-MS/MS	Multiresidue, multiclass, quantitative, confirmatory, 2002/657/EC
	Malone et al.	13 (Growth promoters: anabolic compounds –gestagens, stilbenes, androgens– and corticosteroids)	Muscle (bov)	SPE	HPLC-ESI-MS/MS	Multiresidue, quantitative, confirmatory, 2002/657/EC
	Le Breton et al.	1 (Hormones: rbST)	Serum (bov)	Precipitation + SPE	HPLC-ESI-MS/MS	Unambiguous identification and quantification
	Boison et al.	3 (Antimicrobial growth promoters: feed additives)	Muscle (por)	Digestion + SPE + filter	HPLC-ESI-MS/MS	Quantitative, confirmatory
	van Holthoon et al.	7 (Antimicrobials: aminoglycosides, aminocyclitols)	Kidney, muscle (por)	LE + SPE	HPLC-ESI-MS/MS	Multiresidue, quantitative, confirmatory, 2002/657/EC
	Schmidt et al.	10 (Anabolic growth promoters: estrogenic, androgenic)	Muscle (bov)	Digestion + LLE + SPE	HPLC-APCI-MS/MS	Quantitative, confirmatory, 2002/657/EC
	Destrez et al.	1 (Potential growth promoters: ecdysteroids)	Urine (bov)	SPE + SPE	HPLC-ESI-HRMS	Hybrid mass spectrometry, metabolites elucidation
	Mitrowska et al.	8 (Tranquilizers and sedatives: phenothiazines, butyrophenones, beta-blockers)	Kidney (por, bov)	SLE + filter	HPLC-ESI-MS/MS	Quantitative, confirmatory, 2002/657/EC
	Kinsella et al.	38 (Anthelmintic: benzimidazoles, macrocyclic lactones, flukicides)	Milk, liver (bov)	QuEChERS	HPLC-ESI-MS/MS	Multiresidue, confirmatory, quantitative, 2002/657/EC
	Krebber et al.	2 (Antimicrobials: fluoroquinolones)	Edible tissues (bov, por, turkey, rabbit)	LE + direct injection on TFC column	TFC-MS/MS	Confirmatory, quantitative, EMEA/CVMP/573/00
	Forti and Scortichini	10 (Antimicrobials: sulphonamides)	Egg	LE + SPE	HPLC-ESI-MS/MS	Multiresidue, confirmatory, quantitative, 2002/657/EC
	Hadjigeorgiou et al.	1 (Antimicrobials: sulfones)	Milk, meat (bov)	LE + SPE	HPLC-ESI-MS/MS	quantitative, confirmatory, 2002/657/EC

EuroResidue VIII (2016)	Bovee et al.	6 (Growth promoters: androgenic steroids)	urine (bov)	enzymatic deconjugation + SPE	Yeast bioassay	screening, 2002/657/EC
	Ashwin et al.	16 (Antimicrobials: quinolone, fluoroquinolone)	tissue (bov, fish, chicken), milk	SLE	microbial inhibition test	screening, 2002/657/EC
	Thompson et al.	7 (Antimicrobials: nitroimidazoles)	kidney (por, bov), liver (av), serum (av), egg, milk (bov)	LE	immunobiosensor	multiresidue, screening, 2002/657/EC
	Andersen et al.	3 (Anti-fungal and parasites: triphenylmethane dyes)	muscle (fish)	SLE + LLE + SPE	LC-VIS	multiresidue, screening, validation
	Dubreil et al.	75 (Antimicrobials: beta-lactams, sulphonamides, tetracyclines, macrolides, lincosamides, quinolones, phenicols, pleuromutilins, polypeptides, rifamycins, diaminopyrimidine derivatives, aminocoumarin)	muscle (bov, por, poultry, aquacult)	LE	HPLC-ESI-MS/MS	multiresidue, screening, qualitative approach, validation CRL guidelines
	Cornejo et al.	2 (Antimicrobials: phenicols)	feathers (poultry)	cryogenic grinding + LE + LLE	HPLC-ESI-MS/MS	quantitative, confirmatory, 2002/657/EC
	Rejtharová et al.	5 (Growth promoters: testosterone and estradiol esters)	serum (por, bov)	SPE + SPE	HPLC-ESI-MS/MS	screening, confirmatory, 2002/657/EC
	Fais et al.	2 (Antimicrobials: sulphonamides, diaminopyrimidine derivatives)	muscle (fish)	LE + filter	HPLC-ESI-MS/MS	quantitative, validation Brazilian Ministry of Agriculture guidelines
	Busatto et al.	4 (Anthelmintic: benzimidazole)	muscle (fish)	QuEChERS	HPLC-ESI-MS/MS	quantitative, validation Brazilian Ministry of Agriculture guidelines
	El Hawari et al.	21 (Antimicrobials: sulfonamides, tetracyclines, macrolides, lincosamides, aminoglycosides)	honey	LE + d-SPE	HPLC-ESI-MS/MS	multiclass, quantitative, confirmatory, 2002/657/EC
	Regal et al.	9 (Growth promoters: beta-agonists)	retina, liver (bov)	enzymatic treatment + SPE	HPLC-ESI-MS/MS	multiresidue, quantitative, confirmatory, 2002/657/EC
	Guo et al. Meijer et al.	2 (Antimicrobials: beta-lactams) 18 (Potential growth promoters: SERMs, anti-estrogens, aromatase inhibitors)	milk urine (bov, por)	LE enzymatic treatment + LLE	HPLC-UV UHPLC-ESI-MS/ MS	quantitative, validated quantitative, confirmatory, 2002/657/EC

Dispersive SPE (d-SPE); Immuno-affinity chromatography (IAC); Metal-Chelate Affinity Chromatography (MCAC); turbulent flow chromatography (TFC).

their simplicity and representativeness (Bergwerff, 2016; van Ginkel and Bergwerff, 2008). The determination of residues in edible products cannot be replaced for many reasons, such as the accumulation of residues, and on this basis matrices as liver, muscle, kidney, eggs and milk remain among the most popular options in veterinary drug analysis. Even chicken claw can be used for human consumption and it has been recently used as target sample to determine antimicrobials residues (Cornejo et al., 2017).

Insects are not traditional consumed by the European population, but they are very popular in countries as Mexico, Colombia, Thailand, Indonesia or South Africa. Lately, edible insects have been suggested as novel food alternatives by EU, and already various insect species have been authorized for human consumption. In this context, a new area of action is opening up for multi-residue methods (Poma et al., 2017).

## Multiresidue Techniques

To measure the low concentrations of veterinary drugs that are usually found as residues in food and animal matrices, highly selective, sensitive and accurate analytical methods are needed. Due to the large number of drugs existing on the veterinary market, the use of multi-residue methods capable of analyzing large numbers of analytes and metabolites in one single run has become the most common and most efficient approach for residue monitoring. The term multiresidue was born in the field of pesticide pollutants (insecticides, herbicides, fungicides, rodenticide, molluscicides, nematocides, plant growth regulators and others), possibly due to the great number of compounds included in some groups, such as organochlorines, and their broad distribution in the environment. Because of the cost-effective and time-effective nature of this analytical approach, multiresidue methods rapidly invade other fields.

Traditional methods for single-class/single-residue analysis still exist, but modern multi-class/multiresidue methods, mainly using LC-MS instruments, are now dominant because they are more cost-effective and time-effective, they are selective towards individual drugs and their metabolites, highly sensible (very low LODs and LOQs), and also accurate for identification and confirmation (Frenich et al., 2014; Mainero Rocca et al., 2017; Masiá et al., 2016; Stolker et al., 2007; Toldrá and Reig, 2006). A graphical overview of the most frequent multiresidue analytical workflow nowadays is presented in Fig. 1.

### Multi-residue and Multi-class?

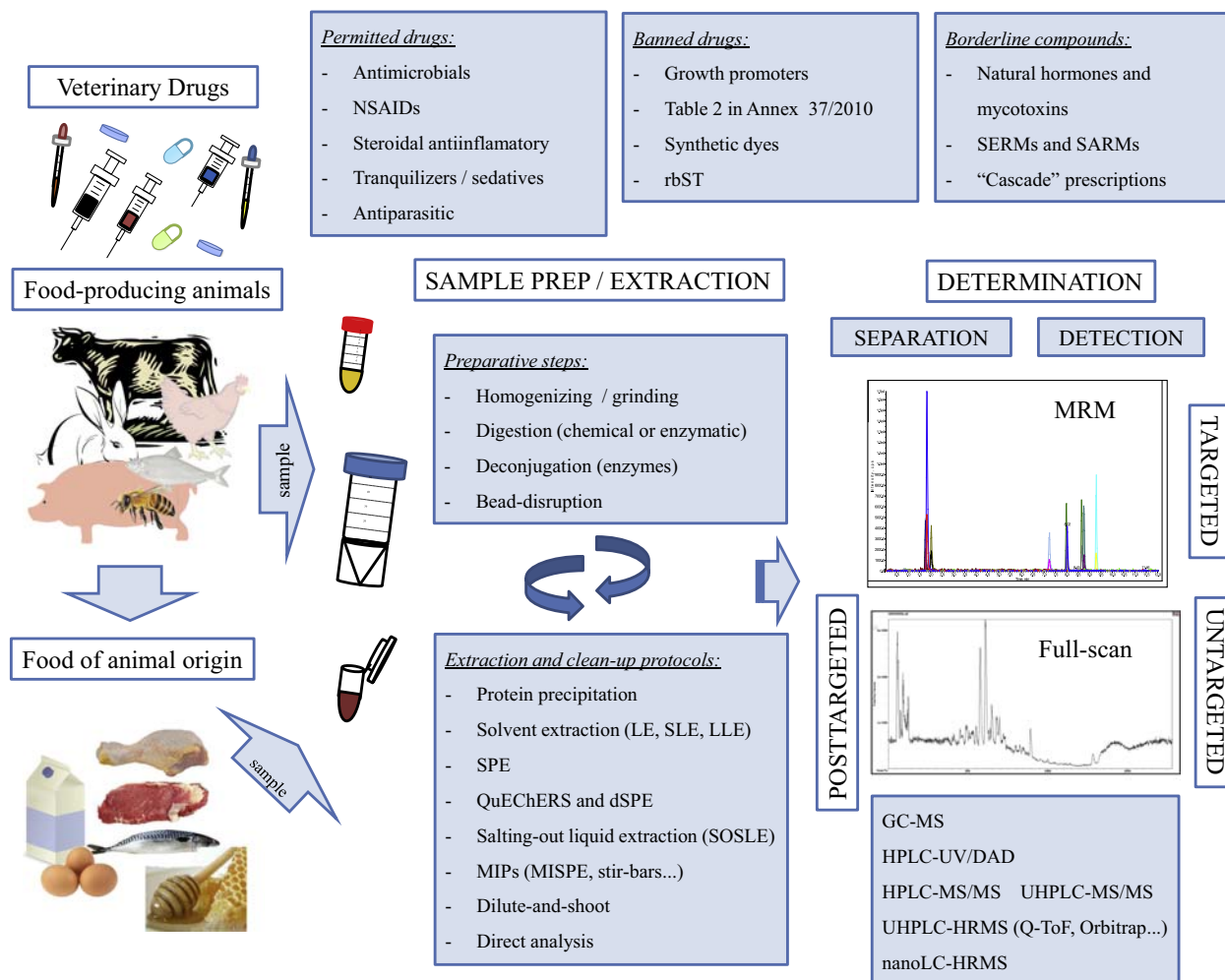
Multi-residue methods include large numbers of compounds, in order to determine the presence of residues of multiple veterinary drugs in a single run, saving sample, solvents, time and analytical costs. In some cases, the specific purpose is covering frequent synergistic/additive combinations of veterinary drugs, such as corticosteroids and beta-agonists, or antimicrobial combinations, amongst others. It is a fact that the terms multi-residue and multi-class are often used interchangeably, but they are not and should not be considered synonyms. Multi-class methods are analytical solutions capable of detecting drugs belonging to different chemical groups or drug categories/classes. Interesting and illustrative examples of a real multiclass, multiresidue (and even multi-matrix) methods have been recently presented, including also food-packaging contaminants, pesticides, mycotoxins and others (Anumol et al., 2017; Ortellì et al., 2009; Piatkowska et al., 2016; Pérez-Ortega et al., 2017; Rizzetti et al., 2018; Zhao et al., 2017). Other frequent term is multi-analyte, referred to a method that may include both multiple residues and natural compounds (or exogenous natural compounds?), from the same veterinary class or from different groups (van Tricht et al., 2018).

## Multiresidue Extraction and Sample Preparation

The selective, accurate and reproducible detection of multiple analytes in different matrices and at low concentrations requires a careful optimization of the analytical method. As some matrix components co-elute with the target compounds and/or interfere with the detector, it is difficult to get a successful multiresidue method without applying an efficient extraction procedure to the sample. Currently, sample preparation is still the most laborious step of the analytical process, especially in the case of multi-analyte analysis. Some of the most common techniques available nowadays for sample clean-up and extraction of veterinary drugs are nicely summarized in a recent review by Mainero Rocca et al. (2017). The commonest reported techniques are solvent extractions (in its various forms), solid-phase extraction (SPE) and the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) approach.

Solvent extractions are still very popular for sample preparation in residue analysis, as they have demonstrated to be both straightforward and effective tools in the laboratory. In many occasions, the solvent effectiveness is enhanced with ultrasound assistance, automatic shaking or mechanical disruption such as bead-disruption. Different variants of solvent extraction are liquid extractions and solid-liquid extractions (LE, SLE) as well as liquid-liquid partitioning (LLE). In the first two options, a solvent (buffers, acetonitrile, methanol, acidic water ...) is added to a liquid or a solid sample, respectively, for different purposes, and the solvent is then recovered mixed with the sample matrix. Liquid-liquid extractions are based on the ability of an analyte to distribute itself between an aqueous solution and an immiscible organic solvent. In this variant, only one phase is recovered from the mixture. Organic phase will retain the lipophilic (hydrophobic) compounds, and aqueous phase will concentrate the lipophobic (hydrophilic) residues. In this context, acetonitrile have been one of the most popular deproteinization solvents and





**Figure 1** Workflow of the most frequent and up-to-date multiresidue analytical strategies used nowadays for confirmatory and screening determinations of veterinary drug residues.

*n*-hexane frequently combined as the degreasing solvent. In LLE, *tert*-butyl methyl ether (TBME), dichloromethane and ethyl acetate are amongst the best options in terms of recovery. These very simple preparative solutions have widely demonstrated to be effective for the simultaneous determination of hundreds of compounds, also in part thanks to their coupling with modern LC and MS instruments (Cao et al., 2018; Ortellì et al., 2009; Wittenberg et al., 2017). In other cases, sample has been simply diluted and shot, a procedure that involves limited sample treatment. Dilute-and-shoot procedures present a high sample throughput, but some important limitations are occurrence of abundant matrix effects, which compromise detection limits, quantitative aspects, method selectivity and instrument maintenance frequency (Mainero Rocca et al., 2017; Masiá et al., 2016).

Salting-out supported liquid extraction (SOSLE) is a rather novel extraction and clean-up technique, based on high salt concentration in the aqueous donor phase, enabling a supported liquid-liquid extraction with a relative polar organic acceptor phase such as acetonitrile. The concept of salting-out in combination with the technique of supported liquid-liquid extraction was proposed by Kaufmann et al. (2014). The proposed technique resulted in milk extracts of equal or superior cleanliness and with higher average recoveries than those obtained with QuEChERS or SPE. Recent research has reaffirmed its soundness in multi-residue analysis for other matrices such as muscle and eggs (Alcántara-Durán et al., 2018). The Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) methodology was originally developed for pesticides, but it was rapidly proposed for the analysis of veterinary drugs in a variety of different matrices. QuEChERS are now a referent to extract simultaneously multiple pesticides and veterinary drugs in food (Mainero Rocca et al., 2017; Masiá et al., 2016). It has also demonstrated to be effective as additional step for removing the fat phase in fatty samples after liquid extractions (Giaccone et al., 2018). It is important to note that QuEChERS is a variant of dispersive solid phase extraction (dSPE) (Socas-Rodríguez et al., 2017). As an example of its great capabilities, d-SPE with in-vial filtration was successfully applied to the high-throughput analytical determination of >100 veterinary drug residues, representing at least 13 different classes, in bovine muscle (Schneider et al., 2015). The novel Enhanced Matrix Removal-Lipid (EMR-Lipid) technology, from Agilent, is a unique sorbent released in 2015 that selectively removes lipids in

complex matrices, using a convenient dispersive solid phase extraction (dSPE) format. The EMR-Lipid sorbent integrates very well with QuEChERS or protein precipitation extracts, collecting the lipids present in aqueous extracts. Various multiresidue methods have implemented this technology, with very little interferences and enhanced time-saving, for hundreds of residues in different animal tissues (Anumol et al., 2017; Rizzetti et al., 2018).

Even now, classical solid phase extraction (SPE) is a frequently used, low-cost and easily automated method to pretreat food samples and it can be coupled to both liquid and gas chromatography. A wide variety of SPE cartridges exist that improve the extraction of analytes from complex matrices. In this sense, new formats have been created, for instance SPE columns for automated protein precipitation and removal of phospholipids (Rejtharová et al., 2017). In addition to this, the 96-well SPE plate formats save the analyst a great deal of time, introducing high-throughput capabilities into SPE field (Blokland et al., 2017; van Tricht et al., 2018). Molecularly imprinted polymers (MIPs) emerged with force a decade ago as promising SPE sorbents, the so-called MISPE cartridges. However, the use of MIPs for SPE is a rather new and scantily explored research domain, particularly for the analysis of certain groups of veterinary drugs. The superiority of MIPs as selective SPE sorbents (MISPE) for the analysis of residues in food has been extensively demonstrated during the last decade; several studies have compared classical SPE sorbents and MISPE sorbents (Regal et al., 2012). Also, novel formats have emerged for multianalyte extraction in food, including for example molecularly imprinted stir-bars, monoliths and/or on-line clean-up columns, suggesting a clear tendency towards miniaturization in MIP technology (Lv et al., 2013; Xu et al., 2011; Zhang et al., 2014).

Last but not least, any sample preparation protocol is usually started with homogenization or grinding of a considerable amount of sample. Hereafter, an aliquot of sample is taken for analysis, in most occasions transferring the desired analyte into a homogenous liquid medium or solvent. Often, the drug is bound to matrix components (protein, keratin, glucuronic acid, sulfate ...) and must be freed by mechanical, chemical (alkaline, acidic) or enzymatic treatments. For example, the metabolism of many veterinary drugs after administration to the animal ( $\beta$ -agonists, NSAIDs, steroids and anabolics, antibacterial ...) includes formation of sulfate and/or glucuronide conjugates that must be hydrolyzed before (or after) further extraction of the target compound (Socas-Rodríguez et al., 2017). The use of  $\beta$ -glucuronidase/arylsulfatase from *Helix pomatia*, for example, is suitable for enzymatic digestion of urine, retina, serum, liver, muscle, kidney and milk samples. Obviously, this deconjugation is occasionally necessary in matrices collected from the animal, but that's never the case in feed or drinking water samples. For hair or similar samples, basic digestions with sodium hydroxide or mechanical pulverization to free the compound from this tight matrix are both acceptable and successful alternatives (Leporati et al., 2014; Stolker et al., 2009). In some situations as determination of steroid esters in hair, chemical digestion of hair at high temperature can easily hydrolyze the analyte, and hence the use of reducing agents such as dithiothreitol and tris(2-carboxyethyl)phosphine hydrochloride or the application of alternative techniques as cryogenic grinding are required (Gratacos-Cubarsi et al., 2006; Regal et al., 2008).

### Multiresidue Separation and Detection

A huge number of multiresidue methods have been developed so far in food chemistry. Classically, the most common strategy is to couple a separation technique (liquid/gas chromatography, LC/GC) with a simple detector such as ultraviolet (UV) or diode array (DAD) detectors, or to a more complicated mass-spectrometer. Actually (ultra-)high performance liquid chromatography (HPLC and UHPLC) with triple-quadrupole tandem mass spectrometry (MS/MS) is the most frequent and effective technique for confirmatory analysis, with proper prior pre-treatment and extraction (Frenich et al., 2014; Masiá et al., 2016; Toldrá and Reig, 2006).

Classic analytical techniques used for multiresidue analysis of veterinary drugs and growth promoters are moving from target methods, mainly liquid chromatography coupled to triple quadrupole mass spectrometers (HPLC-MS/MS platforms), towards untargeted full scan mass spectrometry utilizing accurate high resolution mass spectrometers (HRMS instruments) and ultra-high performance liquid chromatographers (UHPLC systems) (Ferrer and Thurman, 2009). Published methods show that time-of-flight (ToF-MS) and Orbitrap technologies are both very powerful high-resolution options for screening and confirmatory multi-residue analysis of veterinary drugs in food (Jia et al., 2017; Ortelletti et al., 2009; Pérez-Ortega et al., 2017; Stolker et al., 2008). These approaches may be used not only in targeted analysis but also in non-target determinations and retrospective screenings (post-target) of hundreds of compounds, or even in the identification of unknowns (Jia et al., 2017; León et al., 2016; Masiá et al., 2016). Also, nanoflow liquid chromatography (nanoLC) enables the use of high dilution factors (1:100) due to its high sensitivity. This relatively innovative LC system has been coupled to HRMS for the multiresidue determination of almost 100 veterinary drug residues in honey, veal muscle, egg and milk, achieving a complete removal of matrix-effects (Alcántara-Durán et al., 2018). Interestingly, this latter approach would make the use of matrix-matched calibration or standard addition methods no longer necessary.

Still, HPLC-MS/MS analytical platforms are more affordable than UHPLC-HRMS systems, and also provide higher selectivity and sensitivity than LC coupled to conventional detectors. Furthermore, LC-MS/MS alternatives fulfill the requirements demanded by the Commission Decision 2002/657/EC by combining analyte separation and structural information, providing unequivocal detection and confirmation of a substance (EC, 2002). In this context, HPLC-MS/MS methods have been frequently used for determination of various classes of veterinary drugs in different matrices (Piatkowska et al., 2016; Pontes et al., 2017; Rejtharová et al., 2017; Wittenberg et al., 2017). Nevertheless, many UHPLC-MS/MS multiresidue multi-class methods have also been successfully validated and implemented so far (Zhao et al., 2017). Some authors have suggested that, instead of using expensive UHPLC

systems, core-shell columns permit a smooth and easy adaptation of UHPLC protocols into more affordable HPLC instruments (Schneider et al., 2015).

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## Relevant Websites

- U.S. Food & Drug Administration –Laboratory Methods: <https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/default.htm>.
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- Veterinary Medicine Residues Testing - Fera Science: <https://www.fera.co.uk/food-safety/popular-services/veterinary-medicine-residues-testing.html>.

# Endocrine Disrupters: A Review

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## Glossary

**Bioaccumulation** The accumulation of a substance in the tissues of an organism. It is more frequent for fat-soluble (lipophilic) substances.

**Endocrine disrupter/disruptor** A natural and/or man-made chemical that triggers adverse health effects by altering the hormone system.

**Exposure** The qualitative (which way) and/or quantitative (how much) evaluation of the likely intake of a hazard.

**Hazard** Any agent with the potential to cause an adverse health effect.

**Isoflavones** A type of compounds naturally occurring in plants of the bean family: many isoflavones are biologically active, having estrogen-like properties.

**Pesticide** Any substance or mixture of substances intended for preventing, destroying, or controlling any pest that may harm humans, animals, plants or agricultural products.

**Risk assessment** A science-based process to evaluate the severity and probability of a health effect consequential to exposure to a hazard.

## Introduction

Endocrine disrupters (or disruptors: ED) are natural and/or anthropogenic chemicals that trigger adverse health effects by altering the endogenous hormone system (Solecki et al., 2017). Diet is a major exposure route to most ED; four broad categories may be considered from the viewpoint of food chains contamination:

- i. ED that can bioaccumulate in organisms (e.g., polychlorinated biphenyls –PCBs-, polybrominated flame retardants, like PBDE), thereby affecting components of the food chain that are most susceptible to environmental pollution (European Food Safety Authority, 2005a; European Food Safety Authority, 2011a). Perfluoroalkyl substances, like PFOS and PFOA, are emerging persistent ED that contrary to PCB and PBDE are not lipophilic (Wu et al., 2015);
- ii. ED employed in the production of food, such as pesticides (Mantovani et al., 2008), substances used in feeds (Mantovani et al., 2009) and their metabolites. Examples are ethylene bisdithiocarbamates (thyroid inhibiting) and triazoles (impairing the synthesis of steroid hormones). The enactment of up-to-date regulations, as well as the enforcement of appropriate farming practices are critical to control potential risks for consumers. An issue of concern is the presence of multiple pesticide residues in a significant fraction (20% and more in Europe) of fruits and vegetables (European Food Safety Authority, 2013a). The European Food Safety Authority (EFSA) has tackled this issue in 2013: based on the available knowledge, the most robust and conservative approach is the assumption of effect additivity and the formation of “cumulative assessment groups”. Compounds that have the same effect in the same target organ, such as hypothyroidism, are assumed to act in an additive way, even though their chemical structures and molecular mechanisms are different (European Food Safety Authority, 2013a; European Food Safety Authority, 2013b);
- iii. ED released into food from contact materials, processing aids, etc. A well-known example is the plasticizer bisphenol A, an EDC showing mainly estrogen agonism, present in polycarbonate plastics and epoxy resins. In European Union bisphenol A has been forbidden in baby bottles in 2011; due to widespread use, an aggregate exposure of the general population still occurs through migration from food contact materials as well as from non-food sources, e.g. thermal papers (European Food Safety Authority, 2015a). Another main example are several phthalates, used in manufacturing of PVC (Petersen and Jensen, 2010);
- iv. the fourth group of ED includes a variety of bioactive compounds naturally present in food. In such cases, the effects may be beneficial or detrimental, depending on the dose, chemical form and endocrine status of the organism (e.g., age, gender). For instance, the ability of certain phytochemicals (e.g., quercetin, largely present in fruits) to interfere with androgen-related pathways has been shown to depend also from their specific intracellular distribution (Smeriglio et al., 2014). In the case of certain essential nutrients, “endocrine disruption” may be caused by a deficiency, as well as by an excess. Iodine-deficient hypothyroidism is a major endocrine disorder worldwide, but iodine excess may also alter thyroid function; high-dose supplementation of feeds with iodine may increase the risk of iodine excess in consumers through the active carry-over of the element in milk and eggs (European Food Safety Authority, 2005b).

The ensuing paragraphs deal with two main issues: the impact of ED on food safety for developmental lifestages and the influence of dietary styles and food constituents.



## ED and Developmental Lifestages

The exposure to ED in newborns and small infants presents specific features: an early body burden may be derived from in utero exposure: small infants consume a very limited variety of food, only breast milk in many cases, so that contamination of a single item may determine the safety of their entire diet. Furthermore, internal defences of neonates against contaminants are limited by the immaturity of both hepatic detoxification and the blood–brain barrier (Neal-Kluever et al., 2014). Breastfeeding may transfer a mixture of lipophilic compounds (PCBs, brominated flame retardants, dioxins) from the maternal body burden to the newborn: in the 90's it was estimated that approximately 10% of the body burden of dioxin-like substances at 25 years of age may be attributable to breastfeeding during the first six months of life (Patandin et al., 1999), while breast-fed infants are still among the population groups with higher PBDE intake (European Food Safety Authority, 2011a). The possible presence of contaminants in human milk must be balanced against the in disputed benefits of breastfeeding for the child health, that include providing the neonatal intake of iodine which is essential to optimal thyroid function, hence, to the functional development of the brain (Azizi and Smyth, 2009). Because of these enormous benefits, it is not recommendable to reduce exposure to ED by restricting breastfeeding, except in circumstances associated with very high levels of contamination, e.g., contamination by dioxins from e-waste pollution in developing countries (Frazzoli et al., 2010). In most situations, the best way to reduce infant exposure to lipophilic ED is to act at preconceptional and gestational stages, by reducing the exposure and body burden of women at fertile age. For instance, a recent Italian investigation found significant levels of PBDE in Italian girls, especially in those with precocious thelarche: however, no obvious correlations were found with diet or lifestyles of girls, hinting to, yet unrecognized, maternal exposure determinants (Deodati et al., 2016).

Patterns of food consumption are also different in children and adolescents compared to adults; for instance, milk may be a particularly important source of exposure for children to dioxins and PCBs (Fattore et al., 2008) and PBDE (European Food Safety Authority, 2011a). Specific commodities popular among children and adolescents might be an important source of some ED: in California children (2–8 years) the consumptions of hotdogs, chicken nuggets, French fries and chips, and microwave popcorn were significantly associated with serum concentrations of perfluoroalkyl substances, like PFOA (Wu et al., 2015). It is not just a matter of exposure. The period from infancy through to puberty encompasses dynamic processes of growth and development; it may represent a window of specific susceptibility to the effects of ED on the development of reproductive, cognitive, immune and metabolic functions and also on the susceptibility to cancer later in life. Consequently, several papers are calling for a greater attention toward post-natal development in toxicological testing and risk assessment (Mantovani and Fucic, 2014; Narciso et al., 2017).

According to consistent scientific evidence, the prenatal development is the phase most susceptible to ED effects on all body systems and functions regulated by the endocrine networks, such as the reproductive and nervous systems (Latini et al., 2010) as well as body metabolism and composition; indeed, prenatal ED exposure is implicated in the risk of obesity and/or type II diabetes (Heindel et al., 2017). Typically, ED elicit “delayed developmental effects”: interferences with the programming of normal endocrine-signalling pathways during prenatal life lead to adverse consequences later in life on reproductive tissues, thyroid, liver metabolism, etc., as indicated by a wealth of experimental studies (Maranghi et al., 2007, 2010, 2013a ; Kay et al., 2014).

A comprehensive characterization of effects upon intrauterine exposure is pivotal for ED risk assessment; in the meanwhile, some issues deserve close attention. First, attention should be given to the possible differences in toxicokinetics between human and rodents, including those related to the physiology and structure of the placental filter; indeed, when assessing the toxicological studies on BPA, EFSA has translated the dose levels used in rodent developmental toxicity studies into oral human equivalent doses (European Food Safety Authority, 2015a). Second, in general the delayed developmental effects are quite specific and related to the ED mechanisms; moreover, they may appear as subtle changes, however adverse in the long run. New conceptual approaches, such as Adverse Outcome Pathways, will be useful to predict clinically-relevant adverse effects from molecular and cellular biomarkers (Leist et al., 2017).

## ED, Dietary Habits and Food Constituents

Dietary habits are influenced by socioeconomic, cultural and religious factors and individual choices (e.g. vegetarianism/veganism), which may exert a considerable impact on the intake of nutrients, bioactive substances, residues and contaminants. For instance, the extensive consumption of fatty foods of animal origin does increase the intake of lipophilic ED (European Food Safety Authority, 2005a; European Food Safety Authority, 2011a; Fattore et al., 2008). The contributions of individual food commodities to the overall intake of an ED or a group of ED depend on background contamination, as well as on patterns of food preparation, storage and consumption. For instance, management of cereals and nuts before, during and after harvest is critical to the level of contamination by zearalenone, a potent estrogenic mycotoxin (European Food Safety Authority, 2011b). More in general, the preparation and vending of street foods (an important food security component for developing countries) might lead to significant toxicological risks (Proietti et al., 2014).

Main foods, and their production chains, are vulnerable to the contamination by specific ED. Cereals, major food staples worldwide, are vulnerable to contamination by the potent estrogenic ED zearalenone, in particular corn (European Food Safety Authority, 2011b). Cereals may also accumulate from soil and water the toxic heavy metal cadmium which may have estrogen-like effects and specifically impair the production of the erythropoietin hormone within the renal proximal tubular cells, a rather



unique ED effect (European Food Safety Authority, 2009). Cadmium presents specific uptake mechanisms in other edible seeds, such as flaxseeds, which may be added to bread; the presence of antioxidant, and potentially endocrine-active, substances such as lignans in flax seeds appears to counteract the pro-oxidant action of cadmium (Dragone et al., 2016).

The production of fruits and vegetables needs to be closely watched as these commodities are widely recognized as a source of nutrients, fibre and antioxidants, but are also the major source of pesticide residues. Whereas in the European Union the exposure to pesticide residues is well controlled, with just a minor fraction of residues not complying with the legal limits, the presence of a substantial percentage of vegetables with multiple residues still remains a problem (European Food Safety Authority, 2013a; European Food Safety Authority, 2013b). The possibility that certain pesticide metabolites, present as residues, have specific toxicological characteristics must not be overlooked: one example is ethylenethiourea, a metabolite of ethylene bisdithiocarbamate fungicides, and a potent thyrostatic agent (Maranghi et al., 2013a).

In general foods of vegetable origin are not an important source of lipophilic ED, with some exceptions: in Italy vegetable oils accounted for 11%–16% of the dietary intake of PCB, a finding potentially relevant to the protection of the Mediterranean diet (Fattore et al., 2008).

Foods of vegetable origin are also a source of natural endocrine-active substances. For instance, soy is a rich source of isoflavones, such as genistein, daidzein, etc.; they are chemically related to  $17\beta$ -oestradiol and possess oestrogenic properties, as well as interact with the synthesis of thyroid hormone. In Europe the isoflavone intake is highest in consumers of soy-based supplements, vegans/vegetarians and other groups with frequent soy consumption (European Food Safety Authority, 2015b). Soy isoflavones bind to both oestrogen receptors, ER $\alpha$  and ER $\beta$ , their estrogenic activity depending on the distribution of receptors in different tissues, according also to gender and lifestage. When assessing the safety of soy-based supplements in pre- and post-menopausal women, the EFSA considered that a health-based guidance value could not be established and that several rodent studies suggest the capacity to increase proliferation of breast epithelium and endothelium. On the other hand a wealth of human studies did not support that the usual intake of soy isoflavones through supplements (up to 150 mg/day) is associated with increases of uterine or mammary cancer in the general, healthy population (European Food Safety Authority, 2015b). Besides oestrogenic action, isoflavones may inhibit thyroid peroxidase and/or other steps of thyroid hormones iodination (Divi and Doerge, 1996); when iodine status is suboptimal, a high intake may adversely affect thyroid function in children (Milerová et al., 2006).

Since endocrine-active plant compounds and xenobiotics with ED features may be concomitantly present in the diet, some studies investigated whether and how they might interact. Some evidence suggest that isoflavones like daidzein, genistein or quercetin can partially antagonize ED that bind to estrogen receptors (e.g., bisphenol A) by competing for common biological targets (Han et al., 2002; Dolinoy et al., 2007). The available, limited, in vivo studies investigating the co-exposure to isoflavones and xenobiotic ED show dishomogeneous results; for instance, genistein counteracts the antiestrogenic action of persistent organochlorines in testis and liver (Penza et al., 2007), it has a less than additive effect on hypospadias induction with the antiandrogenic fungicide vinclozolin (Vilela et al., 2007), while it has an additive effect with the insecticide methoxychlor, an estrogen agonist (Guo et al., 2002). In addition, even though the actual exposure levels to estrogen-active xenobiotics might be too low to elicit an effect *per se*, they might add up to the estrogenic activity of the dietary isoflavones (van Meeuwen et al., 2007). Possible additivity or interactions, thus, may depend on mechanisms of xenobiotic ED as well as on tissue susceptibility (e.g., distribution and density of receptors). The available data indicate that further research is worthwhile, because the issue may be relevant for consumer's safety.

Other endocrine-active compounds are present in vegetables. Several cyanogenic compounds are recognized goitrogens, such as thiocyanates and isothiocyanates derived from glucosinolates ingested in, among other items, Brassicaceae (turnips, cabbages, etc) (European Food Safety Authority, 2008). Furanocoumarins, present in grapefruit and other citrus fruits, are powerful inhibitors of sulfotransferase enzymes involved in the removal and detoxification of xenobiotics and essential to the metabolism of steroid and thyroid hormones (Harris and Waring, 2008). The risk from the presence of these substances is very low for healthy subjects consuming a balance and varied diet. However, high thiocyanate intake is associated with markers of hypothyroidism in populations with low iodine status (Steinmaus et al., 2013); also, the effects of certain therapeutic drugs could be altered by inhibitors of sulfotransferases (van Ede et al., 2008).

Without direct hormone actions, a diet rich in fruits and vegetables may afford some protection toward environmental ED. Dietary fibre is important in modulating the bioavailability of estrogens and their intestinal metabolism by microbiome (Aubertin-Leheudre et al., 2008). The intake of specific plants may reduce the reproductive effects of ED in rodents, due to their antioxidant properties; indeed, oxidative stress is an event featuring in ED-induced adverse outcomes (Rachid et al., 2016).

As already pointed out, foods of animal origin are the main source for ED able to concentrate in food chains.

Milk and dairy products are vulnerable to the prolonged contamination of pastures by persistent lipophilic ED, especially in contamination hotspots due to long-term disposal of persistent chemicals (Battisti et al., 2013). For the exposure of the general population, the role of milk and dairy products depends also on their lipid content; the habitual consumption of partially skimmed milk in infancy was shown to reduce exposure to dioxin-like compounds and the resulting body burden by 10%–20% (Yaktine et al., 2006).

The ecological and metabolic characteristics of food-producing animal species are important determinants of the contamination of foods. For instance, markedly higher levels of dioxins and dioxin-like PCBs are found in liver from sheep compared to cattle exposed to similar environmental levels. Dioxins from airborne emissions are deposited on soil. Sheep nip herbage close to ground surface, hence the involuntary intake of pasture soil is higher since than in cattle, which normally nip vegetation 5–10 cm above the ground surface. Moreover, compared to cattle sheep may have a lower CYP1A1 activity, which is the key enzyme to metabolize dioxin-like compounds to hydroxy-derivatives (European Food Safety Authority, 2011c).

Seafood, with its variety of species, classes and even phyla, is the paradigm to show the role of the characteristics of the edible organisms. In general, large fatty fishes are more liable to contamination by lipophilic pollutants like PCBs or PBDE; the persistent, but not lipophilic perfluoroalkyl substances are more prevalent in large predatory fishes (like tuna) and in molluscs and crustaceans, which might suggest, respectively, biomagnification in food webs and uptake from sediments (Mantovani et al., 2015). Farmed fish presented background levels of persistent pollutants comparable to caught fish: conventional aquaculture feeds are made with proteins and fats derived from marine organisms, thus reproducing the marine food web in the aquaculture farm (European Food Safety Authority, 2005c). Accordingly, the use of feed ingredients less vulnerable to pollution would facilitate the exploitation of fish as an important source of nutrients such as iodine and polyunsaturated fatty acids (Mantovani et al., 2015).

An interesting aspect of seafood is the concurrent presence of contaminants and nutrients that act on the same pathways, e.g., ED targeting thyroid and iodine. Infants with high dietary intake through fish of PCBs and hexachlorobenzene (another bioaccumulating ED that may alter the metabolism of thyroid hormones) showed only modest changes in thyroid parameters, possibly due to a protective effect of the concomitant iodine intake (Dallaire et al., 2008). However, juvenile female mice exposed to low levels of persistent polyhalogenated ED through a salmon-based rodent diet showed several, however subtle, adverse effects, including on thyroid and steroid hormone balance (Maranghi et al., 2013b). Therefore, a protective effect of fish dietary matrix should not be assumed to occur in all scenarios.

## Conclusions

Diet is recognized as a major route of exposure to ED. The ED issue in food safety can be divided in two broad fields:

- i. regulated substances with ED properties (pesticides, food contact materials, feed additives, etc.), where the testing framework required for authorization should be up-to-date in order to identify and characterize ED effects. The main option to protect consumers is to set and enforce regulations on the use of such substances
- ii. environmental contaminants and natural substances: risk assessment depends on available knowledge and weighing of uncertainties. Protection of consumers could make avail an array of options, including the reduction of environmental emissions, targets control programmes and risk communication.

Whereas the above considerations may apply to all chemicals in foods, ED features such as the potential exposure to multiple chemicals, the high vulnerability of the next generation and the many knowledge gaps call for policy measures which integrate science and precaution. Areas which require increased scientific knowledge include: the mechanisms underlying combined effects, the full characterization of long-term effects upon developmental exposures, the factors modulating dietary exposures, from the ecology of edible organisms through to dietary styles. Last but not least, the interactions between contaminants and natural food components can support the risk-benefit assessment of foods that are rich sources of both ED and nutrients, such as fish. Empowerment about good practices of farmers, food enterprises and consumers can reduce the exposure to, e.g., pesticides residues and food contact materials. Overall, ED call for science supporting the risk assessment, as well as for risk assessment providing clear and fit-for-purpose messages for protecting consumers health.

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## Relevant Websites

- <https://echa.europa.eu/> – European Chemicals Agency (ECHA).
- <http://www.efsa.europa.eu/> – European Food Safety Authority (EFSA).
- <http://www.iss.it/inte> – Italian national website on Endocrine Disruptors.
- <http://www.oecd.org/env/ehs/testing/oecdworkrelatedtoendocrinedisrupters.htm> – Organisation for Economic Co-operation and Development (OECD) - Work Related to Endocrine Disrupters.
- <http://web.unep.org/chemicalsandwaste/> – United Nations Environment Programme (UNEP) - Chemicals and Waste Subprogramme.
- <http://www.who.int/ipcs/en/> – World Health Organization – International Programme on Chemical Safety (WHO-IPCS).

# Acrylamide: US FDA Guidance to Industry

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## Introduction

Acrylamide is a substance that forms in foods from a chemical reaction between asparagine, an amino acid, and reducing sugars such as glucose and fructose. This reaction is part of the Maillard reaction, which leads to color, flavor, and aroma changes in cooked foods (Motttram et al., 2002; Stadler et al., 2002, 2003). Although many of the Maillard reaction products impart favorable characteristics to food, the formation of acrylamide in food is undesirable, because acrylamide is an animal carcinogen and has the potential to cause cancer in humans (National Toxicology Program (NTP), 2011).

Acrylamide formation occurs primarily in plant-based foods, notably potato products such as french fries and potato chips; cereal-grain-based foods such as cookies, crackers, breakfast cereals, and toasted bread; and coffee. Acrylamide formation usually occurs at elevated temperatures used when frying or baking (above 120 °C (248 °F)) and under low moisture conditions, although acrylamide has also been identified in some fruit and vegetable products heated at lower temperatures or higher moisture conditions (Amrein et al., 2007; Roach et al., 2003; FDA, 2006a,b).

Although it would be difficult to remove acrylamide from food completely, reducing acrylamide levels in foods may mitigate human health risks from exposure to acrylamide. For this reason, international efforts have focused on identifying techniques that can reduce acrylamide in foods and disseminating that information in the form of codes of practice, toolboxes, and guidance documents (e.g., FoodDrinkEurope, 2013; Codex Alimentarius, 2009), including the United States Food and Drug Administration (FDA) *Guidance for Industry: Acrylamide in Foods* (FDA acrylamide guidance) (FDA, 2016a).

## FDA Laws, Regulations, and Guidance Documents

To understand the acrylamide guidance, it is helpful to know more about what a guidance document is and what it is not. The Federal Food, Drug, and Cosmetic Act (FD&C Act) and other federal laws establish the legal framework within which FDA operates. FDA can develop regulations based on these laws, typically through a process known as “notice and comment rulemaking,” which allows for public input on a proposed regulation before FDA issues a final regulation. FDA regulations, which are found in Title 21 of the Code of Federal Regulations (CFR), have the force of laws, but they are not part of the FD&C Act.

FDA guidance documents, such as the FDA acrylamide guidance, are neither laws nor regulations and are not intended to be binding on industry or the public. FDA guidance documents describe the agency's current thinking, interpretation of, or policy on a regulatory issue. When issuing guidance, FDA follows procedures required by the FDA Good Guidance Practices regulation (21 CFR 10.115) for developing, issuing, and using guidance documents. Guidance documents do not include mandatory language such as “shall,” “must,” “required,” or “requirement,” unless FDA is using these words to describe a statutory or regulatory requirement.

FDA field investigators are expected to review guidance documents that apply to their work prior to inspections of food facilities, and inspection instructions may reference guidance documents. Investigators may ask about awareness of issues raised in guidance documents and have general discussions about information in guidance with firm management. However, because guidance is not a requirement for firms to follow, any observations investigators make during an inspection must be based on regulations or law, not guidance.

## Acrylamide Guidance Development

The FDA *Guidance for Industry: Acrylamide in Foods* provides information to help growers, manufacturers, and food service operators reduce acrylamide levels in certain foods. This guidance draws on the scientific literature; guidance materials prepared by industry, other governments, and international organizations; and public comments from manufacturers to present approaches to reduce acrylamide in foods at various stages from farm to table.

This guidance is intended to suggest a range of possible approaches to reducing acrylamide levels and not to identify specific recommended approaches. This guidance also does not identify any specific maximum recommended level or action level for acrylamide. Instead growers, manufacturers, and food service operators are encouraged to evaluate approaches that may be relevant to their particular processes and to consider adopting approaches that reduce acrylamide levels in their products. Because acrylamide reduction is an active area of research, the guidance also discusses mitigation methods that were still in the experimental stage when the guidance was developed, in addition to mitigation methods currently in use.

FDA began development of its draft guidance in 2009 with a *Federal Register* (FR) notice (74 FR 43134) that requested scientific data and information on methods for reducing acrylamide levels in food (e.g., changes in ingredients, cooking parameters, and mitigation methods for small manufacturers) and on reductions that manufacturers had achieved in acrylamide levels (e.g., levels



of reduction and factors affecting reductions, including variables affecting the ability to consistently achieve certain levels of acrylamide). Comments received by FDA addressed the many factors contributing to variability in acrylamide levels and the challenges in controlling this variability for the purpose of establishing maximum or recommended acrylamide levels. These comments addressed such factors as seasonal changes in the composition of raw materials, variations in individual product formulation, differences in processing equipment and parameters (e.g., type of cooking apparatus and temperature control), and consumer cooking behavior. Based on these comments and other factors, FDA concluded that it would be appropriate to develop guidance that would include information on reducing acrylamide, rather than developing specific maximum recommended levels for acrylamide in foods.

In 2013, FDA published a draft version of the *Guidance for Industry: Acrylamide in Foods* (78 FR 68852). After considering the public comments received on the draft guidance document, the final guidance was released in 2016 (FDA, 2016a). The guidance is available on the FDA website, along with other information on acrylamide.

Other governments and international organizations have taken similar approaches to providing guidance on mitigation. Examples include the Codex Alimentarius “Code of Practice for Reduction of Acrylamide in Foods” (Codex Alimentarius, 2009), the “Acrylamide Toolbox” produced by FoodDrinkEurope (FoodDrinkEurope, 2013), FoodDrinkEurope Toolbox brochures on selected foods for small- and medium-sized businesses (FoodDrinkEurope, 2014a–e), and “Guidelines to Authorities and Consumer Organisations on Home Cooking and Consumption” (HEATOX Project, 2006a) and “Manual on Strategies to Food Industries [and Restaurants] to Minimize Acrylamide Formation” (HEATOX Project, 2006b) produced by the Heat-Generated Food Toxicants: Identification, Characterization and Risk Minimisation (HEATOX) Project. The European Union Commission Regulation 2017/2158 (EC, 2017) setting benchmark levels for acrylamide in selected foods also establishes mitigation measures. The FDA acrylamide guidance document encourages manufacturers to review all available resources when considering approaches to reducing acrylamide levels in their products.

## Guidance Content

The current version of the FDA acrylamide guidance is organized into six major sections (A through F). The introduction (Section A) includes introductory comments on acrylamide monitoring and analysis, specifically, recommending that manufacturers be aware of acrylamide levels in their products to determine the effectiveness of acrylamide reduction techniques; noting that because acrylamide can vary significantly between identically prepared products, extensive sampling may be required to detect the effects of process changes; and that one approach to reducing analytical testing (e.g., by standard methods such as liquid chromatography/tandem mass spectrometry or gas chromatography/mass spectrometry) is to identify a characteristic that can be monitored as a proxy for acrylamide, such as color or moisture, and to calibrate variation in this characteristic to analytically determined acrylamide levels. To be effective, such analysis may have to be performed on a product-by-product basis.

The technical sections of the guidance cover the following topic areas. Section B covers potato-based foods, including information on raw materials, processing and ingredients, and product-specific information on french fries, sliced potato chips (crisps), and fabricated potato chips and other fabricated potato snacks. Section C covers cereal-based foods, including information on raw materials, processing and ingredients. Additional sections (Sections D through F) cover other foods; preparation and cooking instructions on packaged frozen french fries; and information for food service operations.

Section B,i, “Potato-based foods, raw materials,” focuses on growing and handling conditions that affect reducing sugar levels in potatoes. As stated in the guidance, in potatoes, reducing sugars are present in excess compared with asparagine, and reducing sugar levels are an important factor driving acrylamide formation. Careful control of reducing sugar levels can reduce acrylamide formation in finished potato products such as french fries and potato chips. Among the factors that affect reducing sugar levels are potato variety, growing and harvesting practices, maturity, handling, and storage conditions (including temperature and control of sprouting). This section includes the following recommendations that may reduce acrylamide (FDA, 2016a):

- Selecting potato varieties that are low in acrylamide precursors, keeping in mind seasonal variation.
- Optimizing potato maturity by controlling planting time, harvest time, and input management, and by removing immature tubers before processing.
- Avoiding handling potatoes with excessive roughness, avoiding bruising potatoes, and sorting out or carefully trimming potatoes with defects.
- Avoiding cold temperatures during harvest, transport, delivery, and storage.
- Managing storage conditions to control sprouting and provide ventilation.
- Monitoring reconditioning results and avoiding reconditioning potatoes stored for prolonged periods.
- Assessing reducing sugar levels in incoming potatoes, identifying target levels for incoming potatoes, or using treatments to reduce sugar levels.

Section B,ii, “Potato-based foods, processing and ingredients,” reviews the processing of french fries, sliced potato chips, fabricated potato chips, and other fabricated potato snacks. For french fries, the guidance focuses on production of frozen, par-fried french fries that are cooked to completion in a food-service establishment or in consumer homes. Levels of acrylamide are low in the par-fried fries, but increase significantly in the final cooked product. The guidance focuses on changes in processing by frozen french fry



manufacturers that can affect acrylamide levels in the final products. Specifically, the guidance includes the following processing recommendations that may reduce acrylamide ([FDA, 2016a](#)):

- Cutting fries in shapes with lower surface area to volume ratio and screening out small fragments.
- Changing blanching practices (although such changes may affect product quality).
- Using sugar dips to reduce variability (but using reducing sugars such as fructose in dips may increase acrylamide).
- Using alternative coloration methods to discourage over-baking.
- Using sodium acid pyrophosphate (SAPP), and evaluating other dip or batter ingredients to determine if they contribute to acrylamide formation during frying.

For sliced potato chips, a typical production process consists of peeling, washing (and/or blanching), slicing, frying, sorting, seasoning, and packaging chips. The guidance includes the following processing recommendations that may help reduce acrylamide in sliced potato chips ([FDA, 2016a](#)):

- Increasing peel removal.
- Washing or soaking potato chips before frying (but this may cause unacceptable changes to the chips).
- Cutting thinner potato chip slices.
- Decreasing frying temperatures to 175 °C (347 °F) or below and targeting higher moisture endpoints (but it is important to determine if moisture endpoints provide acceptable product quality).
- Using lower temperatures during final cooking stages and using techniques like flash frying, vacuum frying, or batch frying.
- Sorting by color, by providing a useful indicator of acrylamide levels, especially if correlated with measured levels of acrylamide in specific products.

For fabricated potato chips and other fabricated potato snacks, a typical production process consists of preparation of a dehydrated potato product such as potato flakes or granules, mixing with water and other ingredients to form dough, sheeting the dough, cutting, cooking (frying or baking), sorting, seasoning, and packaging. For extruded potato-based snacks, an extrusion step, with cutting and forming operations, follows dough formation. Some considerations for sliced potato chips also apply to fabricated potato chips or other potato-based snacks. Because of the use of doughs and potato-flake ingredients, other techniques (such as the use of asparaginase to decrease asparagine levels or substitution of potato flakes with other ingredients) may also be useful. Specifically, the guidance includes the following processing recommendations that may help reduce acrylamide in fabricated potato chips and other fabricated potato snacks ([FDA, 2016a](#)):

- Selecting potato flakes with lower levels of reducing sugars, e.g., by specifying maximum sugar levels, buying early in the processing season, or by mixing flakes from different sources. Flakes treated with acidulants, calcium, or asparaginase during flake production may also produce flake-based products with lower acrylamide.
- Partially substituting potato flakes with other ingredients.
- Adding calcium salts to potato doughs.
- Adding acidulants to potato doughs.
- For some products, adding asparaginase to potato doughs.
- In fabricated potato chips, decreasing cooking temperatures, using lower final temperatures in multistage processes, and using higher moisture endpoints.

Section C,i, “Cereal-based foods, raw materials,” reviews information on raw materials and processing approaches that may reduce acrylamide in cereal-based foods. In cereal grains such as wheat, asparagine is present in excess compared with reducing sugars. Therefore, the concentration of asparagine, not reducing sugars, is the important factor driving acrylamide formation. Cereal grain type, grain variety, and growing conditions are some of the factors that affect asparagine levels. For cereal-based foods, the guidance includes the following processing recommendations that may help reduce acrylamide ([FDA, 2016a](#)).

- Using wheat varieties that are lower in asparagine and using wheat grown with adequate soil sulfate and without excessive nitrogen fertilization.
- Partially substituting low-asparagine cereal grains for high-asparagine cereal grains.

Section C,ii, “Cereal-based foods, processing and ingredients,” reviews information on raw materials and processing approaches that may reduce acrylamide in cereal-based foods. For dough-based cereal products, the ability to use asparaginase and substitute ingredients provides additional options for acrylamide mitigation compared with intact potato products. The guidance includes the following specific recommendations for ingredients and processing that may help reduce acrylamide in cereal-based foods ([FDA, 2016a](#)).

- Replacing ammonium bicarbonate in cookies and crackers with alternative leavening agents, while avoiding overall increases in sodium levels.
- Replacing reducing sugars with nonreducing sugars, using reducing sugars with lower fructose content, and only adding sugar coatings to breakfast cereals after toasting.
- Using asparaginase treatment may reduce acrylamide, but asparaginase dose, contact time, dough water content, pH, and water chlorination are important considerations.

- Using calcium supplementation in non-calcium-fortified breads or breakfast cereals (but the addition of calcium propionate may increase acrylamide levels).
- Using yeast fermentation and changing fermentation conditions.
- Lowering thermal input through modifying baking times and temperatures and considering alternative baking technologies.
- Monitoring production by using color as an indicator of acrylamide, but the correlation between color and acrylamide may have to be determined on a product-by-product basis.
- Setting a higher moisture endpoint and monitoring moisture levels in finished products may be useful as an indirect indicator of acrylamide levels (FDA, 2016a).

Section D, “Other foods,” notes that coffee is a significant source of acrylamide exposure for adults, but that only limited information is available on factors known to affect acrylamide concentrations in coffee (FDA, 2016a).

Section E, “Preparation and cooking instructions on packaged frozen french fries,” and Section F, “Information for food service operations,” reflect the fact that final cooking conditions can be the major factor in determining final acrylamide levels in cooked fries prepared from packaged frozen french fries, as well as in other foods prepared in food service operations, such as baked goods. The guidance includes the following recommendations that may help reduce acrylamide in such foods (FDA, 2016a).

- Providing appropriate cooking instructions on frozen french fry packages to guide final preparation by consumers and food service operators.
- Educating food service workers to follow proper frying techniques for french fries.
- Selecting potato varieties that are low in reducing sugars for frying or roasting, properly handling and storing potatoes, and using certain cooking practices for foods made from potatoes.
- Baking and toasting breads and other baked goods to a light brown, not a dark brown color; and avoiding overly dry or crusty cereal-based products.

The final acrylamide guidance is available at [www.fda.gov](http://www.fda.gov). As guidances can be updated, it is good practice to check the FDA website for changes in the future.

## **Food Safety Modernization Act and Acrylamide**

In 2011, the FDA Food Safety Modernization Act (FSMA) (FSMA, 2011) was signed into law. This broad-reaching act gave FDA a legislative mandate to develop comprehensive preventive controls for food and feed facilities and new tools, such as mandatory recall, expanded records access, expanded administrative detention, suspension of facility registration, enhanced product tracing, and third party laboratory testing. To implement the law, FDA has issued regulations on produce safety, accredited third-party certification, mitigation strategies against intentional adulteration, sanitary transportation of human and animal food, foreign supplier verification programs, and preventive controls for human and animal food. Most relevant to acrylamide is the cornerstone prevention rule entitled “Current Good Manufacturing Practice, Hazard Analysis, and Risk-Based Preventive Controls for Human Food” (FDA, 2015). This rule, which became final in September 2015, requires food facilities to have a food safety plan in place that includes an analysis of hazards and implementation of risk-based preventive controls to significantly minimize or prevent identified hazards.

As part of FSMA’s implementation, FDA also has issued multiple guidance documents for industry, including the draft guidance entitled “Hazard Analysis and Risk-Based Preventive Controls for Human Food: Draft Guidance for Industry” (hazard analysis guidance). Chapters 1–5 and several appendices were published in August 2016 for public comment (FDA, 2016b). Chapters 6 and 15 have also been published for comment. The guidance is intended to help manufacturers consider the biological, chemical, and physical hazards that are commonly of concern in food plants and that should be addressed in a hazard analysis. It addresses ingredient-related hazards, process-related hazards, and hazards that may be introduced from the food-production environment (facility-related hazards). The guidance states that is important for manufacturers to understand the potential hazards that may be associated with products using the raw materials and other ingredients, processes, and equipment specific for those products, as well as the environment of their specific facility.

As required under FSMA, if manufacturers identify hazards requiring a preventive control, they have to determine what preventive controls are needed to reduce food safety risks and ensure the safety of their products for human consumption. As examples of chemical hazards requiring a preventive control, the guidance includes pesticides, heavy metals, drug residues, industrial chemicals, environmental contaminants, mycotoxins, allergens, unapproved colors and additives, substances associated with a food intolerance or food disorder, and radionuclides. Some process-related chemical hazards are included, such as hazards related to product formulation (e.g., sulfites that are a hazard for those consumers who are sensitive to them); and hazards that may be unintentionally introduced into food, such as industrial chemicals that are used in a facility for purposes other than food production.

Despite being considered a processing contaminant, acrylamide is not included as a hazard requiring a preventive control in the current draft hazard analysis guidance. As stated in Chapter 3 of the hazard analysis guidance, FDA has “not included such contaminants [i.e., acrylamide] in [a list of Common Sources of Chemical Hazards] as potential process-related chemical hazards that may require a preventive control as part of a food safety plan under part 117 because we believe that more information is

needed regarding appropriate levels and effective controls.” However, the hazard analysis guidance also reiterates the recommendation in the FDA acrylamide guidance that manufacturers evaluate approaches to acrylamide reduction that may be relevant to their particular processes and consider adopting approaches, if feasible, that reduce acrylamide levels in their products. Questions about FSMA and preventive controls, including how preventive controls relate to acrylamide, can be addressed to FDA’s FSMA Technical Assistance Network (TAN).

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## Relevant Websites

- FDA, [www.fda.gov](http://www.fda.gov).
- FDA FSMA and FSMA Technical Assistance Network information, <https://www.fda.gov/Food/GuidanceRegulation/FSMA/default.htm>.
- FDA acrylamide guidance and information, <https://www.fda.gov/Food/FoodbornellnessContaminants/ChemicalContaminants/ucm2006782.htm>.

# Acrylamide: An Overview of the Chemistry and Occurrence in Foods

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## Glossary

**Schiff base** Imine compound, formed from the reaction between an aliphatic or aromatic amine and a carbonyl compound by nucleophilic addition forming a hemiaminal, followed by dehydration.

**5-Hydroxymethyl-2-furfural (HMF)** An organic compound formed by the dehydration of certain sugars or by the Maillard reaction.

**$\alpha$ -Hydroxycarbonyl compound** A class of organic compounds possessing the secondary  $\alpha$ -hydroxy ketone functional group.

**Azomethine ylide** An iminium ion that occurs via decarboxylation of Schiff base during Maillard reaction.

**Amadori compound** Aminodeoxysugar derivatives formed in foods through the Maillard reactions.

**3-Aminopropionamide (3-APA)** An intermediate product in the acrylamide formation during thermal degradation of asparagine.

**Strecker aldehyde** An aldehyde compound formed by the Strecker degradation from an  $\alpha$ -amino acid.

**Electrophile** A compound or functional group accepts electrons.

**Nucleophilic group** Functional group donates electrons.

## Nomenclature

**IARC** International Agency for Research on Cancer

**HMF** 5-hydroxymethyl-2-furfural

**3-APA** 3-aminopropionamide

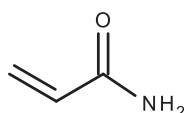
**EFSA** European Food Safety Authority

**WHO** World Health Organization

## Introduction

Acrylamide (or acrylic amide, prop-2-enamide) is an  $\alpha$ ,  $\beta$ -unsaturated carbonyl with a chemical formula  $C_3H_5NO$  (Fig. 1).

Acrylamide had been used as an industrial chemical in paper industry, water treatment, dam, tunnel and sewer construction for different purposes for years, but it was firstly discovered in thermally treated foods in 2002. Acrylamide had been intensively used to repair the water leaks and in October 1997, it was realized that it accidentally leaked to water systems from a railway tunnel in Sweden. It caused to death of the fish living in the river and paralysis of cows drinking from the river. After this incident, researchers in Stockholm University started to study the effects of acrylamide-contaminated water. During their research, they tried to confirm the presence of acrylamide-hemoglobin adducts in exposed animals and they interestingly discovered that acrylamide-hemoglobin adducts was not only found in cows and fish exposed to leak but also in unexposed humans living outside the contaminated area (Mills et al., 2009). Within this information, researchers considered that acrylamide might be a food contaminant. In a conducting study, it was reported that higher amounts of acrylamide-hemoglobin adduct was observed in rats fed with fried feed (Tareke et al., 2000). Eventually in 2002, Swedish National Food Administration (NFA) published that acrylamide was found in different thermally treated foods including potato chips, French fries and bakery products.



**Figure 1** Chemical structure of acrylamide.

## Formation Mechanism

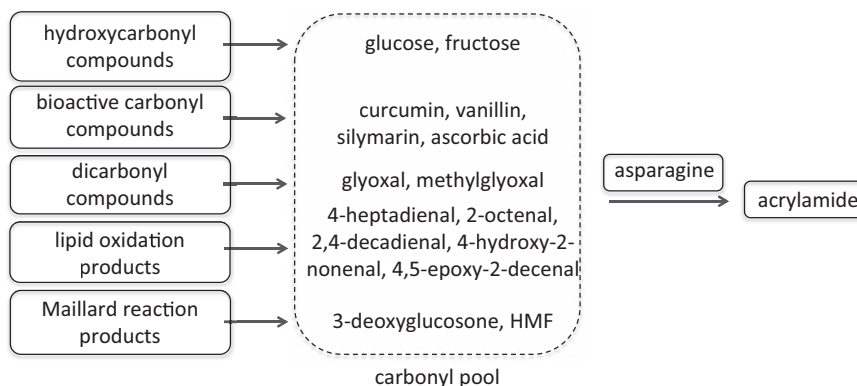
Since acrylamide is classified as a 'probable human carcinogen' by the International Agency for Research on Cancer (IARC), so much effort to evaluate its formation mechanism has been done so far (WHO, 1994; Eriksson, 2005; Manson et al., 2005). It has been found out that the Maillard reaction, the reaction between amino acids and reducing sugars as a result of heating food, was found to be the key responsible mechanism for the formation of acrylamide in certain starch-based foods, such as potato chips, French fries, bread and cereal products (Mottram et al., 2002b; Stadler et al., 2002; Tareke et al., 2002).

Experiments conducted by Zyzak et al. (2003) using labeled asparagine and glucose revealed that both carbon atoms and the nitrogen of acrylamide were derived from asparagine. As asparagine forms the backbone of acrylamide, it was confirmed that asparagine was the responsible amino acid for the formation of acrylamide. However, it was stated that formation rate of acrylamide is faster when asparagine is heated with carbonyl compounds such as reducing sugars (Yaylayan et al., 2003). Even asparagine itself is converted to acrylamide; studies carried out with different carbonyl compounds showed that carbonyl compounds react rapidly with asparagine yielding acrylamide (Stadler et al., 2002; Stadler and Scholz, 2004; Schieberle et al., 2005; Hamzalioglu and Gokmen, 2012). In this manner, reaction yield depends on carbonyl source and its ability to decarboxylate Schiff base (Blank, 2005).

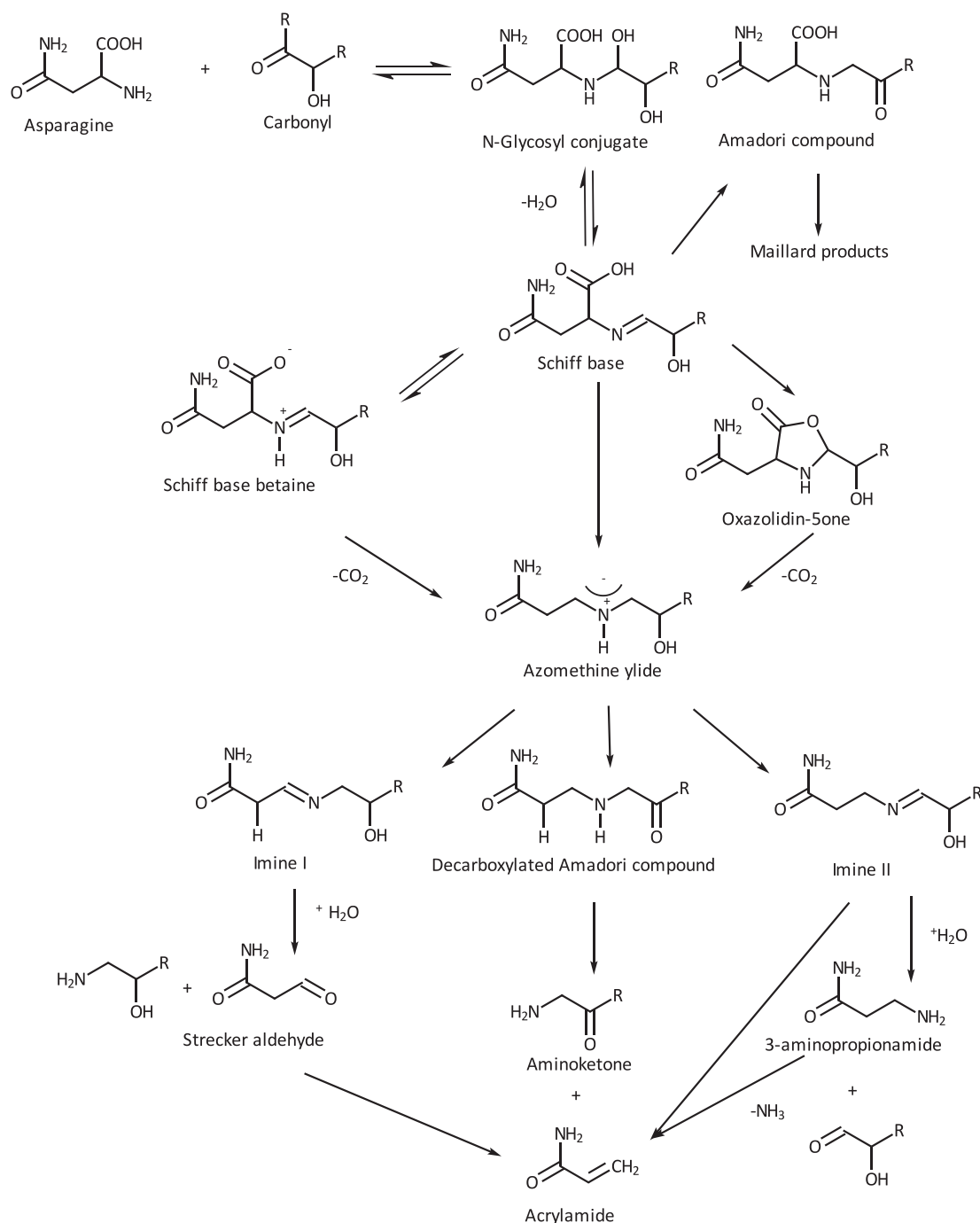
In addition to significant contribution of mostly known  $\alpha$ -hydroxycarbonyl compounds such as fructose and glucose to acrylamide formation (Yaylayan and Stadler, 2005), glyoxal, methylglyoxal, 5-hydroxymethyl-2-furfural (HMF) and other sugar degradation products bearing carbonyl groups have also been reported to trigger acrylamide formation during heating (Gokmen et al., 2012; Amrein et al., 2004; Amrein et al., 2006). Studies carried out with galactose, lactose, and even non-reducing sugar sucrose resulted in comparable amounts of acrylamide when heated with asparagine at 180 °C (Stadler et al., 2003). Sucrose is assumed to lead to acrylamide formation through its hydrolysis to reducing sugars, glucose and fructose, during thermal treatment. Besides these carbonyl compounds, it was shown by Hamzalioglu and Gokmen (2012) that bioactive carbonyl compounds also participate in Maillard reaction yielding to acrylamide formation. Among the bioactive carbonyl compounds, it was revealed that curcumin, ascorbic acid, dehydroascorbic acid, silymarin and vanillin react with asparagine for the formation of acrylamide. Additionally, some carbonyl compounds of virgin olive oil phenolic extracts and pyridoxal also act like precursor of acrylamide (Kotsiou et al., 2010; Zeng et al., 2009). Moreover, carbonyl compounds produced during processing of foods might contribute to acrylamide formation. Zamora and Hidalgo (2008) showed that several carbonyl compounds formed as a result of lipid oxidation contributed to the conversion of asparagine into acrylamide. Other researchers also investigated that oil oxidation level affected carbonyl formation and positively influenced the formation of acrylamide in dry fat-rich model systems (Capuano et al., 2010). All these carbonyl compounds, present in the food or formed during processing, create a carbonyl pool and involve in acrylamide formation as summarized in Fig. 2.

Formation of acrylamide is given in Fig. 3. Firstly,  $\alpha$ -amino group of asparagine reacts with the carbonyl group of carbonyl source yielding N-glycosyl-asparagine (carbinolamine), which then forms Schiff base through the removal of one molecule of water as a result of heating under dry conditions at temperatures above 120 °C. The moisture content of system determines the direction of the reaction. When the reaction condition is high-moisture, Schiff base may rearrange to form Amadori compounds. Since these stable compounds cannot decarboxylate, rearrangement of Schiff base is not prerequisite for the formation of acrylamide in this mechanism. Lately they decompose for the formation of color and flavor compounds (Stadler et al., 2004; Yaylayan et al., 2003; Ledl and Schleicher, 1990).

Decarboxylation of Schiff base, yielding an azomethine ylide (decarboxylated Schiff base) that may form imine I and II, is the second step of acrylamide formation. It may also proceed through Schiff base betaine or oxazolidin-5-one (Zyzak et al., 2003; Yaylayan et al., 2003). Hydrolyses of imine I leads to the Strecker aldehyde (3-oxopropanamide) formation, which does not release



**Figure 2** A reactive carbonyl pool converting asparagine into acrylamide. Modified from Kocadağlı, T., Göncüoğlu, N., Hamzalioglu, A., Gökmen, V., 2012. In depth study of acrylamide formation in coffee during roasting: role of sucrose decomposition and lipid oxidation. *Food Funct.* 3 (9), 970–975 and Zhang, Y., Jin, C. Chapter 17 Relationship between antioxidants and acrylamide formation. In: Gökmen, V. (Ed.), *Acrylamide in Food*, 2016, Academic Press, pp. 325–353



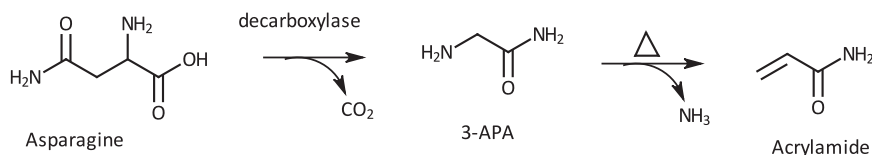
**Figure 3** Formation pathway of acrylamide. Adapted from Stadler, R.H., Robert, F., Riediker, S., Varga, N., Davidek, T., Devaud, S., Goldmann, T., Hau, J. and Blank, I., 2004. In-depth mechanistic study on the formation of acrylamide and other vinylogous compounds by the Maillard reaction. *J. Agric. Food Chem.* 52, 5550–5558.

high amounts of acrylamide (Stadler et al., 2004; Blank et al., 2005). Imine II could form acrylamide directly through 1,2-elimination. It also could form acrylamide through  $\beta$ -elimination of decarboxylated Amadori compound (Wedzicha et al., 2005).

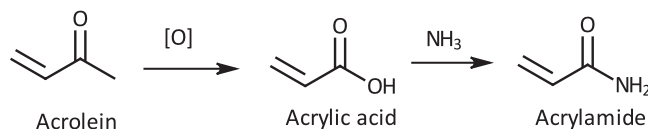
Additionally, formation of 3-aminopropionamide, which then deaminates to form acrylamide, as a result of hydrolysis of imine II is also possible (Zyzak et al., 2003). On the other hand, it is believed that Strecker aldehyde, which is formed through hydrolysis of imine I, is one of the direct precursors of acrylamide (Mottram et al., 2002a).

Even Maillard reaction was proposed as the major route for acrylamide formation, there are also some minor routes. Alternatively to acrylamide formation from asparagine through Maillard reaction, it was shown that asparagine might be converted to acrylamide in the absence of any carbonyl compound (Fig. 4). This is a non-Maillard pathway and 3-aminopropionamide (3-APA) is the major precursor in this route, which is generated directly from asparagine (Granvogl and Schieberle, 2007; Granvogl et al., 2004).





**Figure 4** Formation of acrylamide through 3-APA. Modified from Granvogl, M., Jezussek, M., Koehler, P., Schieberle, P., 2004. Quantitation of 3-Aminopropionamide in Potatoes: A Minor but Potent Precursor in Acrylamide Formation. *J. Agric. Food Chem.* 52, 4751–4757.



**Figure 5** Formation of acrylamide from acrolein. Adapted from Stadler, R.H., Studer, A., Chapter 1 Acrylamide formation mechanisms, In: Gökmen, V. (Ed.), *Acrylamide in Food*, 2016, Academic Press, pp. 1–17.

Formation of acrylamide through 3-APA proceeds in a lower extent. However, 3-APA might also be generated in foods under enzymatic action on asparagine, and rapidly converted to acrylamide even under aqueous conditions and at relatively low temperatures (Becalski et al., 2011).

Acrolein is also one of the precursors in acrylamide formation and could be formed through oxidative lipid degradation, carbohydrate degradation and Maillard reaction (Stadler and Studer, 2016). As shown in Fig. 5, acrolein is oxidized to acrylic acid and then reacts with ammonia (produced from  $\alpha$ -amino acids via Strecker degradation in the presence of carbonyl compounds) yielding acrylamide (Yasuhara et al., 2003). However, since acrolein is highly reactive, it prefers reacting with food components than oxidation to acrylic acid. Acrylic acid could also be formed from thermal decomposition of aspartic acid, carnosine and  $\beta$ -alanine (Stadler et al., 2003; Yaylayan et al., 2004; Yaylayan and Stadler, 2005).

### Further Reactions of Acrylamide With Other Food Components

As stated before, acrylamide is an  $\alpha,\beta$ -unsaturated carbonyl and these structural properties make it electrophile. Due to its highly electrophilic nature, it readily reacts with nucleophilic groups such as amino and thiol groups in foods through Michael type addition. Hidalgo and others carried out the studies investigating the Michael type reactions of both thiol and amino compounds with acrylamide (Hidalgo et al., 2010; Hidalgo et al., 2011; Zamora et al., 2010). They reported that thiol compounds are easily added to carbon–carbon double bond of acrylamide. Similarly, they showed that acrylamide could react with amino compounds as well. However, reaction yield with thiol compounds were 100–300 times higher than with amino compounds. In addition, formation of Michael adducts of acrylamide with thiol compounds takes place even at low temperatures in a very high extent. Also, reactions of acrylamide with thiol compounds are encouraged in the presence of amino compounds (Hidalgo et al., 2010; Zamora et al., 2010; Hidalgo et al., 2011).

### Factors Affecting Acrylamide Formation

Extensive studies carried out on the formation kinetics of acrylamide formation since its discovery in heated foods provided the important factors affecting its formation in detail (Martins and Van Boekel, 2005; Claeys et al., 2005a,b; Granda and Moreira, 2005). As stated before, asparagine is the main responsible amino acid from acrylamide formation and reducing sugars highly affect its conversion rate to acrylamide. Physical state of precursors in food has also been found to be important for the reaction rate (Van der Fels-Klerx et al., 2014). Asparagine is abundantly found in potatoes and concentration of reducing sugar limits the conversion of asparagine into acrylamide in potato products. Contrary to reducing sugars, asparagine concentration is limited in cereals thus it determines the acrylamide formation in cereal products. Accordingly, reduction in precursors results in limiting acrylamide content in heated potato and cereal products. For this, use of low-asparagine and/or low carbonyl crop varieties or controlling growth and storage conditions of crops were found to be effective (Haase et al., 2004; Haase and Weber, 2003; De Wilde et al., 2006; Muttucumaru et al., 2013). Selection of low-asparagine varieties was not only recommended for potatoes and cereals but also for chicory, which is the raw material of coffee substitutes and leads to acrylamide formation during roasting (Loaec et al., 2014). However, this could not be recommended for coffee beans since they already contains low levels of asparagine ( $0.3\text{--}0.9\text{ g kg}^{-1}$ ) (Stadler and Scholz, 2004). Asparaginase enzyme was found to greatly affect acrylamide formation. Asparaginase catalyzes the conversion reaction of asparagine into ammonia and aspartic acid, which makes asparagine unable to react to form acrylamide. Using this enzyme in different foods as ingredients, e.g. mashed potato, bakery products, or as dipping solution, e.g. for French fry, effectively decreased the final acrylamide content of these foods.

Presence or incorporation of compounds such as amino acids, bioactive carbonyl compounds that might compete with the precursors affects the reaction rate (Rydberg et al., 2003; Hamzalioglu and Gokmen, 2012). Incorporation of inorganic salts to the recipe was found as effective in controlling acrylamide content in foods by blocking the formation of some intermediates or accelerating the polymerization of acrylamide (Simko et al., 2009; Gokmen and Senyuva, 2007; Sadd et al., 2008; Amrein et al., 2004; Kolek et al., 2007). As stated before, formation of Schiff base between the carbonyl and asparagine is the first step in acrylamide formation and it was proposed that presence of  $\text{Ca}^{2+}$  prevented the formation of Schiff base and thus of acrylamide (Gokmen and Senyuva, 2007). In addition, it was confirmed by Kolek et al. (2007) that acrylamide polymerization was strongly accelerated by the presence of NaCl yielding polyacrylamide, which is biologically inactive. Several carbonyl compounds formed through the lipid oxidation contribute to the formation of acrylamide (Zamora and Hidalgo, 2008). Therefore, acrylamide formation might be affected by the addition of antioxidants, which avoid lipid oxidation and consequently formation of carbonyl compounds. On the other hand, Ou et al. (2010) proposed that antioxidants could attack acrylamide when they are in oxidized form. They found that acrylamide content decreased due to radical-induced elimination in the presence of antioxidant compounds both in the acrylamide and in the asparagine-glucose model systems. Oxidized antioxidant addition could cause significant reduction in acrylamide content of both asparagine-glucose and acrylamide model system.

Apart from the precursors, process conditions have strong influence on acrylamide formation. Formation of acrylamide is directly related to the amount of heat (temperature–time) applied and final moisture of the food (Claeys et al., 2005b; Hedegaard et al., 2007; Knol et al., 2005). Kinetic studies in model systems showed that the increase in acrylamide was relatively moderate in the case of temperature applied ranging between 150 and 170 °C (Gertz and Klostermann, 2002). However, in French fries, it was reported that acrylamide linearly increased by increasing frying temperature (especially higher than 175 °C) (Matthaus et al., 2004; Taeymans et al., 2004). For this reason, FoodDrinkEurope recommended a maximum frying temperature as 175 °C in the Acrylamide Toolbox (FoodDrinkEurope, 2013). Furthermore, the amount of acrylamide in these French fries also increased when the frying time was extended at the same temperature. From the moisture point of view, the suitable regions for acrylamide formation were discovered as the surface and a thin layer just below the surface. Since these regions were exposed to higher temperatures, acrylamide was mostly concentrated in these regions (Acar and Gokmen, 2009). Accordingly, Gokmen et al. (2006) indicated that acrylamide was mostly found in the outer parts of French fries.

## Occurrence in Foods

After the discovery of presence of acrylamide in heated foods, European Commission started to have data about acrylamide concentrations in foods. Within the acquired data, basically, foods rich in carbohydrate and asparagine, processed at high temperature, and containing low moisture have the highest potential to form acrylamide. Boiling doesn't lead to acrylamide formation, whereas baking, deep-fat frying, roasting cause to form acrylamide in certain foods (Tornqvist, 2005). In June 2015, the European Food Safety Authority (EFSA) published a report including the acrylamide levels in monitored foods. As indicated in this report, acrylamide is formed in baked or fried carbohydrate-rich foods mostly in French fries, potato chips, breads, biscuits and coffee. **Table 1** gives the summary of the recent results published by EFSA (EFSA, 2015). Indicative values for some foods determined by European Commission were also given in **Table 1**. Indicative values are not legal limits but further investigation is recommended if an indicative value is exceeded. Additionally, in 2017, EU Commission prepared a recent regulation proposal in which benchmark acrylamide levels were specified (**Table 1**) (EU, 2017). It was stated that benchmark levels could be used to test the performance of mitigation measures that will be used by the industry. The Council of European Parliament will review draft of regulation and after its approval, European food operators will likely be enforced by the mandatory limits on acrylamide in food and this will lead them to review their food operations.

According to these data, French fries, potato crisps, coffee and coffee substitutes were found to be containing the highest amounts of acrylamide. However, all these acrylamide content of foods should be considered together with the consumption amount in order to evaluate the total risk and exposure to acrylamide.

Acrylamide amounts in foods greatly varied depending on both process conditions and precursor concentrations. Accordingly, considering the bakery products, no acrylamide was detected in bread crumbs (Ahrne et al., 2007) whereas acrylamide concentration was more than 2000  $\mu\text{g kg}^{-1}$  in crackers (EFSA, 2015) due to the differences in moisture contents. Since potato is rich in asparagine, fried potato products, including French fries, potato crisps and snacks, have also been reported to contain high levels of acrylamide with mean values of 332–580  $\mu\text{g kg}^{-1}$ .

As reported by EFSA, mean acrylamide concentration was found to be as 578  $\text{ng g}^{-1}$  in roasted coffee (EFSA, 2015). Differences in species of roasted coffee were found to strongly affect acrylamide formation. Acrylamide content of roasted Robusta coffee beans was analyzed to be 45%–55% higher than that of Arabica coffee beans under the same roasting conditions (Summa et al., 2007). Higher acrylamide levels were detected in instant coffee and cereal based coffee substitutes but, chicory-based coffee substitutes showed the highest levels of indicative value of 4000  $\mu\text{g g}^{-1}$ .

Roasted nuts, such as almond, have been reported to contain acrylamide. Acrylamide concentration given in literature was 260  $\text{ng g}^{-1}$  for roasted almonds and 2147  $\text{ng g}^{-1}$  in dark roasted almonds (Amrein et al., 2005a). On the contrary, very little acrylamide was detected in both roasted hazelnuts (16–56  $\text{ng g}^{-1}$ ) and chestnuts (90  $\text{ng g}^{-1}$ ) since they contain relatively low amounts of asparagine in raw (Zilic, 2016; Amrein et al., 2005b).

**Table 1** Levels, indicative values and benchmark levels ( $\mu\text{g kg}^{-1}$ ) of acrylamide in foods (EFSA, 2015; EU, 2017)

	<i>n</i>	<i>Median</i> ( $\mu\text{g kg}^{-1}$ )	<i>Mean</i> ( $\mu\text{g kg}^{-1}$ )	<i>P95</i> ( $\mu\text{g kg}^{-1}$ )	<i>Indicative value</i> ( $\mu\text{g kg}^{-1}$ )	<i>Benchmark level</i> ( $\mu\text{g kg}^{-1}$ )
French fries	1378	196	332	1115	600	500
Potato crisp and snacks	800	389	580	1841	1000	750
<b>Soft bread</b>						
Wheat based bread	302	15	38	120	80	50
Other	99	25	46	203	150	100
<b>Breakfast cereals</b>						
Bran products and whole grain cereals	151	135	164	413	400	300
Non-whole grain or non-bran based wheat and rye based products	33	140	142	–	300	300
Non-whole grain or non-bran based maize, oat, spelt, barley and rice based products	149	50	73	230	200	150
Biscuits and wafers	682	103	201	810	500	350
Crackers	162	183	231	590	500	400
Crisp bread	437	89	149	428	450	350
Ginger bread	693	155	407	1600	1000	800
Roasted coffee	566	203	244	563	450	400
Instant coffee	116	620	674	1333	900	850
<b>Coffee substitutes</b>						
Based on cereals	20	522	510	–	2000	500
Chicory	37	3100	2942	–	4000	4000
Baby foods (other than processed cereal based)	348	15	24	70	50–80	–
Cereal based baby foods	394	15	103	200	50	40
Roasted nuts	40	25	93	–	–	–
Black olives in brine	3	313	454	–	–	–

Mean, median, P95: mean, median and 95th percentile contamination level presented as the middle bound (MB) estimate.

As stated above, formation of acrylamide is strongly dependent to excessive thermal treatment at high temperatures. Even though such conditions are not generally applied to table olives, acrylamide content of canned black ripe olives was found around  $2000 \mu\text{g kg}^{-1}$ . Recent studies have also confirmed that acrylamide has been only detected in California-style black ripe and California-style green ripe olives, not detected in Spanish-style and Greek-style olives. Sterilization was proposed to be responsible step from the formation of acrylamide in canned black ripe olives. Acrylamide was not detected in olives without thermal treatment (Casado and Montano, 2008). Additionally, acrylamide content of Californian style green ripe olives were comparably lower than black ripe olives. This was due to formation of acrylamide precursors during darkening, which then will be converted to acrylamide in the course of sterilization (Charoenprasert and Mitchell, 2014).

Apart from the thermally treated foods, acrylamide was reported to be present in drinking water due to the contamination during the water treatment process. During this process, acrylamide is released from the residual monomer of polyacrylamide. The World Health Organization (WHO) announced a value of  $0.5 \mu\text{g L}^{-1}$  for residual acrylamide concentration in drinking waters, whereas this limit value was specified as  $0.1 \mu\text{g L}^{-1}$  by European Union. However, acrylamide levels in potable waters coming from the rivers treated with polyacrylamides were analyzed as lower than  $5 \mu\text{g L}^{-1}$ . Similarly, drinking waters analyzed from different countries all contained acrylamide in lower amounts (Chu and Metcalfe, 2007; Brown and Rhead, 1979). Consequently, acrylamide content of common foods are greatly higher than the maximum residual acrylamide concentrations in water.

## Summary

Acrylamide, a classified probable carcinogen to humans, is primarily formed through the Maillard reaction and mostly found in carbohydrate-rich foods. Asparagine is the main responsible precursor for its formation, and several carbonyl compounds contribute to its higher conversion to acrylamide. Acrylamide is dense in foods exposed to excessive heat treatment and having high amounts of asparagine together with reducing sugars. Potato chips, French fries, pan-fried potato products, biscuits, crisp bread, coffee and coffee substitutes are some of the examples of these foods.

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## Relevant Website

[http://www.fooddrinkeurope.eu/uploads/publications\\_documents/AcrylamideToolbox\\_2013.pdf](http://www.fooddrinkeurope.eu/uploads/publications_documents/AcrylamideToolbox_2013.pdf).



# Dietary Acrylamide: An Update on the Chronic Risks

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## Nomenclature

AA acrylamide  
APC adenomatous polyposis coli  
BMDL10 bench mark dose lower limit 10  
BMI body mass index  
CI confidence interval  
CONTAM panel contaminants in the Food Chain panel  
CYP2E1 cytochrome P4502E1  
DNA deoxyribonucleic acid  
EFSA European Food Safety Authority  
FFQ food frequency questionnaire  
FSH follicle-stimulating hormone  
GA glycidamide  
KRAS Kirsten rat sarcoma viral oncogene homolog  
µg/kg bodyweight microgram per kilogram bodyweight  
MoBa study Norwegian Mother and Child Cohort Study  
MOE margin of exposure  
NHANES National Health and Nutrition Examination Survey  
NOAEL no observed adverse effect level  
OR odds ratio  
RR relative risk  
SGA small for gestational age  
SH-group sulfhydryl group

## Introduction

Due to its presence in many every-day foods, virtually every person is exposed to acrylamide on a daily basis. When ingested, acrylamide is well absorbed from the stomach and gastrointestinal tract and is distributed with the blood to all organs and tissues (EFSA, 2015). Thus, it has the potential to cause adverse effects throughout the whole body.

Acrylamide is a soft electrophile which means that it can bind with soft nucleophiles such as cysteine residues in proteins (EFSA, 2015). Proteins with a diversity of functions, such as antibodies, receptors, hormones, and enzymes, often contain cysteine residues. Cysteine residues consist of intra- and inter-molecular disulfide bonds, important for protein folding and stability, or of free thiols (SH-groups). Free thiols are often part of the catalytic activity site in enzymes. Thus, by binding to these cysteine residues, acrylamide can potentially affect many proteins with a diversity of functions. Interference with the function of proteins can lead to the disturbance of various physiological processes.

Acrylamide itself has low reactivity towards hard nucleophiles such as DNA (EFSA, 2015). In contrast, the epoxide metabolite of acrylamide, glycidamide, has the potential to alkylate DNA due to its epoxide moiety (EFSA, 2015). Through this alkylation, glycidamide can lead to DNA point mutations, which can initiate tumors by changing the DNA sequence, potentially activating oncogenes or inactivating tumor suppressor genes. In addition, glycidamide is a clastogen, which means that it can cause breakages in DNA that can also lead to cancer (EFSA, 2015).

Acrylamide has been proven to cause cancer, neurotoxicity, reproductive toxicity and developmental toxicity in experimental animal studies (EFSA, 2015). No experimental studies have been performed on humans because they are deemed unethical. The available epidemiological evidence comes from observational studies.

In humans, occupational exposure to acrylamide has not clearly been shown to be associated with an increased risk of cancer, even though the exposure in occupational settings was higher than exposure through diet (EFSA, 2015). However, it has to be kept in mind that exposure routes in occupational settings are mostly inhalation and dermal absorption whereas dietary exposure is through ingestion. In addition, the historical occupational cohorts were mainly made up of men, lacked thorough data to adjust for potentially important other risk factors for cancer and estimated individual acrylamide exposure rather crudely. Epidemiological data on neurotoxic health effects of acrylamide stem from case studies and occupational exposure studies, in which effects on both



the peripheral and central nervous system were observed, at doses considerably higher than the doses to which we are exposed through diet.

There is scarce data, both from animal and epidemiological studies, on possible effects of acrylamide on the immune, metabolic and cardiovascular system.

In 2015, the European Food Safety Authority (EFSA) performed a full risk assessment for dietary acrylamide intake. It concluded that the thus far available epidemiological studies did not yield sufficient evidence to be able to be used in risk characterization and it thus based its risk assessment on experimental animal data only. The outcome of the risk assessment was that there is a high level of concern with regard to dietary intake for neoplastic (carcinogenic) effects but a low level of concern for non-neoplastic effects (neurotoxicity, and developmental and reproductive toxicity) (EFSA, 2015).

In the following sections, the different chronic health risks of dietary acrylamide are discussed based on results from experimental animal studies and epidemiological studies, with a focus on the latter.

## Carcinogenicity

In 2-year experimental studies with rats, acrylamide repeatedly proved to cause tumors in the thyroid and mammary gland (latter in females) and the scrotal mesothelioma in male rats. In mice, acrylamide causes cancer in among other the lungs, skin and Harderian gland (EFSA, 2015). It can thus be concluded that acrylamide is a multispecies, multisite carcinogen. The results of these studies are summarized in **Tables 1** and **2**. It has been argued that acrylamide behaves in human bodies the same way it does in animals in terms of uptake, metabolism and genotoxicity, and therefore it was classified as a probable human carcinogen by the International Agency for Research on Cancer in 1994 (IARC, 1994).

Initially, all attention with regard to the carcinogenic mechanism of acrylamide was focused on the genotoxicity of acrylamide's metabolite glycidamide. In recent years, alternative mechanisms for carcinogenesis are considered: increasing oxidative stress, interference with cell division by forming adducts on meiotic/mitotic spindle kinesin proteins causing aneuploidy, and interfering with sex hormone systems (Besaratnia and Pfeifer, 2007). Alternative mechanisms were put forward for several reasons. In rodent studies, DNA adduct formation and DNA damage alone could not account for the observed organ specificity for tumor induction in the rodents (Segeberback et al., 1995). In addition, because of the difference in the efficiency to metabolize acrylamide to glycidamide in mice and rats, it is unlikely that all tumors resulting from acrylamide exposure in rats and mice occur by the same mechanism. Furthermore, some of the tumors observed in rodents upon acrylamide exposure are known or hypothesized to be caused by non-genotoxic mechanisms, such as sex hormone-induced tissue proliferation (Haber et al., 2009; Maier et al., 2012).

For its 2015 acrylamide risk assessment, EFSA based its estimation of the risk of cancer related to dietary acrylamide exposure on the animal studies. They calculated the bench mark dose lower limit 10 (BMDL10), which is the lower limit of the confidence interval around the modeled estimated dose that increased the incidence of the most sensitive cancer (occurring at the lowest dose) outcome (Harderian gland adenomas and adenocarcinomas in male B6C3F1 mice) by 10% over the incidence in controls. In this way, they arrived at a reference point value of 0.17 mg/kg bodyweight per day. This reference value is then divided by the estimated dietary exposure of humans and the so-called margin of exposure (MOE) is calculated. For genotoxic carcinogenic effects, it is determined that the MOE has to be 10000 or more. Because the MOE for cancer ranged from only 50 to 425 in different age groups, EFSA arrived at the conclusion that there is important cause for concern with regard to the carcinogenic risks of dietary acrylamide intake (EFSA, 2015).

For humans, the body of evidence for dietary acrylamide intake and cancer is less clear than for experimental animals, as might be expected because the doses in humans are lower and harder to assess, and observational studies cannot be controlled for interfering or confounding factors to the extent that experimental studies can be.

The association between dietary acrylamide intake and the risk of several cancers has been studied in several epidemiological studies, both retrospective (case-control studies) and prospective (prospective cohort studies) in nature, and using both food frequency questionnaire-derived acrylamide intake and acrylamide biomarkers. The studies with their characteristics and results are summarized in **Table 3**.

For some cancers, an association between increased acrylamide intake and increased risk was observed in some studies but not in other studies on the same cancers. This is not unusual for epidemiological research. In situations where there is no agreement between studies, such as is the case here with studies showing an association while other studies do not, a meta-analysis that statistically pools all the available evidence is useful. In 2015, a meta-analysis was performed (Pelucchi et al., 2015). A relative risk (RR) of borderline statistical significance for renal cancer (RR = 1.20; 95% confidence interval (CI), 1.00–1.45) comparing the highest category of acrylamide intake to the lowest was calculated. Among never-smokers, comparing the highest category of acrylamide intake to the lowest, borderline statistically significant associations were observed for endometrial (RR = 1.23; 95% CI, 1.00–1.51) and ovarian (RR = 1.39; 95% CI, 0.97–2.00) cancers. The authors of the meta-analysis concluded that dietary acrylamide is not related to the risk of most common cancers but a modest association for kidney cancer, and for endometrial and ovarian cancers in never smokers only, cannot be excluded (Pelucchi et al., 2015).

With regard to breast cancer, the picture emerging from the epidemiological studies is heterogeneous with some studies showing indications for an increased risk of estrogen receptor-positive breast cancer (Olesen et al., 2008; Pedersen et al., 2010) or for premenopausal women (Burley et al., 2010) while other studies show no associations or indications for an inverse association (**Table 3**).

**Table 1** Tumor incidence derived from the 2-year NTP carcinogenicity assays with acrylamide in B6C3F1 mice (NTP, 2012)

<i>Tumor</i>	<i>Sex</i>	<i>Dose (mg/kg b.w. per day)</i>	<i>Incidence (%)<sup>a</sup></i>
Harderian gland adenoma	Female	0	0
		<b>1.10</b>	<b>18</b>
		<b>2.23</b>	<b>42</b>
		<b>4.65</b>	<b>68</b>
		<b>9.96</b>	<b>72</b>
Mammary gland adenoacanthomas and adenocarcinoma	Female	0	0
		1.10	9
		<b>2.23</b>	<b>15</b>
		4.65	9
		<b>9.96</b>	<b>41</b>
Lung alveolar, bronchiolar adenoma	Female	0	2
		1.10	9
		2.23	13
		<b>4.65</b>	<b>24</b>
		<b>9.96</b>	<b>42</b>
Ovary granulosa cell tumors (benign)	Female	0	0
		1.10	2
		2.23	0
		4.65	2
		<b>9.96</b>	<b>12</b>
Skin, various types of sarcoma	Female	0	0
		1.10	0
		2.23	6
		<b>4.65</b>	<b>22</b>
		<b>9.96</b>	<b>14</b>
Stomach, forestomach squamous cell papilloma	Female	0	9
		1.10	0
		2.23	4
		<b>4.65</b>	<b>11</b>
		<b>9.96</b>	<b>19</b>
Harderian gland adenoma and adenocarcinoma	Male	0	4
		<b>1.04</b>	<b>28</b>
		<b>2.20</b>	<b>57</b>
		<b>4.11</b>	<b>79</b>
		<b>8.93</b>	<b>83</b>

<sup>a</sup>Incidence is given in bold when statistically significantly higher than incidence at dose 0.

Adapted from EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2015. Scientific Opinion on acrylamide in food.

EFSA Journal 2015; 13(6):4104, 321 pp. <https://doi.org/10.2903/j.efsa.2015.4104>. Number of mice per dose group: 42–48

For esophageal cancer, 2 studies suggest that there may be an increased risk in overweight persons (Hogervorst et al., 2008a,b; Lin et al., 2010).

In addition, positive associations with acrylamide intake were observed for some cancers but only 1 study so far has been performed for those cancers: colorectal cancer with KRAS mutations in men (Hogervorst et al., 2014), lung cancer in smoking men (Hirvonen et al., 2010), oral cavity cancer in women (Schouten et al., 2009), lymphatic malignancies in men (Bongers et al., 2012), and melanoma in men (Lipunova et al., 2017). For these cancers, more epidemiological studies are needed.

Several studies have been performed for prostate, pancreatic, stomach, bladder and laryngeal cancer but no clear increased risks were observed in relation to acrylamide intake (Table 3).

In addition, for lung cancer in women (Hogervorst et al., 2009a,b) and colorectal with a truncating APC mutation in women (Hogervorst et al., 2014), inverse associations were observed but these cancers were only once so far. Also for these cancers, more epidemiological studies are needed.

If it is true that acrylamide exerts both a risk-increasing and protective effect with regard to cancer, then studies on total cancer incidence and mortality are important to judge the net effect of acrylamide. There recently was 1 study on cancer mortality in Chinese men and women that showed increased risks of cancer mortality (total, respiratory system, and digestive tract cancer) in Chinese elderly (Liu et al., 2017b). However, the main dietary sources of acrylamide exposure in this study (stir-fried vegetables) were very different from those in Western countries (e.g., fried potatoes, coffee) and it would be interesting to know whether associations are also observed in Western populations.

**Table 2** Tumor incidence derived from the 2-year carcinogenicity assays with acrylamide in F344 rats

<i>Tumor</i>	<i>Gender</i>	<i>Dose (mg/kg b.w. per day)</i>	<i>Incidence (%)<sup>a</sup></i>	<i>References</i>
Mammary gland adenoma, fibroadenoma or fibroma	Female	0	17	Johnson et al., 1986
		0.01	18	
		0.1	15	
		0.5	33	
		<b>2.0</b>	<b>38</b>	
Mammary gland fibroadenoma	Female	0	11	Friedman et al. 1995
		<b>1.0</b>	<b>21</b>	
		<b>3.0</b>	<b>27</b>	
Mammary gland fibroadenoma	Female	0	33	NTP 2012
		0.44	38	
		<b>0.88</b>	<b>52</b>	
		<b>1.84</b>	<b>47</b>	
		<b>4.02</b>	<b>65</b>	
Thyroid gland follicular cell adenoma or carcinoma	Female	0	2	Johnson et al., 1986
		0.01	0	
		0.1	2	
		0.5	2	
		<b>2.0</b>	<b>8</b>	
Thyroid gland follicular cell adenoma or carcinoma	Female	0	2	Friedman et al. 1995
		1.0	10	
		<b>3.0</b>	<b>23</b>	
Thyroid gland follicular cell adenoma or carcinoma	Female	0	0	NTP 2012
		0.44	0	
		0.88	4	
		1.84	6	
		<b>4.02</b>	<b>9</b>	
Thyroid gland follicular cell adenoma	Male	0	2	Johnson et al., 1986
		0.01	0	
		0.1	3	
		0.5	2	
		<b>2.0</b>	<b>12</b>	
Thyroid gland follicular cell adenoma	Male	0	2	Friedman et al. 1995
		0.1	4	
		0.5	5	
		<b>2.0</b>	<b>16</b>	
Thyroid gland follicular cell adenoma or carcinoma	Male	0	2	NTP 2012
		0.33	6	
		0.66	9	
		1.32	13	
		<b>2.71</b>	<b>19</b>	
Mesothelioma of the testes tunica albuginea	Male	0	5	Johnson et al., 1986
		0.01	0	
		0.1	12	
		<b>0.5</b>	<b>18</b>	
Mesothelioma of the testes tunica	Male	<b>2.0</b>	<b>17</b>	Friedman et al. 1995
		0	4	
		0.1	4	
		0.5	8	
Mesothelioma of the epididymis or testes tunica vaginalis	Male	<b>2.0</b>	<b>17</b>	NTP 2012
		0	4	
		0.33	6	
		0.66	2	
		1.32	10	
		<b>2.71</b>	<b>17</b>	

Number of mice per dose group: 46–204.

<sup>a</sup>Incidence is given in bold when statistically significantly higher than incidence at dose 0.

Adapted from EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2015. Scientific Opinion on acrylamide in food. EFSA Journal 2015; 13(6):4104, 321 pp. <https://doi.org/10.2903/j.efsa.2015.4104>.

**Table 3** Associations between dietary acrylamide intake and cancer risk

References	Method acrylamide intake assessment	Study design and size	Range of intake	Overall results: OR/RR (95% CI)	Subgroup analyses
<b>Breast cancer</b>					
Mucci et al., 2005	FFQ	Cohort: 667 mainly premenopausal cases; 43404 cohort members	12–44 µg/day (mean Q1-Q5)	Q2: 0.9 (0.7–1.1) Q3: 1.0 (0.8–1.3) Q4: 1.0 (0.8–1.3) Q5: 1.2 (0.9–1.6)	n.a.
Pelucchi et al., 2006	FFQ	Case-control study: 2900 pre and postmenopausal cases; 3122 controls	11–34 µg/day (p20-p80)	Q2: 1.01 (0.85–1.20) Q3: 1.01 (0.85–1.20) Q4: 1.09 (0.92–1.31) Q5: 1.06 (0.88–1.28) p for trend: 0.37	n.a.
Hogervorst et al., 2007	FFQ	Cohort: 1835 postmenopausal cases; 62573 cohort members	10–37 µg/day (median Q1-Q5)	Q2: 0.80 (0.64–1.02) Q3: 0.92 (0.72–1.17) Q4: 0.86 (0.67–1.10) Q5: 0.93 (0.73–1.19) p for trend: 0.79	Comparable results for never-smokers
Olesen et al., 2008	Hemoglobin adducts of AA and GA	Nested case-control study in a cohort; 374 cases; 374 controls	AA adducts: 20–209 pmol/g Hb GA adducts: 9–99 pmol/g Hb (p5–p95)	Per 10-fold increase: AA adducts: 1.9 (0.9–4.0) GA adducts: 1.3 (0.6–2.8)	<b>ER + tumors:</b> <b>2.7 (1.1–6.6) per 10-fold increase AA-Hb adducts</b> Stronger associations for smokers
Larsson et al. 2009c	FFQ	Cohort: 2592 pre and postmenopausal cases; 61433 cohort members	20–29 µg/day (p25–p75)	Q2: 1.02 (0.92–1.14) Q3: 0.95 (0.85–1.06) Q4: 0.91 (0.80–1.02) p for trend: 0.06	Comparable results for ER- and PR-subgroups and according to smoking status
Wilson et al., 2009b	FFQ	Cohort: 1179 premenopausal cases; 90628 cohort members	11–38 µg/day (mean Q1-Q5)	Q2: 0.95 (0.79–1.14) Q3: 0.94 (0.78–1.13) Q4: 1.03 (0.87–1.24) Q5: 0.92 (0.76–1.11) p for trend: 0.61	Comparable results according to hormone receptor and smoking status
Pedersen et al., 2010	FFQ	Cohort: 2225 postmenopausal cases; 62573 cohort members	10–37 µg/day (median Q1-Q5)	Q2: 0.91 (0.73–1.23) Q3: 0.96 (0.76–1.19) Q4: 0.89 (0.72–1.12) Q5: 0.92 (0.73–1.15) p for trend: 0.48	Positive, not statistically significant, associations for never-smokers and for ER+, PR+ and ER + PR+

Wilson et al., 2010	FFQ	Cohort: 6301 postmenopausal cases; 88672 cohort members	9–26 µg/day (mean Q1–Q5)	Q2: 0.93 (0.86–1.01) Q3: 0.98 (0.91–1.06) Q4: 0.98 (0.90–1.06) Q5: 0.95 (0.87–1.03) p for trend: 0.50	Comparable results in strata of smoking, menopausal status, and BMI
Burley et al., 2010	FFQ	Cohort: 1084 cases; 33731 cohort members	6–32 µg/day (mean Q1–Q5)	Q2: 1.06 (0.83–1.35) Q3: 1.05 (0.82–1.34) Q4: 1.12 (0.87–1.45) Q5: 1.16 (0.88–1.52) p for trend: 0.10	<b>Premenopausal cases:</b> Q2: 1.06 (0.71–1.59) Q3: 1.15 (0.77–1.71) Q4: 1.15 (0.76–1.73) Q5: 1.47 (0.96–2.27) <b>p for trend: 0.008</b> No association for postmenopausal cases <b>Statistically significant associations ER + cases:</b> <b>2.23 (1.38–3.61) per 25 pmol/g Hb</b> No or slightly weaker associations in smoking women
Olsen et al., 2012	Hemoglobin adducts of AA and GA	Breast cancer survival cohort: 420 postmenopausal cases; 80 postmenopausal breast cancer deaths	AA and GA-Hb adducts: Non-smokers: 30–137 pmol/g Hb Smokers: 60–389 pmol/g Hb (p5–p95)	<b>Non-smoking women:</b> AA adducts: 1.21 (0.98–1.50) per 25 pmol/g globin <b>GA adducts:</b> <b>1.63 (1.06–2.51) per 25 pmol/g globin</b>	<b>Statistically significant associations ER + cases:</b> <b>2.23 (1.38–3.61) per 25 pmol/g Hb</b> No or slightly weaker associations in smoking women
Kotemori et al., 2017	FFQ	Cohort: 792 pre and postmenopausal cases; 48910 cohort members	0–63 µg/day	T2: 1.00 (0.84–1.18) T3: 0.95 (0.79–1.14) p for trend: 0.58	No associations in strata of smoking, coffee and alcohol consumption, BMI, menopausal status, ER and PR receptor status
Hogervorst et al., 2018	FFQ	Update from <a href="#">Hogervorst et al., 2007</a> : Cohort: 844 ER + breast cancer cases; 62573 cohort members	10–37 µg/day (median Q1–Q5)	20.3 yrs of follow-up: Q2: 0.88 (0.69–1.11) Q3: 1.01 (0.79–1.29) Q4: 0.93 (0.73–1.20) Q5: 0.85 (0.66–1.09) p for trend: 0.37 Per 10 µg/day increment of acrylamide intake: 0.94 (0.88–1.00)	20.3 yrs of follow-up: Never-smokers: Q2: 1.08 (0.78–1.49) <b>Q3: 1.44 (1.04–2.01)</b> Q4: 1.34 (0.96–1.86) Q5: 1.18 (0.85–1.64) p for trend: 0.17 Per 10 µg/day increment of acrylamide intake: 1.02 (0.93–1.11) <b>FDR-adjusted statistically significant interaction between acrylamide intake and rs1056827 in CYP1B1, rs2959008 and rs7173655 in CYP11A1, the GSTT1 deletion, and rs1052133 in hOGG1</b>

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**Table 3** Associations between dietary acrylamide intake and cancer risk—cont'd

References	Method acrylamide intake assessment	Study design and size	Range of intake	Overall results: OR/RR (95% CI)	Subgroup analyses
<b>Endometrial cancer</b>					
Hogervorst et al., 2007	FFQ	Cohort: 221 cases; 62573 cohort members	10–37 µg/day (median Q1–Q5)	Q2: 0.95 (0.59–1.54) Q3: 0.94 (0.56–1.56) Q4: 1.21 (0.74–1.98) Q5: 1.29 (0.81–2.07) p for trend: 0.18	<b>Never-smokers:</b> Q2: 1.16 (0.63–2.15) Q3: 1.35 (0.73–2.51) Q4: 1.30 (0.69–2.46) <b>Q5: 1.99 (1.12–3.52)</b> <b>p for trend: 0.03</b>
Larsson et al. 2009e	FFQ	Cohort: 687 cases; 61226 cohort members	17–33 µg/day (median Q1–4)	Q2: 1.10 (0.89–1.36) Q3: 1.08 (0.88–1.34) Q4: 0.96 (0.59–1.78) p for trend: 0.72	Never-smokers: Q2: 1.31 (0.85–2.04) Q3: 1.30 (0.83–2.02) Q4: 1.20 (0.76–1.90) p for trend: 0.52
Wilson et al., 2010	FFQ	Cohort: 484 cases; 88672 cohort members	9–26 µg/day (mean Q1–Q5)	Q2: 1.12 (0.83–1.50) Q3: 1.31 (0.97–1.77) Q4: 1.35 (0.99–1.84) <b>Q5: 1.41 (1.01–1.97)</b> <b>p for trend: 0.03</b>	<b>Never-smokers:</b> Q2: 0.97 (0.64–1.46) Q3: 1.35 (0.90–2.02) Q4: 1.47 (0.97–2.24) Q5: 1.43 (0.90–2.28) <b>p for trend: 0.04</b> Comparable results in strata of menopausal status <b>Statistically significant association in women with normal BMI (&lt; 25 kg/m2)</b>
Obon-Santacana et al., 2014	FFQ	Cohort: 1382 cases; >500000 cohort members	10–41 µg/day (p10–p90)	Q2: 1.05 (0.86–1.29) Q3: 1.11 (0.90–1.36) Q4: 0.88 (0.71–1.10) Q5: 0.98 (0.78–1.25)	Comparable results across strata of smoking. <b>An excess risk of type-I endometrial cancer in never-smoking women not using OAC: RR Q5 vs Q1: 1.97 (1.08–3.62)</b>
Pelucchi et al., 2016	FFQ	Case-control study: 454 cases; 908 controls	Not reported	Q2: 1.02 (0.67–1.54) Q3: 1.20 (0.80–1.80) Q4: 1.00 (0.65–1.54) Q5: 1.17 (0.73–1.85) p for trend: 0.59	Never-smokers: Q2: 1.21 (0.75–1.95) Q3: 1.24 (0.76–2.01) Q4: 1.02 (0.60–1.73) Q5: 1.28 (0.73–2.25) p for trend: 0.60 No associations in strata of menopausal status, Stronger association in normal-weight women. <b>Statistically significant positive association between intake of fried/baked potatoes and endometrial cancer risk.</b>



Obon-Santacana et al., 2016a	Hemoglobin adducts of AA and GA	Nested case-control study: 383 non-smoking cases; 385 non-smoking controls	AA adducts: 31–4–52.4 pmol/g Hb (quartile range) GA adducts: 24.6–44.6 pmol/g Hb (quartile range)	AA adducts: Q2: 0.82 (0.49–1.37) Q3: 0.96 (0.57–1.61) Q4: 0.87 (0.51–1.48) Q5: 0.85 (0.49–1.46) p for trend: 0.94 GA adducts: Q2: 1.28 (0.76–2.15) Q3: 1.20 (0.71–2.04) Q4: 1.06 (0.62–1.83) Q5: 0.94 (0.54–1.63) p for trend: 0.74 Similar ORs for sum AA + GA	Similar ORs for type I endometrial cancer No clearly increased risks in strata of weight, alcohol; drinking or oral contraceptives use
Hogervorst et al., 2016	FFQ	Update from Hogervorst et al., 2007: Cohort: 393 endometrial cancer cases; 62573 cohort members	10–37 µg/day (median Q1–Q5)	20.3 yrs of follow-up: Q2: 0.87 (0.60–1.27) Q3: 0.86 (0.58–1.28) Q4: 0.95 (0.64–1.41) Q5: 1.03 (0.71–1.51) p for trend: 0.77	20.3 yrs of follow-up: Never-smokers: Q2: 1.07 (0.67–1.70) Q3: 1.14 (0.70–1.86) Q4: 1.08 (0.66–1.77) Q5: 1.44 (0.90–2.28) p for trend: 0.17 <b>11.3 yrs of follow-up:</b> Positive association in <b>homozygous wildtypes rs915906</b> , not in women with variant alleles. <b>T2: 1.28 (0.74–2.20)</b> <b>T3: 1.90 (1.15–3.12)</b> <b>p for trend: 0.01,</b> <b>nominally significant p interaction: 0.02</b> <b>Never-smokers:</b> <b>T2: 1.40 (0.71–2.75)</b> <b>T3: 2.31 (1.26–4.21)</b> <b>p for trend: 0.006</b> Similar for rs2480258 Stronger positive associations in women with at least 1 copy of GSTM1 and GSTT1
<b>Ovarian cancer</b> Pelucchi et al., 2006	FFQ	Case-control study: 1031 cases; 2411 controls	10–32 µg/day (p20–p80)	Q2: 1.03 (0.79–1.34) Q3: 1.09 (0.83–1.44) Q4: 1.01 (0.76–1.34) Q5: 0.97 (0.73–1.31) p for trend: 0.80	n.a.

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**Table 3** Associations between dietary acrylamide intake and cancer risk—cont'd

References	Method acrylamide intake assessment	Study design and size	Range of intake	Overall results: OR/RR (95% CI)	Subgroup analyses
Hogervorst et al., 2007	FFQ	Cohort: 195 cases; 62573 cohort members	10–37 µg/day (median Q1–Q5)	Q2: 1.22 (0.73–2.01) Q3: 1.12 (0.65–1.92) Q4: 1.28 (0.77–2.13) <b>Q5: 1.78 (1.10–2.88)</b> <b>p for trend: 0.02</b>	<b>Never-smokers:</b> Q2: 1.60 (0.85–3.02) Q3: 1.64 (0.84–3.19) Q4: 1.86 (1.00–3.48) <b>Q5: 2.22 (1.20–4.08)</b> <b>p for trend, 0.01</b>
Larsson et al. 2009d	FFQ	Cohort: 368 cases; 61057 cohort members	17–33 µg/day (median Q1–Q4)	Q2: 0.91 (0.68–1.21) Q3: 0.97 (0.73–1.29) Q4: 0.86 (0.63–1.16) p for trend: 0.39	No association for serous ovarian cancer cases
Wilson et al., 2010	FFQ	Cohort: 416 cases; 88672 cohort members	9–26 µg/day (mean Q1–Q5)	Q2: 0.93 (0.68–1.29) Q3: 1.29 (0.94–1.76) Q4: 1.17 (0.84–1.64) Q5: 1.25 (0.88–1.77) p for trend: 0.12	Never-smokers: Q2: 1.17 (0.72–1.88) Q3: 1.04 (0.63–1.74) Q4: 1.11 (0.63–1.94) Q5: 1.19 (0.66–2.15) p for trend, 0.63 Comparable results in strata of menopausal status. <b>Statistically significant association in women with normal BMI (&lt;25 kg/m2)</b>
Xie et al., 2013	Hemoglobin adducts of AA and GA	Nested case-control study: 263 cases; 526 matched controls	AA + GA adducts 74–226 pmol/g Hb (p10–p90)	T–2: 0.83 (0.56–1.24) T–3: 0.79 (0.50–1.24) p for trend: 0.08	Comparable results in non-smokers and for histological subtypes
Obon-Santacana et al., 2015	FFQ	Cohort: 1191 cases; >500000 cohort members	14.7–30.4 µg/day (p25–p75)	Q2: 0.89 (0.72–1.11) Q3: 0.87 (0.70–1.09) Q4: 1.08 (0.87–1.34) Q5: 0.97 (0.76–1.23)	Comparable results for various subtypes and across strata of smoking
Obon-Santacana et al., 2016b	Hemoglobin adducts of AA and GA	Nested case-control study: 334 cases; 417 controls	AA adducts: 33.8–54.8 pmol/g Hb (quartile range) GA adducts: 26.0–49.9 pmol/g Hb (quartile range)	AA adducts: Q2: 1.25 (0.75–2.10) Q3: 1.01 (0.58–1.76) Q4: 1.20 (0.69–2.06) Q5: 1.19 (0.67–2.11) p for trend: 0.86 <b>GA adducts:</b> Q2: 1.23 (0.72–2.11) <b>Q3: 2.14 (1.27–3.60)</b> Q4: 1.32 (0.75–2.33) Q5: 1.63 (0.92–2.86) <b>p for trend: 0.04</b>	ORs of AA + GA comparable to GA. No interaction with weight, alcohol drinking, oral contraceptives use but risks stronger in normal weight women and oral contraceptives users Comparable ORs for serous ovarian cancer

Hogervorst et al., 2017	FFQ	Update from Hogervorst et al., 2007: Cohort: 252 ovarian cancer cases; 62573 cohort members	10–37 µg/day (median Q1–Q5)	20.3 yrs of follow-up: Q2: 1.07 (0.73–1.54) Q3: 1.10 (0.75–1.61) Q4: 1.05 (0.71–1.53) Q5: 1.38 (0.95–1.99) p for trend: 0.13	<b>20.3 yrs of follow-up:</b> <b>Never-smokers:</b> Q2: 1.37 (0.85–2.21) Q3: 1.61 (0.98–2.65) Q4: 1.50 (0.92–2.44) <b>Q5: 1.85 (1.15–2.95)</b> <b>p for trend: 0.01</b> <b>Nominally statistically significant interaction for HSD3B1/2 SNPs rs4659175, rs10923823, rs7546652, rs1047303, and rs6428830. Only clear positive association women with 1 or 2 variant alleles.</b> Positive association in <b>homozygous wildtypes rs2480258 CYP2E1</b> , not in women with variant alleles: T2: 1.03 (0.66–1.62) T3: 1.40 (0.93–2.13) p for trend: 0.10 <b>Never-smokers:</b> T2: 1.52 (0.87–2.64) <b>T3: 1.75 (1.04–2.97)</b> <b>p for trend: 0.04</b> Similar for rs915906. Stronger positive associations in women with at least 1 copy of GSTT1
<b>Oesophageal cancer</b> Pelucchi et al., 2006	FFQ	Case-control study: 395 cases; 1066 controls	13–40 µg/day (p20–p80)	Q2: 1.16 (0.75–1.81) Q3: 1.20 (0.75–1.93) Q4: 0.74 (0.44–1.24) Q5: 1.10 (0.65–1.86) p for trend: 0.67	n.a.
Hogervorst et al., 2008b	FFQ	Cohort: 216 cases; 120852 cohort members	Men: 10–42 µg/day Women: 9–40 µg/day (median Q1–Q5)	Q2: 0.73 (0.47–1.15) Q3: 0.86 (0.56–1.33) Q4: 0.83 (0.54–1.28) Q5: 0.83 (0.54–1.30) p for trend: 0.68	Comparable results in men and women, never and former smokers, and in oesophageal adenocarcinomas or squamous cell carcinomas <b>Increased risks in overweight and obese persons</b>
Lin et al. 2010	FFQ	Case-control study: 618 cases; 820 controls	27–44 µg/day (p25–p75)	Q2: 1.35 (0.96–1.99) Q3: 1.12 (0.91–1.58) <b>Q4: 1.23 (1.02–1.75)</b> p for trend: 0.46	Comparable results for adenocarcinoma, squamous cell carcinoma and gastroesophageal junction <b>Stronger associations in overweight persons, and non-smokers</b>
Lujan-Barroso et al., 2014	FFQ	Cohort: 341 cases; >500000 Cohort members	14–37 µg/day (p20–p80)	<b>Q2: 1.75 (1.12–2.74)</b> <b>Q3: 1.66 (1.05–2.61)</b> Q4: 1.41 (0.86–2.71)	Comparable results for adenocarcinoma and squamous-cell carcinoma, and never-smokers. Attenuated HRs using energy-adjusted AA intake

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**Table 3** Associations between dietary acrylamide intake and cancer risk—cont'd

References	Method acrylamide intake assessment	Study design and size	Range of intake	Overall results: OR/RR (95% CI)	Subgroup analyses
<b>Stomach cancer</b>					
Hogervorst et al., 2008b	FFQ	Cohort: 563 cases; 120852 cohort members	Men: 10–42 µg/day Women: 9–40 µg/day (median Q1–Q5)	Q2: 1.09 (0.81–1.47) Q3: 1.09 (0.81–1.48) Q4: 1.18 (0.87–1.60) Q5: 1.06 (0.78–1.45) p for trend: 0.77	No increased risks in men or women, never or former smokers, and in gastric cardia or other stomach cancers
Hirvonen et al., 2010	FFQ	Cohort of male smokers: 224 cases; 27111 cohort members	22–56 µg/day (median Q1–Q5)	Q2: 1.42 (0.94–2.13) Q3: 0.78 (0.49–1.26) Q4: 1.34 (0.88–2.05) Q5: 0.96 (0.60–1.53) p for trend: 0.78	n.a.
<b>Colorectal cancer</b>					
Mucci et al., 2003	FFQ	Case-control study: 591 cases; 538 controls	28 (0.6) µg/day mean (SE)	Q2: 0.9 (0.6–1.3) Q3: 0.6 (0.4–0.9) Q4: 0.6 (0.4–1.0) p for trend: 0.01	Comparable results for nonsmokers and current smokers
Mucci et al. (2006) Mucci et al. 2006	FFQ	Cohort: 741 female cases; 61467 female cohort members	13–38 µg/day (mean Q1–Q5)	Q2: 1.1 (0.9–1.4) Q3: 1.2 (0.9–1.5) Q4: 1.1 (0.8–1.4) Q5: 0.9 (0.7–1.3) p for trend: 0.85	Comparable results for colon and rectal cancer
Pelucchi et al., 2006	FFQ	Case-control study: 2280 cases; 4765 controls	12–40 µg/day (p20–p80)	Q2: 0.89 (0.75–1.05) Q3: 1.06 (0.89–1.26) Q4: 1.05 (0.88–1.26) Q5: 0.97 (0.80–1.18) p for trend: 0.56	Comparable results for colon and rectal cancer
Hogervorst et al., 2008b	FFQ	Cohort: 2190 cases; 120852 cohort members	Men: 10–42 µg/day Women: 9–40 µg/day (median Q1–Q5)	Q2: 0.96 (0.81–1.15) Q3: 1.06 (0.89–1.27) Q4: 0.96 (0.80–1.14) Q5: 1.00 (0.84–1.20) p for trend: 0.94	No increased risks in men or women, never-smokers, and in colon or rectal cancer
Larsson et al. 2009a	FFQ	Cohort: 676 male cases; 45306 male cohort members	25–49 µg/day (median Q1–Q4)	Q2: 1.02 (0.83–1.25) Q3: 1.03 (0.83–1.28) Q4: 0.95 (0.74–1.20) p for trend: 0.69	Comparable results for different subsites of colorectal cancer, and in never-, past and current smokers
Hirvonen et al., 2010	FFQ	Cohort of male smokers: 316 cases; 27111 cohort members	22–56 µg/day (median Q1–Q5)	Q2: 0.93 (0.66–1.32) Q3: 0.89 (0.62–1.26) Q4: 0.95 (0.67–1.36) Q5: 0.93 (0.65–1.34) p for trend: 0.75	n.a.

Hogervorst et al., 2014	FFQ	Cohort: 733 cases; 120852 cohort members	Men: 10–42 µg/day Women: 9–40 µg/day (median Q1–Q5)	Subset of cases from Hogervorst et al. (2008a,b)	<b>AA intake is statistically significantly positively associated with CRC with an activating mutation in the KRAS gene among men, and has a statistically significant inverse association with the risk of tumors with a truncating mutation in the APC gene among women</b>
<b>Pancreatic cancer</b> Hogervorst et al., 2008a	FFQ	Cohort: 349 cases; 120852 cohort members	Men: 10–42 µg/day Women: 9–40 µg/day (median Q1–Q5)	Q2: 1.02 (0.72–1.44) Q3: 0.96 (0.66–1.38) Q4: 0.87 (0.60–1.27) Q5: 0.98 (0.68–1.40) p for trend: 0.75	No increased risks in men or women, never- and former smokers, and in microscopically confirmed cancers
Hirvonen et al., 2010	FFQ	Cohort of male smokers: 192 cases; 27111 cohort members	22–56 µg/day (median Q1–Q5)	Q2: 0.98 (0.61–1.56) Q3: 1.08 (0.69–1.71) Q4: 1.06 (0.66–1.69) Q5: 1.00 (0.62–1.62) p for trend: 0.89	n.a.
Pelucchi et al., 2011	FFQ	Case-control study 326 cases; 652 controls	32 (20) µg/day Mean (SD)	Q2: 1.48 (0.88–2.50) Q3: 1.57 (0.91–2.69) Q4: 1.70 (0.98–2.96) Q5: 1.49 (0.83–2.70) p for trend: 0.21	n.a.
Obon-Santacana et al., 2013	FFQ	Cohort: 865 cases; >500000 cohort members	14–37 µg/day (p20–p80)	Q2: 0.90 (0.71–1.15) Q3: 0.78 (0.60–1.01) Q4: 0.68 (0.52–0.90) Q5: 0.77 (0.58–1.04)	Comparable results across strata of smoking Lower risks in women and in obese persons (BMI ≥30 kg/m <sup>2</sup> )
Pelucchi et al., 2017	FFQ	Case-control study: 1975 cases; 4239 controls	8.3–39.6 µg/day (p25–p75)	Q2: 0.97 (0.79–1.19) Q3: 0.91 (0.71–1.16) Q4: 0.92 (0.66–1.28) Per 10 µg/day increment of acrylamide intake: 0.96 (0.87–1.06)	No associations in strata of sex, age, smoking, BMI, diabetes, study area
<b>Prostate cancer</b> Pelucchi et al., 2006	FFQ	Case-control study: 1294 cases; 1451 controls	12–36 µg/day (p20–p80)	Q2: 1.00 (0.77–1.30) Q3: 1.22 (0.94–1.58) Q4: 1.01 (0.77–1.33) Q5: 0.92 (0.69–1.23) p for trend, 0.65	n.a.
Hogervorst et al., 2008a	FFQ	Cohort: 2246 cases; 58279 male cohort members	10–42 µg/day (median Q1–Q5)	Q2: 1.07 (0.88–1.31) Q3: 1.01 (0.82–1.24) Q4: 1.02 (0.83–1.26) Q5: 1.06 (0.87–1.30) p for trend: 0.69	Comparable results in never and former smokers, possibly an inverse association with advanced cancer in never-smokers

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**Table 3** Associations between dietary acrylamide intake and cancer risk—cont'd

<i>References</i>	<i>Method acrylamide intake assessment</i>	<i>Study design and size</i>	<i>Range of intake</i>	<i>Overall results: OR/RR (95% CI)</i>	<i>Subgroup analyses</i>
Larsson et al., 2009b	FFQ	Cohort: 2696 cases; 45306 cohort members	28–43 µg/day (p20–p80)	Q2: 0.86 (0.71–1.04) Q3: 1.02 (0.84–1.23) Q4: 0.90 (0.73–1.10) Q5: 0.88 (0.70–1.09) p for trend: 0.34	Comparable results in never-smokers, in localized and in advanced cancer
Wilson et al., 2009a	Hemoglobin adducts of AA + FFQ	Case-control study: AA adducts: 170 cases; 161 controls FFQ data: 1499 cases; 1118 controls	AA adducts: 32–56 pmol/g Hb (median Q1–Q4) FFQ: 33–56 µg/day (p20–p80)	AA adducts: Q2: 0.74 (0.37–1.49) Q3: 0.98 (0.50–1.93) Q4: 0.93 (0.47–1.85) FFQ: Q2: 1.14 (0.89–1.47) Q3: 0.99 (0.76–1.28) Q4: 1.06 (0.82–1.37) Q5: 0.97 (0.75–1.27)	Comparable results in advanced and localized cancers and in high- and low-grade cancers
Hirvonen et al., 2010	FFQ	Cohort of male smokers: 799 cases; 27111 cohort members	22–56 µg/day (median Q1–Q5)	Q2: 0.95 (0.76–1.19) Q3: 1.03 (0.83–1.29) Q4: 1.06 (0.84–1.33) Q5: 1.05 (0.83–1.32) p for trend: 0.43	n.a.
Wilson et al., 2012	FFQ	Cohort: 5025 cases; 47896 cohort members	12–35 µg/day (median Q1–Q5)	Q2: 1.10 (1.01–1.20) Q3: 1.08 (0.99–1.18) Q4: 1.06 (0.97–1.16) Q5: 1.02 (0.92–1.13) p for trend: 0.90	Comparable results in advanced and localized cancers and in high- and low-grade cancers
<b>Bladder cancer</b>					
Mucci et al., 2003	FFQ	Case-control study: 263 cases; 538 controls	28 (0.6) µg/day Mean (SE)	Q2: 1.1 (0.7–1.8) Q3: 0.7 (0.4–1.3) Q4: 0.8 (0.5–1.5) p for trend: 0.26	Comparable results for nonsmokers and no association in smokers
Hogervorst et al., 2008a	FFQ	Cohort: 1210 cases; 120852 cohort members	Men: 10–42 and Women: 9–40 µg/day (median Q1–Q5)	Q2: 0.96 (0.77–1.20) Q3: 0.89 (0.71–1.12) Q4: 1.01 (0.81–1.26) Q5: 0.91 (0.73–1.15) p for trend: 0.60	Indications for increased risk in heavy smokers Indications for decreased risks in women
Hirvonen et al., 2010	Hemoglobin adducts of AA and GA	Cohort of male smokers: 365 cases; 27111 cohort members	22–56 µg/day (median Q1–Q5)	Q2: 0.91 (0.65–1.27) Q3: 1.06 (0.77–1.47) Q4: 0.78 (0.55–1.11) Q5: 0.99 (0.71–1.39) p for trend: 0.71	n.a.
<b>Renal cell cancer</b>					



Mucci et al., 2003	FFQ	Case-control study: 133 cases; 538 controls	28 (0.6) µg/day Mean (SE)	Q2: 1.0 (0.6–1.9) Q3: 1.1 (0.6–2.0) Q4: 0.8 (0.4–1.7) p for trend: 0.64	Slightly increased risks for smokers, although not statistically significant
Mucci et al., 2004	FFQ	Case-control study: 379 cases; 353 controls	20–32 µg/day (p25–p75)	Q2: 1.1 (0.7–1.8) Q3: 1.0 (0.7–1.6) Q4: 1.1 (0.7–1.8) p for trend: 0.8	No associations in strata of smoking status
Pelucchi et al., 2007	FFQ	Case-control study: 767 cases; 1534 controls	20–44 µg/day (p25–p75)	Q2: 1.21 (0.94–1.57) Q3: 1.14 (0.86–1.51) Q4: 1.20 (0.88–1.63) p for trend: 0.35	n.a.
Hogervorst et al., 2008a	FFQ	Cohort: 339 cases; 120852 cohort members	Men: 10–42 and Women: 9–40 µg/day (median Q1–Q5)	Q2: 1.25 (0.86–1.83) Q3: 1.48 (1.02–2.15) Q4: 1.23 (0.83–1.81) <b>Q5: 1.59 (1.09–2.30)</b> <b>p for trend: 0.04</b>	Similar results for men and women <b>Stronger association in long-term smokers</b>
Hirvonen et al., 2010	FFQ	Cohort of male smokers: 184 cases; 27111 cohort members	22–56 µg/day (median Q1–Q5)	Q2: 0.94 (0.55–1.62) <b>Q3: 1.65 (1.02–2.67)</b> Q4: 1.47 (0.89–2.41) Q5: 1.28 (0.76–2.15) p for trend: 0.12	n.a.
<b>Oral cavity and pharyngeal cancer</b>					
Pelucchi et al., 2006	FFQ	Case-control study: 749 cases; 1772 controls	13–40 µg/day (p20–p80)	Q2: 1.10 (0.78–1.57) Q3: 1.27 (0.89–1.81) Q4: 1.04 (0.72–1.51) Q5: 1.12 (0.76–1.66) p for trend: 0.70	n.a.
Schouten et al., 2009	FFQ	Cohort: 101 oral cavity cases; 120852 cohort members	Men: 10–42 and Women: 9–40 µg/day (median Q1–Q5)	Q2: 0.70 (0.37–1.33) Q3: 0.77 (0.39–1.52) Q4: 0.77 (0.39–1.53) Q5: 0.72 (0.36–1.42) p for trend: 0.49	<b>Statistically significant positive association in non-smoking women (21 cases): HR 1.28 (1.01–1.62) per 10 µg/day increment of acrylamide intake</b>
Schouten et al., 2009	FFQ	Cohort: 83 oro- and hypopharynx cases; 120852 cohort members	Men: 10–42 and Women: 9–40 µg/day (median Q1–Q5)	T–2: 0.44 (0.23–0.85) T–3: 0.61 (0.33–1.12) p for trend: 0.17	No significant differences between men and women
<b>Laryngeal cancer</b>					

(Continued)

**Table 3** Associations between dietary acrylamide intake and cancer risk—cont'd

References	Method acrylamide intake assessment	Study design and size	Range of intake	Overall results: OR/RR (95% CI)	Subgroup analyses
Pelucchi et al., 2006	FFQ	Case-control study: 527 cases; 1297 controls	13–38 µg/day (p20–p80)	Q2: 1.04 (0.70–1.57) Q3: 0.85 (0.56–1.29) Q4: 0.89 (0.59–1.36) Q5: 1.23 (0.80–1.90) p for trend: 0.54	n.a.
Schouten et al., 2009	FFQ	Cohort: 180 cases; 120852 cohort members	Men: 10–42 and Women: 9–40 µg/day (median Q1–Q5)	Q2: 0.66 (0.38–1.16) Q3: 1.06 (0.62–1.80) Q4: 1.02 (0.60–1.74) Q5: 0.93 (0.54–1.58) p for trend: 0.85	No significant differences between men and women
<b>Lung cancer</b> Hogervorst et al., 2009b	FFQ	Cohort: 1600 male lung cancer cases; 58279 male cohort members	10–42 and (median Q1–Q5)	Q2: 1.05 (0.81–1.38) Q3: 0.94 (0.71–1.26) Q4: 1.00 (0.75–1.34) Q5: 1.03 (0.77–1.39) p for trend: 0.85	No significant differences between histological subtypes
	FFQ	Cohort: 295 female lung cancer cases; 62573 female cohort members	9–40 µg/day (median Q1–Q5)	Q2: 0.66 (0.42–1.04) Q3: 0.60 (0.38–0.96) Q4: 0.58 (0.36–0.95) Q5: 0.45 (0.27–0.76) p for trend: 0.01	Strongest inverse association observed for adenocarcinomas Similar associations in never-smoking women
Hirvonen et al., 2010	FFQ	Cohort of male smokers: 1703 cases; 27111 cohort members	22–56 µg/day (median Q1–Q5)	Q2: 1.01 (0.86–1.18) Q3: 1.11 (0.95–1.29) Q4: 0.93 (0.79–1.10) <b>Q5: 1.18 (1.01–1.38)</b> p for trend: 0.11	n.a.
<b>Brain cancer</b> Hogervorst et al., 2009b	FFQ	Cohort: 216 cases; 120852 cohort members	Men: 10–42 and Women: 9–40 µg/day (median Q1–Q5)	Q2: 0.92 (0.59–1.44) Q3: 1.20 (0.78–1.83) Q4: 1.07 (0.68–1.68) Q5: 0.87 (0.54–1.41) p for trend: 0.61	No association in men or women, never-smokers and subgroups of microscopically verified cancers, and astrocytic gliomas
<b>Thyroid cancer</b> Schouten et al., 2009	FFQ	Cohort: 66 cases; 120852 cohort members	Men: 10–42 and Women: 9–40 µg/day (median Q1–Q5)	T–2: 1.14 (0.58–2.26) T–3: 1.33 (0.70–2.53) p for trend: 0.42	No significant associations in women or in non-smokers
<b>Lymphatic malignancies</b> Hirvonen et al., 2010	FFQ	Cohort of male smokers: 175 lymphoma cases; 27111 cohort members	22–56 µg/day (median Q1–Q5)	Q2: 0.93 (0.56–1.53) Q3: 1.17 (0.73–1.88) Q4: 0.98 (0.59–1.61) Q5: 1.10 (0.67–1.80) p for trend: 0.67	n.a.

Bongers et al., 2012	FFQ	Cohort: 170 male cases of multiple myeloma; 58279 male cohort members	10–42 µg/day (median Q1–Q5)	Q2: 0.65 (0.36–1.16) Q3: 1.14 (0.67–1.94) Q4: 1.14 (0.67–1.94) Q5: 1.54 (0.92–2.58) <b>p for trend: 0.02</b>	<b>Statistically significant positive association in never-smoking men: 1.98 (1.38–2.85) per 10 µg/day increment of acrylamide intake</b>
	FFQ	Cohort: 153 female cases of multiple myeloma; 62573 female cohort members	9–40 µg/day (median Q1–Q5)	Q2: 1.46 (0.85–2.49) Q3: 1.19 (0.67–2.12) Q4: 0.73 (0.39–1.37) Q5: 0.93 (0.50–1.73) p for trend: 0.22	No clear association in never-smoking women
	FFQ	Cohort: 159 male cases of diffuse large-cell lymphoma; 58279 male cohort members	10–42 µg/day (median Q1–Q5)	Q2: 0.93 (0.54–1.59) Q3: 1.23 (0.74–2.04) Q4: 1.26 (0.74–2.17) Q5: 1.06 (0.61–1.38) p for trend: 0.73	n.a.
	FFQ	Cohort: 100 female cases of diffuse large-cell lymphoma; 62573 female cohort members	9–40 µg/day (median Q1–Q5)	Q2: 1.05 (0.51–2.15) Q3: 1.71 (0.87–3.36) Q4: 1.72 (0.84–3.50) Q5: 1.38 (0.63–3.02) p for trend: 0.43	No association in never-smoking women
	FFQ	Cohort: 134 male cases of chronic lymphocytic leukaemia; 58279 male cohort members	10–42 µg/day (median Q1–Q5)	0.88 (0.74–1.09) per 10 µg/day	No association in never-smoking men
	FFQ	Cohort: 66 female cases of chronic lymphocytic leukaemia; 62573 female cohort members	9–40 µg/day (median Q1–Q5)	0.83 (0.64–1.09) per 10 µg/day	No association in never-smoking women
	FFQ	Cohort: 42 male follicular lymphoma and 47 female follicular lymphoma cases; 58279 male and 62573 female cohort members	Men: 10–42 µg/day Women: 9–40 µg/day (median Q1–Q5)	<b>Men: 1.28 (1.03–1.61)</b> Women: 1.12 (0.80–1.57) per 10 µg/day	No association in never-smoking women

(Continued)

**Table 3** Associations between dietary acrylamide intake and cancer risk—cont'd

References	Method acrylamide intake assessment	Study design and size	Range of intake	Overall results: OR/RR (95% CI)	Subgroup analyses
<b>Cutaneous malignant melanoma (CMM)</b> <a href="#">Lipunova et al., 2017</a>	FFQ	Cohort: 54 male Waldenström macroglobulinemia and immunocytoma, and 35 female Waldenström macroglobulinemia and immunocytoma, cases, 58279 male and 62573 female cohort members	Men: 10–42 µg/day Women: 9–40 µg/day (median Q1–Q5)	Men: 1.21 (0.93–1.50) Women: 1.21 (0.88–1.66) per 10 µg/day	No association in never-smoking women
	FFQ	Cohort: 224 male CMM cases; 58279 male subcohort members	10–42 µg/day (median Q1–Q5)	Q2: 1.14 (0.73–1.80) Q3: 0.90 (0.55–1.45) Q4: 1.03 (0.64–1.66) Q5: 1.52 (0.98–2.33) p for trend: 0.12 <b>Per 10 µg/day increment in acrylamide intake: 1.13 (1.01–1.26)</b>	Less strong (not statistically significant) association in never-smoking men for overall melanoma <b>Stronger, statistically significant, association for nodular melanoma: Per 10 µg/day increment in acrylamide intake: 1.36 (1.11–1.67) Non-smokers, nodular melanoma: 1.60 (1.19–2.15)</b>
<b>Cancer mortality</b> <a href="#">Liu et al., 2017b</a>	FFQ	Cohort: 224 female CMM cases; 62573 female subcohort members	9–40 µg/day (median Q1–Q5)	Q2: 1.10 (0.71–1.72) Q3: 1.25 (0.80–1.96) Q4: 1.11 (0.71–1.73) Q5: 0.91 (0.57–1.44) p for trend: 0.71 Per 10 µg/day increment in acrylamide intake: 0.97 (0.86–1.08)	Higher relative risks but not statistically significant and with no clear linear dose-response in never-smoking women
	FFQ	330 total cancer deaths; 39271 person-years	8.2–21.4 µg/day (median Q1–Q5)	Q2: 1.2 (0.8–1.6) Q3: 1.4 (1.0–2.0) <b>Q4: 1.9 (1.3–2.8)</b> <b>p for trend: 0.001</b>	Similar results in strata of smoking, sex, obesity and overall lifestyle pattern scores
	FFQ	106 respiratory system cancer deaths; 39271 person-years	8.2–21.4 µg/day (median Q1–Q5)	Q2: 1.2 (0.7–2.2) Q3: 1.3 (0.7–2.5) Q4: 2.0 (1.0–4.0) p for trend: 0.06	n.a.
	FFQ	131 digestive tract cancer deaths; 39271 person-years	8.2–21.4 µg/day (median Q1–Q5)	Q2: 1.3 (0.8–2.2) Q3: 1.4 (0.8–2.5) Q4: 1.9 (1.0, 3.6) p for trend: 0.05	n.a.

AA: Acrylamide; BMI: Body Mass Index; ER: Estrogen receptor; GA: Glycidamide; Hb: Hemoglobin; n.a.: not applicable; OAC: Oral contraceptives; OR: Odds Ratio; PMH: post-menopausal hormones; pmol: picomole; PR: Progesterone receptor; Q1–Q4: quartile 1–4; Q1–Q5: Quintile 1–5; RR: relative risk; SES: Social Economic Status. T: tertile.

Adapted from EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2015. Scientific Opinion on acrylamide in food. EFSA Journal 2015;13(6):4104, 321 pp. doi:10.2903/j.efsa.2015.4104.

Based on the experimental animal data, it was calculated that the relative risk for cancer in humans would only range between approximately 1.015 and 1.05 when comparing the part of the population with extremely high acrylamide intake to the part of the population with a mean intake (Hagmar and Tornqvist, 2003). Detecting risks that low would require hundreds of thousands of cases in an epidemiological study, which is practically unfeasible. However, the fact that statistically significant risks and higher risks than calculated from animal studies were observed for some cancers in epidemiological studies may indicate that humans are more susceptible to the carcinogenic effect of acrylamide than rodents (Hogervorst et al., 2010).

Two epidemiological studies on ovarian cancer (Obon-Santacana et al., 2016b; Xie et al., 2013) and 1 for endometrial cancer (Obon-Santacana et al., 2014) have investigated the association with acrylamide and glycidamide to hemoglobin adducts (medium to chronic term biomarkers (approximately 4 months) of internal exposure to acrylamide), but observed no associations. Some would argue that this renders strong evidence against a causal association between dietary acrylamide intake and these cancers but it is important to keep in mind that this biomarker also has drawbacks when it comes to representing long-term dietary acrylamide intake, which has to be assessed accurately in order to be able to link it to diseases that take a long time to develop, such as cancer (Hogervorst et al., 2010). For example, the biomarkers acrylamide and glycidamide to hemoglobin adducts still only represent a relatively short exposure period and may be susceptible to seasonal influences. In addition, it is not specific for dietary acrylamide intake and also represents acrylamide exposure through active or passive smoking. Furthermore, this biomarker is expressed per gram of hemoglobin in the blood sample and there are many factors that influence hemoglobin levels. Finally, the biomarker does not allow discrimination between long-term low, with incidentally high intake levels of acrylamide, and long-term moderate intake levels, while the latter is expected to be more relevant for carcinogenesis.

From the epidemiological work described above, it can be concluded that there may be a positive association between acrylamide intake and renal, endometrial and ovarian cancers.

In light of the still thin evidence there is that acrylamide at current dietary levels causes renal, endometrial and ovarian cancer, we need a way to better understand the causality of this link. Although experimental studies on acrylamide and cancer in humans are off-limits, it should be possible, in the era of molecular epidemiology using omic techniques, to investigate the biological plausibility for acrylamide at current dietary levels to cause cancer.

One example of such research is to study whether genetic variability in genes involved in acrylamide metabolism modifies the association between acrylamide and cancer. In recent analyses from a Dutch prospective cohort study, there were indications that genetic variants in cytochrome P4502E1 (CYP2E1: the enzyme responsible for the metabolism of acrylamide to glycidamide) modified the association between acrylamide intake and endometrial and ovarian cancer risk, which supports the idea that these associations are causal (Hogervorst et al., 2016, 2017).

In future studies, it seems justified to analyze associations separately for men and women because some tumors were only associated to acrylamide intake in one of the sexes, which may be due to the potential effect of acrylamide on sex hormone systems.

To conclude, due to the potential carcinogenic risks associated with dietary acrylamide intake, be it the risks estimated from animal studies or the risks shown from the epidemiological studies, there is every reason to try and reduce the intake of acrylamide through food.

## Neurotoxicity

Experimental animals exposed to acrylamide exhibited both peripheral and central nervous system toxicity, such as hind-limb paralysis, reduction in rotorod performance and histopathological changes in peripheral nerves and central nervous system structures (EFSA, 2015). As the most sensitive neurotoxic effect, the Contaminants in the Food Chain (CONTAM) panel of EFSA that performed the 2015 acrylamide risk assessment selected peripheral nerve (sciatic) axonal degeneration in male F344 rats exposed to AA in drinking water for two years. For this endpoint, they calculated the so-called bench mark dose lower limit 10 (BMDL10) which is the lower limit of the confidence interval around the modeled estimated dose that increased the incidence of the health outcome (peripheral nerve (sciatic) axonal degeneration in this case) by 10% over the incidence in controls. For this endpoint, they calculated the so-called they arrived at a reference point value of 0.43 mg/kg bodyweight per day. This reference value was divided by the estimated dietary exposure of humans to calculate the MOE for neurotoxicity. Taking into account differences between rodents and humans and differences between humans, it is determined that the MOE for non-neoplastic effects has to be 100 or more. Because the MOE for neurotoxicity ranged from 126–1075 in different age groups, EFSA arrived at the conclusion that no neurotoxicity is expected to follow from dietary acrylamide exposure (EFSA, 2015).

However, some experts have concerns that low levels of exposure may be cumulative in nature and just take a longer time to sort effects (Erkekoglu and Baydar, 2014). Since rats only live two years and humans around 80 years, it is not inconceivable that low level acrylamide exposure has effects on the nervous system later on in life.

So far, there has only been one epidemiological study on the association between dietary acrylamide intake and neurotoxicity. In a prospective cohort study of elderly Chinese, increased acrylamide intake was associated with mild cognitive decline and with an increased risk of poor cognitive function after 4 years of follow-up in men but not in women (Liu et al., 2017a). However, the main dietary sources of acrylamide exposure in this study (stir-fried vegetables) were very different from those in Western countries.

In addition, because of the exquisite sensitivity of fetal and neonatal brain development to toxicological insults, research on the possible association between gestational and neonatal acrylamide exposure and neurotoxicity in the developing child seems warranted.

## Reproductive Toxicity

In rodents, acrylamide has effects on various endocrine and reproductive functions, especially on male reproduction. Acrylamide causes among other perturbed sperm functionality and sperm reservoirs in the epididymis, genotoxicity in several types of testicular cells, Leydig cell toxicity, and changes in sex hormones such as testosterone and prolactin (EFSA, 2015). These effects may be caused by acrylamide-induced damage of proteins and/or by damage of nucleic acids and proteins by acrylamide's metabolite glycidamide. The most sensitive reproductive effects selected by EFSA were reduced sperm counts and effects on sperm and testis morphology with a no observed adverse effect level (NOAEL), the dose at and below which no adverse effects are seen anymore, of approximately 2 mg/kg bodyweight per day. This dose is higher than the reference dose determined for neurotoxicity and therefore EFSA concluded that no reproductive effects of dietary acrylamide intake are to be expected either (EFSA, 2015).

There are no epidemiological studies on the reproductive toxicity of acrylamide apart from 3 studies that investigated the association between acrylamide exposure and blood or urinary sex hormone levels in different study populations.

In a cross-sectional study from the United States that used a food frequency questionnaire (FFQ) to assess acrylamide intake, there were some statistically significant associations between acrylamide intake and plasma sex hormone levels in both pre and postmenopausal women (Hogervorst et al., 2013). Plasma luteal estradiol and free estradiol levels were statistically significantly increased in normal weight premenopausal women with higher acrylamide intake. In postmenopausal women, statistically significant inverse associations were observed between AA intake and plasma estrone, free estradiol and prolactin levels in normal weight women. In overweight postmenopausal women, statistically significant positive associations were observed between AA intake and plasma testosterone and androstenedione levels. In a cross-sectional Japanese study in premenopausal women, also using an FFQ to estimate acrylamide intake, a higher acrylamide intake was associated with lower plasma total and free estrogen levels and a higher follicle-stimulating hormone (FSH) level. The association with total and free estrogen levels was only present in women with a body mass index (BMI) higher than 20.5 kg/m<sup>2</sup> (Nagata et al., 2015).

In another Japanese cross-sectional study by the same authors but this time in preschool children (aged 3–6 years), acrylamide intake was significantly positively associated with testosterone and androstenediol levels in urine in boys (Nagata et al., 2018). There were no statistically significant associations with sex hormones in girls. The authors concluded that acrylamide exposure of preschool-age boys may alter their androgen metabolism.

In all 3 studies, there were some statistically significant associations between acrylamide intake and sex hormones. However, the results come from very diverse populations, using different bodily fluids, and with widely varying dietary acrylamide sources, which makes it hard to draw conclusions at this time. In addition, the results on adult women from the American and Japanese study seem to partly contradict each other. More epidemiological studies in this area, also studies using acrylamide biomarkers, are needed. It would also be of interest to have epidemiological studies on the association between dietary acrylamide and fertility.

## Developmental Toxicity

In experimental studies, rats and mice exposed prenatally and/or neonatally to acrylamide showed signs of developmental toxicity, such as skeletal variations, impaired body weight gain, histological central nervous system changes, and neurobehavioral effects (EFSA, 2015). Some effects did not occur without maternal toxicity also taking place. The lowest NOAEL reported for developmental toxicity was 1.0 mg/kg bodyweight per day for neurodevelopmental toxicity (reduced learning task performance) from studies in rats exposed gestationally and neonatally. This NOAEL is higher than the reference dose that was calculated for neurotoxicity and therefore EFSA, in their latest acrylamide risk assessment (2015), arrived at the conclusion that no adverse developmental effects of acrylamide are expected to occur at dietary intake levels (EFSA, 2015).

However, 3 recent epidemiological birth cohort studies have consistently shown an association between increased maternal acrylamide exposure during pregnancy and a reduced fetal growth (Duarte-Salles et al., 2013; Kadawathagedara et al., 2016; Pedersen et al., 2012). These epidemiological studies on acrylamide exposure and birth outcomes are summarized in Table 4 and 5. Every one of these studies used an FFQ to estimate acrylamide intake and 1 study additionally measured acrylamide and glycidamide to hemoglobin adduct levels in umbilical cord blood.

All 3 studies observed a statistically significant association with an increased risk of the newborn being small for-gestational age (SGA), both in the whole study population and in the subpopulation of neonates from non-smoking mothers. These associations are in line with experimental animal studies in which increased acrylamide exposure led to a reduced birth weight of the animals. However, the dose levels at which this occurred in animals were so high that they also caused toxicity to the mother (EFSA, 2015). Thus, it is questionable whether the same mechanism of action operates in humans at dietary levels (if it is a causal effect) and animals at massively high dose levels. EFSA did not give the epidemiological findings (of 2 studies published at the time) any weight in their risk assessment, mainly because of lack of a known mechanism for these associations in humans. As mentioned above, based on the animal data, they ruled developmental effects at dietary doses to be unlikely (EFSA, 2015).

Although it is known that reduced fetal growth predisposes for adverse health outcomes later in life, such as cardiovascular disease, diabetes and overweight (Eriksson, 2016), it remains to be studied further whether gestational acrylamide exposure is associated with those health outcomes through effects on fetal growth.

In one of the cohorts (the Norwegian Mother and Child Cohort Study (MoBa)) in which an inverse association with fetal growth was seen, the association between prenatal dietary acrylamide exposure and postnatal growth was investigated (Kadawathagedara et al., 2018). Increased acrylamide intake was statistically significantly associated with a higher risk of overweight/obese status at 3, 5



**Table 4** Characteristics of the epidemiological studies on the association between dietary acrylamide intake and birth outcomes

References	Name of cohort	Country	Method for acrylamide intake assessment	Study size	Range of intake
Pedersen et al. (2012)	NewGeneris cohort	Denmark, England, Greece, Norway, and Spain	Hemoglobin adducts of AA and GA + FFQ	1101	AA: 4.4–147.6 pmol/g Hb GA: 2.0–117.6 pmol/g Hb
Duarte-Salles et al. (2013)	The Norwegian Mother and Child Cohort Study (MoBa)	Norway	FFQ	50 651	Q1: < 8.5 Q2: 8.5–11.1 Q3: 11.1–14.3 Q4: > 14.3 ng/kcal/day
Kadawathagedara et al. (2016)	EDEN Cohort	France	FFQ	1471	1.8–30.3 µg/day (interquartile range)

and 8 years of age. In contrast, an increase in the level of the exposure biomarker acrylamide to hemoglobin adducts was associated with reduced BMI and body fat in a cross-sectional analysis among adults from the National Health and Nutrition Examination Survey (NHANES) (Chu et al., 2017). However, the association was only observed in persons who actively smoked themselves and in persons who were exposed to environmental tobacco smoke, so confounding by tobacco smoke cannot be excluded.

The epidemiological findings summarized above and in Tables 4 and 5 indicate the urgent need to gather further data on the potential important effects of acrylamide on human prenatal and postnatal development. More studies along the line of the presented studies should be performed and it would also be helpful to try and interrogate the causality of the observed inverse association by investigating the biological plausibility (mechanism of action) using biomarkers.

### Other Possible Chronic Health Effects of Dietary Acrylamide Intake

Whereas it might be expected that acrylamide, due to its potential adverse on fetal growth, may lead to increased risks of metabolic disease such as diabetes in later life, an opposite association was seen in the NHANES study (Lin et al., 2009). Adults with a higher level of acrylamide to hemoglobin adducts had lower serum levels of insulin and insulin resistance. However, this association was only statistically significant among smokers, which again raises concern for confounding by smoking. Obviously, more epidemiological research on this topic is needed.

Immunotoxicity has so far hardly been studied as a possible health outcome of acrylamide exposure in animals and is therefore not a part of the risk assessments that have been performed for acrylamide.

In a study on mice (oral dose range: 12.5–50 mg/kg body weight per day), acrylamide caused immunotoxicity as shown by a reduction in immune blood cells, pathological changes in the spleen, and reduced body and organ weights, potentially by inducing apoptosis or oxidative stress (Zamani et al., 2017). Again in mice, orally exposed to acrylamide for 30 days, spleen and thymus weights and lymphocyte counts were decreased, and pathological changes were observed in spleen, lymph glands and thymus upon acrylamide exposure (Fang et al., 2014).

In rats exposed to acrylamide through gavage (dose range: 0–60 mg/kg bodyweight per day) for 5 days or to 0–175 mg/kg by a single oral dose, a dose-dependent decrease was observed in peripheral blood lymphocytes and the gut-associated lymphoid tissue, which is a part of the immune system that protects the body from invasion through the gut (Yener et al., 2013). In another study on rats, acrylamide caused a decrease in spleen, thymus and mesenteric lymph nodes weights. In addition, a decrease in cellularity of the spleen, thymus, bone marrow and the population of circulating blood lymphocytes population was observed (Zaidi et al., 1994).

A recent cross-sectional analysis from the epidemiological NHANES study showed increased risks of eczema and itchy rash associated with increased hemoglobin adducts levels of acrylamide and glycidamide (Guo et al., 2017). So far, this is the only epidemiological study related to immunotoxic effects of acrylamide. Clearly, more research is needed on the potential immunotoxic effects of acrylamide.

### Conclusions

With regard to the carcinogenic effects of acrylamide, experimental rodent studies unequivocally show that acrylamide causes cancer in different species and in different tissues. Epidemiological studies so far have resulted in a less strong body of evidence but there are indications for increased risks of some cancers (renal, endometrial and ovarian). Should those risks be true, then they are considerably higher than the risks based on extrapolation from the experimental animal studies. Thus, there clearly is every reason to be concerned about current dietary exposure levels with regard to cancer.

For other health effects than cancer, the indications that dietary acrylamide intake is a reason for concern are fewer. However, several epidemiological studies have consistently shown inverse associations between gestational acrylamide exposure and fetal

**Table 5** Associations between dietary acrylamide intake and birth outcomes

References	Effect size (95% confidence interval)	Subgroup analyses	Covariables in the analysis
Pedersen et al. (2012)	<p><b>Birth weight (g):</b>  <b>AA adducts:</b>            Q2: −65 (−139, 19)  <b>Q3: 110 (−207, −48)</b>  <b>Q4: 157 (−256, −58)</b>            Per 10 pmol/g Hb increment: −23 (−51, 5)  <b>GA adducts:</b>            Q2: −80 (−159, −1)            Q3: −50 (−131, −31)            Q4: −110 (−207, −12)            Per 10 pmol/g Hb increment: −22 (−67, 23)  <b>Risk of small for gestational age (SGA):</b>  <b>AA adducts:</b>  <b>Per 10 pmol/g Hb increment: 1.20 (1.08, 1.33)</b>  <b>GA adducts:</b>  <b>Per 10 pmol/g Hb increment:</b>  <b>1.36 (1.13, 1.64)</b>            Birth head circumference (cm):  <b>AA adducts:</b>            Q2: −0.08 (−0.37, 0.21)            Q3: −0.08 (−0.37, 0.21)            Q4: −0.22 (−0.59, 0.14)            Per 10 pmol/g Hb increment: 0.02 (−0.08, 0.12)  <b>GA adducts:</b>            Q2: −0.08 (−0.36, 0.21)            Q3: −0.07 (−0.36, 0.23)            Q4: −0.26 (−0.62, 0.09)            Per 10 pmol/g Hb increment: −0.01 (−0.17, 0.16)            Birth weight (g):            Per 1-unit increase in acrylamide food score            (ranging from 0 to 8):            −16 (−33, 1)</p>	<p><b>Non-smokers:</b>  <b>Birth weight (g):</b>  <b>AA adducts:</b>            Q2: −19 (−102, 64)  <b>Q3: 132 (−216, −49)</b>  <b>Q4: 149 (−248, −50)</b>            Per 10 pmol/g Hb increment: −34 (−72, 4)  <b>GA adducts:</b>            Q2: −67 (−150, 16)  <b>Q3: 89 (−173, −4)</b>  <b>Q4: 97 (−193, −1)</b>            Per 10 pmol/g Hb increment: −52 (−112, 8)  <b>Risk of small for gestational age (SGA):</b>  <b>AA adducts:</b>  <b>Per 10 pmol/g Hb increment: 1.35 (1.10, 1.65)</b>  <b>GA adducts:</b>  <b>Per 10 pmol/g Hb increment: 1.42 (1.00, 2.02)</b>            Birth head circumference (cm):  <b>AA adducts:</b>            Q2: 0.01 (−0.30, 0.32)            Q3: −0.10 (−0.41, 0.21)            Q4: −0.21 (−0.57, 0.16)            Per 10 pmol/g Hb increment: −0.05 (−0.09, 0.19)  <b>GA adducts:</b>            Q2: −0.08 (−0.38, 0.22)            Q3: −0.21 (−0.52, 0.10)            Q4: −0.23 (−0.58, 0.12)            Per 10 pmol/g Hb increment: −0.05 (−0.27, 0.17)</p>	<p>Gestational age, country, maternal smoking at the end of pregnancy, passive smoking, sex, prepregnancy BMI, parity, maternal age, maternal ethnicity, maternal education, maternal consumption of fruit and vegetables, fish, and soft drinks</p>

Duarte-Salles et al. (2013)

**Birth weight (g):**  
**Q2: 13.0 (–23.19, –2.81)**  
**Q3: 20.8 (–31.05, –10.65)**  
**Q4: 25.7 (–35.89, –15.44)**  
**Per 1 SD increment:**  
**–9.9 (–13.50, –6.27)**  
**Risk of small for gestational age (SGA):**  
**Q2: 1.05 (0.96, 1.14)**  
**Q3: 1.08 (0.99, 1.18)**  
**Q4: 1.11 (1.02, 1.21)**  
**Per 1 SD increment:**  
**1.03 (1.00, 1.06)**

Kadawathagedara et al. (2016)

Per 10 µg/day increment of acrylamide intake:  
 Birth weight (g):  
 –9.79 (–21.3, 1.69)  
 Birth length (cm):  
 –0.05 (–0.11, 0.001)  
**Risk of small for gestational age (SGA) OR:**  
**1.11 (1.03, 1.21)**  
 Birth head circumference (cm):  
 –0.002 (–0.04, 0.04)

**Non-smoking mothers:**

**Birth weight: (g)**  
**Q2: 15.9 (–26.17, –4.94)**  
**Q3: 19.8 (–30.55, –9.31)**  
**Q4: 25.1 (–35.97, –14.73)**  
**Per 1 SD increment:**  
**–9.6 (–13.48, –5.84)**  
 Risk of small for gestational age (SGA):  
 Q2: 1.08 (0.99, 1.18)  
**Q3: 1.09 (1.00, 1.19)**  
**Q4: 1.13 (1.03, 1.23)**  
**Per 1 SD increment:**  
**1.03 (1.00, 1.06)**

There was only an inverse association between acrylamide intake and birth length in girls, not in boys  
 Non-smokers:  
 Per 10 µg/day increment of acrylamide intake:  
 Birth weight (g):  
 –7.69 (–23.0, 7.67)  
 Birth length (cm):  
 –0.01 (–0.08, 0.05)  
**Risk of small for gestational age (SGA) OR:**  
**1.16 (1.04, 1.30)**  
 Birth head circumference (cm):  
 0.04 (–0.02, 0.10)  
 Smokers:  
 Per 10 µg/day increment of acrylamide intake:  
 Birth weight (g):  
 –9.85 (–27.8, 8.11)  
 Birth length (cm):  
 –0.09 (–0.18, 0.00)  
 Risk of small for gestational age (SGA) OR:  
 1.05 (0.92, 1.19)  
 Birth head circumference (cm):  
 –0.06 (–0.13, 0.01), **p interaction**  
**for smoking: 0.01**

Gestational age, parity, sex of the child, age of the mother, maternal BMI, maternal gestational weight gain, and smoking during pregnancy

Model for birth weight, length and head circumference: study centre, maternal age at delivery, parity, height, maternal education level, tobacco consumption during pregnancy, gestational age at delivery, sex  
 Model for SGA: study centre, maternal age at delivery, maternal education level, tobacco consumption during pregnancy, maternal weight gain during pregnancy

growth, which can have important repercussions on children's development and future health. The fact that developmental effects in humans were not expected based on extrapolation from animal studies should not be a reason to classify those epidemiological findings as less credible. Humans are not rodents and human data, if of high quality, are preferable over animal data. Therefore, more epidemiological research on the possible effects of dietary acrylamide exposure on fetal growth and neonatal development (and on mechanisms of action) is urgently needed.

In the mean time, every possible effort should be taken by the food industry to lower acrylamide levels. In addition, more advice for the general public on dietary acrylamide intake reduction is warranted, especially during sensitive time windows, such as pregnancy and childhood.

Furthermore, more epidemiological research is needed on possible effects of acrylamide on the nervous system, both in elderly and childhood populations. Lastly, I encourage epidemiological studies on reproductive effects of acrylamide, considering that associations between acrylamide intake and sex hormone levels have been observed in various populations, on immunotoxic and cardiotoxic effects, and on the incidence of metabolic disease. For all these latter health effects, the evidence for their occurrence at dietary level exposure is still flimsy. In the era of omics research possibilities, it would be helpful to conduct those studies with collection of omics data in order to try to elucidate the biological plausibility of observed associations.

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# Advanced Glycation End Products (AGEs): Occurrence and Risk Assessment

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## Overview

The research on chemical reactions between carbohydrates and amino compounds was established by Louis-Camille Maillard over one century ago (Maillard, 1912). The complexity of the reaction has been the subject of countless works and the chemistry, physiology and technology of the Maillard reaction have been reviewed frequently (see for instance Ledl and Schleicher, 1990; Hellwig and Henle, 2014; Lund and Ray, 2017).

According to the fundamental Hodge scheme (Hodge, 1953), which is still valid today, the Maillard reaction is subdivided into three stages (Fig. 1): In the first stage, Amadori rearrangement products (ARPs) are formed from reducing sugars and amino groups of free amino acids, peptides, and proteins. The ARPs are the first stable intermediates of the Maillard reaction. In the second stage, ARPs are degraded via enolization and elimination reactions to 1,2-dicarbonyl compounds, which may react again with free amino groups or with the guanidino group of arginine. The complex spectrum of Maillard reaction products (MRPs) can thus be subdivided into various classes of compounds, namely (i) Schiff bases and Amadori rearrangement products (ARPs), (ii) dicarbonyl compounds and their N-free reaction products, and (iii) glycated amino acids and polymerized brown pigments. Examples of glycated amino acids are shown in Fig. 2. Resulting from lysine, main representatives are carboxyalkylated derivatives such as N- $\epsilon$ -carboxymethyllysine (CML) and N- $\epsilon$ -carboxyethyllysine (CEL), and pyrrole derivatives incorporating the  $\epsilon$ -amino group of lysine such as pyrroline and formyllysine (Hellwig and Henle, 2014). Further important products are pyrrolinones such as pronyllysine and amides such as lactoyllysine. At arginine residues, hydroimidazolones such as G-H1, MG-H1 and 3-DG-H are the main reaction products. Pentosidine and glucosepane were described as the most important crosslinking structures in glycation reactions beneath the imidazolium salts GOLD, MOLD, and DOLD.

## “AGE” – Still a Reasonable Expression?

The fact that Maillard reactions also occur *in vivo* was first demonstrated by Rahbar (1968) and Bunn et al. (1975) with the characterization of HbA<sub>1c</sub>, a hemoglobin variant containing an N-terminal fructosylvaline moiety at the  $\beta$ -chain. In order to differentiate between such non-enzymatically formed sugar adducts to proteins and the well-known enzymatic glycosylation of proteins, the expression “glycation” was proposed by the IUPAC in 1984 (IUPAC, 1984) and is now synonymously used with the term “Maillard reaction”. The expression “advanced glycosylation end products”, later termed “advanced glycation end products” (AGEs), was introduced into biomedical glycation research by Brownlee et al. (1984). This term was needed in order to distinguish reversibly bound glycation adducts on proteins (Schiff bases, Amadori products) from irreversibly covalently modified protein structures (“brown fluorescent pigments which crosslink proteins”; Brownlee et al., 1984). As a first structural example for a possible AGE, the crosslinking structure 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI) was isolated and characterized (Brownlee et al.,

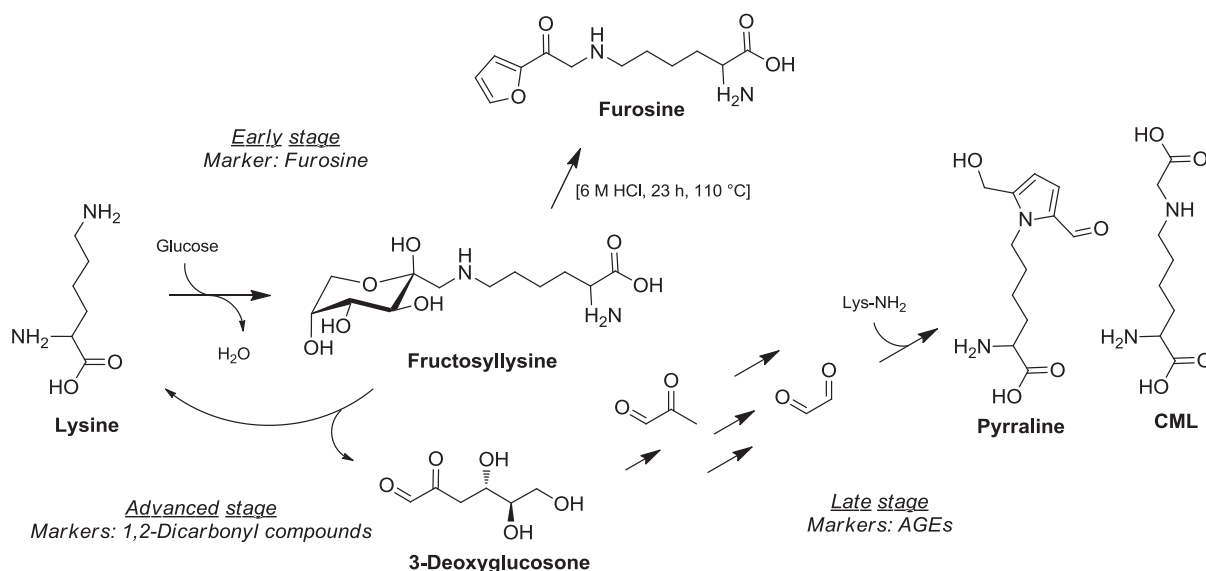
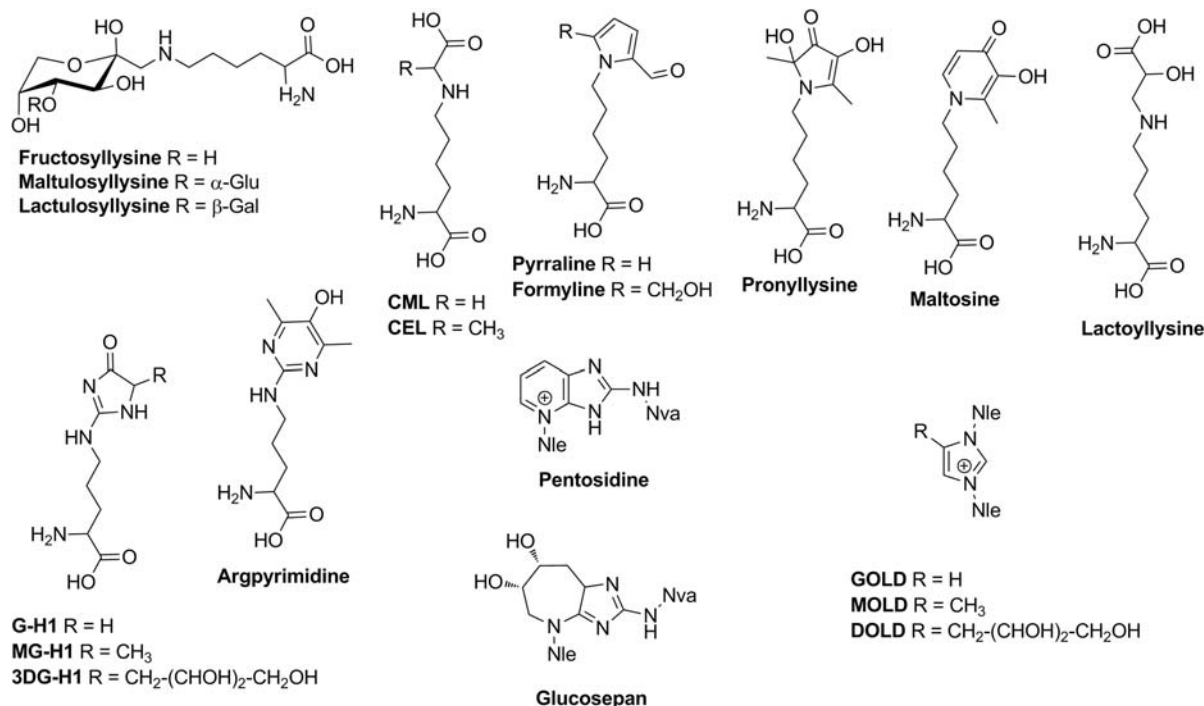


Figure 1 General scheme of the Maillard reaction (CML, N- $\epsilon$ -carboxymethyllysine).



**Figure 2** Glycated amino acids (“advanced glycation end products”). Nle, norleucyl residue; Nva, norvalyl residue.

1984), but later turned out to be an artifact of acid hydrolysis (Gerhardinger et al., 1990). In the following decades, the expression “AGE” became generally applied to stable products formed during the late stage of the Maillard reaction independently on the molecular weight.

From the term “advanced glycation end products”, it becomes apparent that the reaction products must be linked to the attachment of sugars or their degradation products to proteins such as those compiled in Fig. 2. These structures comprise a huge structural diversity. Glycation can modify the polarity of the side-chains of the parent amino acids lysine and arginine to more polarity (Amadori products) or less polarity (pyrrole derivatives). Positive charges of lysine and arginine side-chains can not only be masked by glycation but even inverted by carboxyalkylation. The overall accessibility of side-chains can be reduced by crosslinking. All these chemical modifications together change the physico-chemical properties of proteins, for example the solubility and the isoelectric point.

Despite this structural diversity, several sum methods for the determination of the “AGE concentrations” of foods and the “AGE status” in physiological samples have been established and were applied widely in the literature. These methods are based on the measurement either of “AGE fluorescence” or immunoreactivity of AGEs (Meerwaldt et al., 2004; Uribarri et al., 2010; Fernandez-Gomez et al., 2015; Takeuchi et al., 2015). From a chemical point of view, however, it must be noted that statements about an “AGE concentration” resulting from such methods are scientifically incorrect. A specific “AGE fluorescence” has been postulated based on the fluorescence of the above mentioned compound FFI (excitation wavelength, 370 nm; emission wavelength, 440 nm). It is obvious, since many fluorescent MRPs are known, that this “AGE fluorescence” does not represent one defined structure, but is always the sum of different individual structures with varying spectroscopic properties, whose composition may change depending on the composition of the matrix. Furthermore, as not all AGEs are fluorescent, calculation of an “AGE content” based on fluorescence measurements must lead to a false estimation of the modification of proteins by the Maillard reaction. Nevertheless, the measurement of skin autofluorescence, which was attributed to glycation, has been proposed as a diagnostic tool in the monitoring of diabetic complications (Meerwaldt et al., 2004). The same problems arise for immunological methods used for quantification of AGEs. Such methods make use of antibodies that have been raised against glycated proteins, with the glycation being induced for example by glucose or 3-DG (Papanastasiou et al., 1994; Takeuchi et al., 2001). The proteins used for antibody production are often poorly characterized in terms of the formation of individual MRPs, whereas experience of the Maillard reaction teaches that the preparations must contain a broad spectrum of individual amino acid derivatives. Poor specificity and comparability as well as crossreactivity between antibodies are, therefore, inevitable (Morioka et al., 2017). Data collections, reporting on the “AGE content” of foods, therefore, must be handled with care, as the concentration values based on immunological techniques (mostly reported in “AGE units per kg protein” or similar) are generally not validated by alternative techniques and very often are merely impossible (see below). Today, it must be state of the art to use specific and more reliable methods for the quantification of MRPs, primarily HPLC-MS/MS, to overcome the shortcomings of the sum methods based on fluorescence and immunobiology (Teerlink et al., 2004;

Assar et al., 2009; Scheijen et al., 2016). The use of such methods is called for by the scientific community (Calder et al., 2011; Tessier and Birlouez-Aragon, 2012; Kellow and McCoughlan, 2015; DeChristopher, 2017).

The uncritical use of effect-based analytical tools mainly in studies dealing with physiological aspects has led to much confusion in glycation research in the last decades, raising the question as to whether the use of the expression “AGE” continues to make sense. In the opinion of the authors, structure-based analytical means must be applied to approach the actual concentrations of individual glycation-derived structures in food products and physiological media. This becomes clear when concentrations of individual glycated amino acids and “AGE concentrations” are compared.

## Formation and Occurrence of Individual MRPs in Food

Only few individual MRPs have been reliably quantified in foods up to now. Consistently, the most important MRPs in foods are the Amadori products, accounting for >90% of the total amount of MRPs. While ARPs have been quantified mostly after conversion to furosine by acid hydrolysis (Fig. 1), recent works based on HPLC-MS/MS methods reveal differences in the patterns of individual ARPs in individual foodstuffs: Fructosyllysine is the most relevant ARP in the honey proteome, whereas maltosyllysine predominates in the beer proteome (Hellwig et al., 2016, 2017). Food systems containing reducing sugars and free amino groups react very sensitively even to comparatively mild heat treatments such as the UHT process in dairy processing. The concentrations of ARPs in UHT-treated milk can be 10 times as high as those in pasteurized or raw milk (Henle et al., 1995). Particularly high concentrations of Amadori products can be found in more strongly heat-treated foods such as bread, breakfast cereals and processed meat products (Table 1). However, a comparison of ARP concentrations in bread crust, bread crumb, and pasta products, which differ strongly in their heating conditions, reveals that these differences are no longer mirrored in the ARP concentrations. This is due to the progress of the Maillard reaction which entails the degradation of the thermodynamically unstable ARPs. The patterns of glycated amino acids formed in the final stage of the Maillard reaction strongly depend on the heating conditions (temperature/time) and often also on the water activity. Moreover, oxidation reactions may play a role as is known from the conversion of fructosyllysine to CML. The water activity is decisive for the formation of pyrrole compounds such as pyrroline and formylpyrroline. Correspondingly, food products that have undergone strong heat treatment always contain higher amounts of late-stage MRPs than mildly treated products (cf. milk and evaporated milk). This is also valid for different heat impact regions in the same food product (cf. whole bread and bread crust).

Due to the pathways of formation of MRPs described in the preceding sections, it is not intriguing that protein- and carbohydrate rich foods will contain the highest amounts of these substances (Table 1). Counterintuitively, databases compiling “AGE concentrations” based on an ELISA method have reported high AGE concentrations especially in fat-rich foods such as processed meat and fish products and butter, whereas the concentrations in protein-rich food such as eggs, bread, and pasta products were low (Goldberg et al., 2004; Uribarri et al., 2010). It has been argued that it is very difficult to bring in line the data obtained with the unvalidated ELISA methods with the chemistry of the Maillard reaction (Calder et al., 2011), and that the semi-quantitative data need to be interpreted with caution (Scheijen et al., 2016). Moreover, these data stand in contrast to the outcome of several structure-based works on MRPs of different groups (Assar et al., 2009; Scheijen et al., 2016; Hellwig et al., 2016, 2017) and do not withstand a closer examination by superior validated analytical techniques such as HPLC-MS/MS.

## Physiological Relevance of Dietary AGEs

Glycation reactions are a common side-reaction in biological systems and occur in the human body throughout the lifespan. It is indisputable that protein modifications brought about by glycation reactions may alter the functionality of proteins and other

**Table 1** Concentrations of individual Maillard reaction products in food

Food product	ARP <sup>[a]</sup> [g kg <sup>-1</sup> ]	CML [mg kg <sup>-1</sup> ]	Pyrroline [mg kg <sup>-1</sup> ]	MG-H1 [mg kg <sup>-1</sup> ]	AGE Units [MU kg <sup>-1</sup> ]
Milk (pasteurized, UHT)	0.003–0.1 <sup>[c]</sup>	0.2–2.6 <sup>[j,m]</sup>	n.d. <sup>[i]</sup>	0.8–3.0 <sup>[m]</sup>	0.01–0.1 <sup>[h]</sup>
Evaporated milk	0.4–1.1 <sup>[c]</sup>	4.7–46.2 <sup>[g,m]</sup>	0.4–8.5 <sup>[b,i]</sup>	0.9–5.4 <sup>[m]</sup>	–
Bread (loaf)	0.4–0.5 <sup>[e]</sup>	3.1–38.8 <sup>[j,m]</sup>	6.3–69.3 <sup>[i]</sup>	20.9–60.1 <sup>[m]</sup>	0.2–1.5 <sup>[h]</sup>
Bread crust	0.2–0.6 <sup>[d]</sup>	37.1–46.1 <sup>[g]</sup>	60–260 <sup>[b,i]</sup>	–	0.4–0.7 <sup>[h]</sup>
Pasta products	0.2–3.2 <sup>[c,e]</sup>	2.4–8.2 <sup>[j,m]</sup>	n.d.–12.0 <sup>[b,i]</sup>	14.1–30.2 <sup>[m]</sup>	1.1–2.5 <sup>[h]</sup>
Breakfast cereals	0.2–2.8 <sup>[f]</sup>	4.6–19.6 <sup>[m]</sup>	27–87 <sup>[i]</sup>	36–416 <sup>[m]</sup>	0.2–20 <sup>[h]</sup>
Meat and fish, fried, boiled, baked	0.4–1.1 <sup>[j]</sup>	0.5–42.2 <sup>[j,k,l,m]</sup>	–	1.1–134.8 <sup>[m]</sup>	2–916 <sup>[h]</sup>
Chocolate	3.1 <sup>[e]</sup>	5.2–35.1 <sup>[m]</sup>	–	4.2–32.3 <sup>[m]</sup>	–

[a] Amadori products are calculated as fructosyllysine. [b] Henle et al., 1994. [c] Henle et al., 1995. [d] Ramírez-Jiménez et al., 2000. [e] Erbersdobler and Faist, 2001. [f] Delgado-Andrade et al., 2005. [g] Assar et al., 2009. [h] Uribarri et al., 2010. [i] Hellwig and Henle, 2012. [j] Hull et al., 2012. [k] Chen and Smith, 2015. [l] Roldan et al., 2015. [m] Scheijen et al., 2016. n.d., not detectable. –, no data available.

biomolecules. On a molecular level, modification of particular arginine residues was shown to negatively influence collagen functionality (Dobler et al., 2006), and crosslinking reactions due to *in vivo* glycation may deteriorate the biomechanical properties of cartilage (Bank et al., 1998).

Lots of studies have been conducted in order to estimate to what extent dietary MRPs contribute to the *in vivo* concentrations, and if diets rich in MRPs lead to physiologically negative effects such as inflammatory processes, oxidative stress, insulin resistance, or endothelial dysfunction (for reviews, see Kellow and Savage, 2013; Van Puyvelde et al., 2014; Kellow and McCoughlan, 2015; Clarke et al., 2016). Several studies agree that (i) dietary AGEs are taken up into circulation after ingestion and contribute to the “AGE pool” of the body, (ii) these AGEs may accumulate in the body thereby prolonging their negative impact (“glycotoxins”, Koschinsky et al., 1997), (iii) AGEs exert negative effects on human health through interaction with a special “receptor for advanced glycation end products (RAGE)”. This has led to a widely accepted narrative in glycation research (“AGEs are a risk for human health”) that may have led to publication bias precluding researchers from submitting data not confirming this hypothesis (Kellow and Savage, 2013).

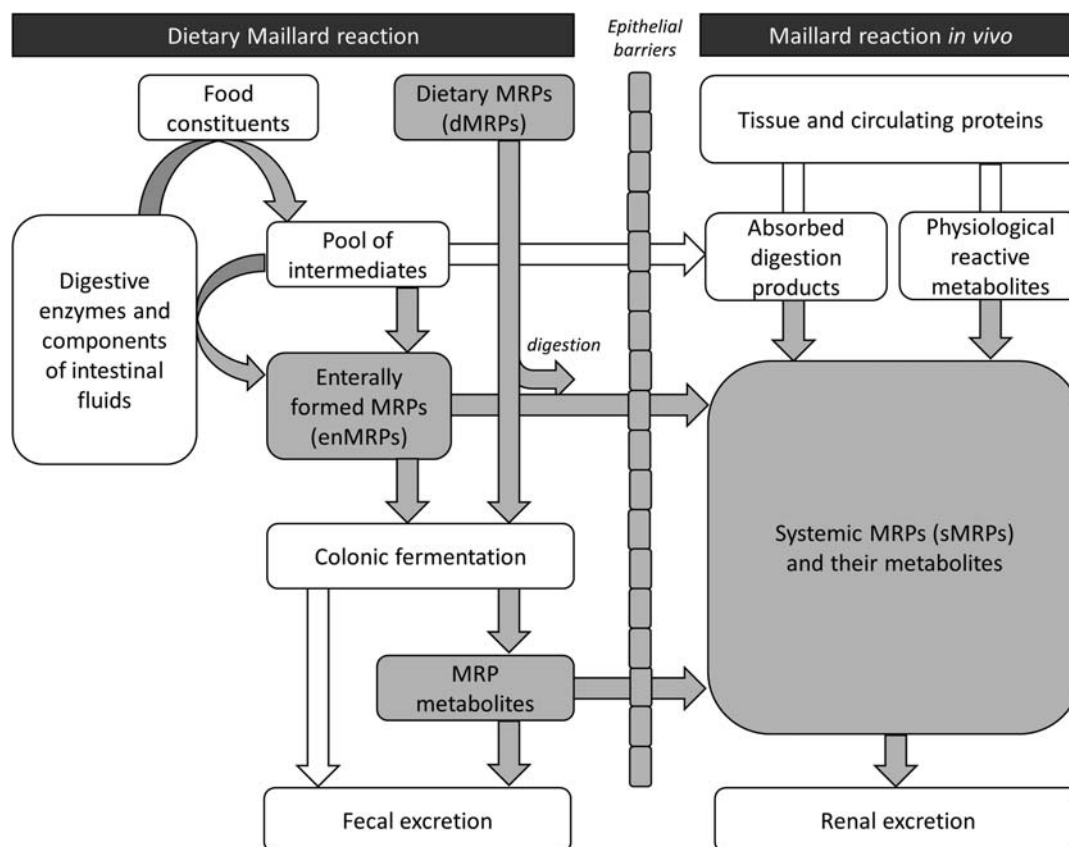
All three points have been challenged in the last years, and it becomes clear that general conclusions on the biological effect of AGEs cannot be made due to their strong structural diversity. The absorption of AGEs is precluded by digestion and transport processes. MRPs in food are mostly protein-bound and need to be released from proteins during digestion. Amino acids with a basic or unpolar side-chain such as fructosyllysine or pyrraline are more easily released from proteins than amino acids with an acidic side-chain such as CML (Hellwig et al., 2014). Transport via the small intestinal epithelial membrane is only possible for free amino acids, di- and tripeptides. Free glycated amino acids cannot pass this barrier due to the narrow substrate specificity of amino acid transporters. Some glycated dipeptides, however, were shown to be transported into cells expressing the peptide transporter PEPT1. After intracellular cleavage, free glycated amino acids may pass into the systemic circulation, but they are rapidly excreted via the urine, because they are no substrates of renal amino acid transporters (Bergmann et al., 2001; Förster et al., 2005; Hellwig et al., 2011). Therefore, dietary glycated amino acids cannot accumulate in the body.

Moreover, the design of the studies was critically reviewed: “AGEs” in the experimental diets are often generated by extreme heating methods, and it cannot be excluded that essential micronutrients, for example thiamine, decompose simultaneously (Buetler and Henle, 2009). From a mechanistic point of view, it must be argued as to whether the same MRPs are formed under preparation conditions such as roasting and deep-frying as compared to boiling, implying that strong qualitative differences may be existent behind a different quantitative “AGE concentration” as measured by ELISA or fluorescence spectrometry. Nevertheless, differences observed in studies comparing “AGE-rich” and control diets are often traced back to a single MRP, very often CML. However, owing to the commonly practiced use of the databases of Goldberg et al. (2004) and Uribarri et al. (2010), it is largely uncertain which individual MRPs have been taken up in which concentrations.

### **Risk Assessment of Dietary AGEs – Is This Even Possible?**

Regarding the study design discussed above, it must be concluded that up to now—to the best of our knowledge—only one study is available that links a single glycated amino acid to an adverse effect. In this preliminary study, 5-hydroxymethylfurfural (HMF) and pyrraline (Fig. 2) were identified as mutagenic substances in the Ames test (Omura et al., 1983). Nevertheless, the exposure to HMF from food was shown not to be of toxicological relevance (Janowski et al., 2000). On the other hand, positive effects of glycated proteins and “maillardized” food items such as malt and bread crust were identified in animal studies as judged from an increase in the activity of renal and hepatic chemoprotective enzymes (glutathione-S-transferase, UDP-glucuronyl transferase). These chemopreventive effects were partly traced back to the defined substances *N*-methylpyridinium and pronyllysine (Fig. 2, Lindenmeier et al., 2002; Somoza et al., 2003). As defined in Article 1 (9) of regulation (EC) No 178/2002 of the European Parliament and of the Council, laying down, *inter alia*, procedures in matters of food safety, ‘risk’ means a function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard. At present, neither adverse health effects nor any negative effect resulting from, e.g., cell or animal studies can be unequivocally linked to the dietary uptake of glycated amino acids. Therefore, for reasons of semantics as well as due to basic considerations with respect to risk theory, it is not possible to calculate a “risk” for dietary AGEs. This, of course, must not prevent food scientists to study possible adverse as well as positive effects of process-induced protein modifications.

With respect to MRPs, research may not solely regard the concentrations of individual compounds in the diet, but must take into account the individual bioavailability of compounds, their reactivity during intestinal digestion and their metabolism by the colonic microbiota. Research in food chemistry has brought forward our understanding of these issues in recent years, starting from the question as to how dietary MRPs or dietary constituents can influence the “pool” of MRPs in the circulation or in tissues. In the opinion of the authors, there are at least three types of MRPs that are of relevance when the physiological role of the Maillard reaction is discussed (Fig. 3). The first type of MRPs are those that form in foods and that can be quantified by chemical analysis of foods, the “dietary MRPs” (dMRPs). The second type are the MRPs that form under physiological conditions in tissues and on circulating proteins, collectively termed “systemic MRPs” (sMRPs). Dietary glycated amino acids cannot simply cross the epithelial barrier, as they are mostly protein-bound. However, some of them may appear in free or peptide-bound form during intestinal proteolysis. Glycated dipeptides may then be transported into enterocytes and further pass into circulation thereby contributing to the pool of sMRPs (Hellwig et al., 2011, 2014). However, the chemistry during digestion may be even more complex, because the pool of digestion intermediates that is present postprandially and the different pH and ionic environment may enable new



**Figure 3** Proposed pathways for the metabolic fate of MRPs.

chemical reactions. 1,2-Dicarbonyl compounds such as methylglyoxal and 3-deoxyglucosone are not stable during simulated intestinal digestion (Degen et al., 2013, 2014). It must be supposed that these compounds react with digestive enzymes, unabsorbed dietary protein fragments or other components of intestinal fluids, thereby generating a third class of MRPs, namely enterally formed MRPs (enMRPs). These products may be structurally identical to the dMRPs known up to now, but may just as well represent novel structures. In this context, the consumption of high amounts of fructose may be of particular relevance with respect to the formation of enMRPs (DeChristopher, 2017). A recent animal study has shown that long-term feeding of mice with excessive glucose caused an increase in methylglyoxal-derived MRPs, whereas excessive fructose consumption caused an increase in glyoxal-derived MRPs such as CML in liver tissue (Mastrocola et al., 2013). We conclude that the intestinal tract is a reaction space whose significance has been overlooked up to now. Research on the occurrence of dietary MRPs must further consider the potential of enMRP formation after ingestion.

MRPs are subject to colonic fermentation (Hellwig et al., 2015), but apart from reported changes in the composition of the microbial consortium (Tuohy et al., 2006), the formation of novel MRP metabolites is completely underexplored. Metabolites may be formed both from dMRPs and enMRPs. As such metabolites may theoretically be taken up into circulation and show bioactivity, research in this area is urgently needed.

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# Furan and Alkylfurans: Occurrence and Risk Assessment

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## Glossary

**BMD, benchmark dose** The BMD is the minimum dose of a substance that produces a clear, low level health risk, usually in the range of a 1%–10% change in a specific toxic effect such as cancer induction.

**BMDL10, benchmark dose lower confidence limit** The lower confidence level of the dose that results in an increase of cancer incidence by 10% above background.

**Exposure assessment** One of the key steps in risk assessment, consisting of a thorough evaluation of exposure to a hazard and a quantification of the amounts involved.

**Deterministic exposure assessment** Exposure assessments using point values of occurrence and intakes to produce a point estimate of individual or population exposure.

**HBGV, health-based guidance value** Guidance on safe consumption of substances that takes into account current safety data, uncertainties in these data, and the likely duration of consumption.

**MOE, margin of exposure** In toxicology, the MOE of a substance is the ratio of its PoD (BMDL<sub>10</sub> or similar) to its estimated dose of human intake. It is used in risk assessment to explore safety concerns arising from the presence of potentially toxic substances in food.

**PoD, point of departure** In toxicology the PoD is defined as the point on a toxicological dose-response curve established from experimental data generally corresponding to an estimated low effect level or no effect level. It is used to derive a safe level of exposure.

**LoD, limit of detection** The LoD is the lowest concentration of a substance that can be detected using standard tests but which is too small to be measured with certainty.

**LoQ, limit of quantification** the LoQ is the lowest concentration of a substance that can be measured with certainty using standard tests.

**LB, lower bound estimate** An estimate of the minimum exposure to a potentially harmful substance, normally zero, which takes into account normal consumption of food which contains negligible amounts of the substance.

**UB, upper bound estimate** A way of estimating exposure to a particular compound from analytical data by assigning the lowest value which can be detected (or quantitated) to all samples with levels below this value. For a toxic chemical this gives the most pessimistic estimate of exposure (i.e. the real level of exposure will always be below the upper bound estimate).

**Risk assessment** A specialised field of applied science that involves reviewing scientific data and studies in order to evaluate risks associated with certain hazards. It involves four steps: hazard identification, hazard characterisation, exposure assessment and risk characterisation.

(for further definitions, the reader is referred to the EFSA glossary <https://www.efsa.europa.eu/en/glossary-taxonomy-terms>)

## Nomenclature

°C degree Celsius

μg (kg bw)<sup>−1</sup> day<sup>−1</sup> dose on a body weight basis

## Introduction

Furan and a number of furan derivatives such as 2- and 3-methylfuran are known to be present in certain heat processed foods where they contribute to flavor and aroma. First described in coffee (Maga, 1979), food analytical data on furan were published in 2004 when its presence was described in a variety of foods including canned and jarred foods and coffee (FDA, 2004; EFSA, 2004). Furan is a volatile heterocyclic organic compound used as a chemical intermediate in the chemicals industry. It can also be generated and released by human activities (combustion, fires, exhausts) and is present in cigarette smoke. Human exposure, however, occurs mainly via food. Furan is a potent liver toxin, induces liver cancer in rodents and is classified as ‘reasonably anticipated to be a human carcinogen’ (NTP, 2016) and as ‘possibly carcinogenic to humans’ (group 2B; IARC, 1995). Evidence is accumulating that furan induced carcinogenicity involves mainly non-genotoxic mechanisms of action. The current thinking is that methylfurans

(MeF) share toxic and mechanistic properties with furan suggesting dose additivity. Exposure to furan(s) via the diet, particularly in high intake consumers including infants receiving baby foods in jars may represent a health concern.

## Analytical Methods

Several analytical methods to quantify furan in different foodstuffs have been described in the literature over the past decade, mostly based on headspace (HS)-GC-MS, due to the volatility of the analyte, with a boiling point at 32 °C (FDA, 2004; Nyman et al., 2006, 2008). Furan needs to be volatilised from the food or beverage matrix, requiring certain conditions (e.g. sample fluidity, equilibrium temperature and time, addition of inorganic salts such as ammonium sulfate) that promote release of the analyte. The initial HS-GC-MS method published by the FDA in 2004 required certain improvements, particularly related to headspace oven temperature and risk of furan forming adventitiously during analysis at temperatures at 80 °C (Morehouse et al., 2008; Nyman et al., 2008). Today, most methods apply equilibration temperatures in the range of 40–50 °C, which provides comparable results as judged from different literature surveys on furan in food. The contribution of the food matrix and food components to the generation of furan during analysis may, therefore, be significant, and together with other parameters impact the partition coefficient of furan (Senyuva and Gokmen, 2005; Huang and Barringer, 2016).

Many of the analytical methods published to date apply an SPME (solid phase microextraction) GC-MS, with the advantage that SPME in general enables superior sensitivity by concentrating the analyte on the fiber (Table 1). In fact, many different surveys on furan in foods are based on methods utilizing SPME, covering several different fiber types that have been assessed and used for analysis, e.g. carboxen/polydimethylsiloxane (CAR/PDMS), divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), polydimethylsiloxane/divinylbenzene (PDMS/DVB), polyacrylate, and carbowax/divinylbenzene (CW/DVB) (Goldmann et al., 2005; Altaki et al., 2017). However, care must be taken to avoid *in situ* formation of furan during thermal desorption, that takes place at temperatures ranging from 200–300 °C (Adams et al., 2012).

Mass spectrometry with the addition of isotope labelled internal standards is the best choice for the reliable quantification of furan (and MeF) in foods, using characteristic ions (e.g. furan molecular ion at  $m/z = 68$  as quantifier,  $m/z = 39$  and  $m/z = 69$  as qualifiers;  $d_4$ -furan at  $m/z = 72$ ) in the selective ion monitoring (SIM) mode. For the analysis of MeF, Becalski et al. (2010, 2016) utilized  $d_4$ -furan, 2-methylfuran- $d_3$  (methyl- $d_3$ ) and 3-methylfuran (methyl- $d_4$ ) as internal standards for a wide range of jarred and canned foodstuffs, including coffee (roast and ground coffee, instant coffee). Several investigations have, however, quantified MeF based on the response of  $d_4$ -furan (Chaichi et al., 2015; Fromberg et al., 2014), noting that the boiling points are somewhat higher for MeF (63–65 °C).

Recently, a GC-FID (flame ionization detection) based method was developed and applied to determine furan, 2-MeF and pentylfuran in fruit juices. The method achieves a comparable LoQ to mass spectrometry based approaches (Hu et al., 2016). However, FID is non-specific, and thus the identification of the analytes is dependent on retention time, requiring clean chromatograms with no interference in the regions where the analytes elute. The applicability of FID is therefore most probably limited to only a few 'cleaner' food matrices.

In 2015, the European Standard EN 16620 was published that describes a HS-GC-MS method to determine furan in coffee and coffee products (CEN (European Committee for Standardization), 2015). However, one major gap today from the analytical perspective is the lack of an official or agreed standard method encompassing furan as well as the alkylfurans for the concerned foodstuffs, which is important in case that risk management measures for certain foods are proposed or implemented. Certified reference materials would be a major advantage, to ensure appropriate laboratory performance.

**Table 1** Selected single and multimethod for the determination of furan and MeF in different foodstuffs

Foodstuff	Analytes	Principle of the method	LoQ (ng/g)	References
Coffee	Furan, 2-MeF, 3-MeF	HS-GC-MS; isotope labelled standards for all analytes	3 (roast and ground coffee)	Becalski et al. (2016)
Fruit juices	Furan, 2-MeF, 2-pentylfuran	HS-SPME-GC-FID	0.18 (furan); 0.14 (2-MeF)	Hu et al. (2016)
All foodstuffs	Furan, 2-MeF, 3-MeF	HS-GC-MS; isotope labelled standards for all analytes	>1 (all foodstuffs)	Becalski et al. (2010)
Baby foods	Furan	HS-SPME-GC-MS; isotope labelled standard	0.04–0.11 (depending on foodstuff)	Altaki et al. (2017)
All foodstuffs	Furan, 2-MeF, 2,5-diMeF, 2-ethylfuran, 2-pentylfuran	HS-GC-MS; isotope labelled furan	2.4 (all foods except crisps) 2.9 (crisps)	Fromberg et al. (2014)
Bread (crust)	Furan	Automatic HS-trap GC-MS; isotope labelled standard	0.1	Huault et al. (2016)

## Occurrence

### Formation

Research over the past decade has revealed several pathways to the formation of furan, mainly via thermal reactions (e.g. sugar decomposition, Maillard reaction) and oxidative processes. Much of the earlier work was done in model systems, revealing the importance of substances such as ascorbic acid, selected amino acids (e.g. serine, cysteine), carbohydrates (pentoses, hexoses), polyunsaturated fatty acids (PUFAs) and carotenoids as precursors to furan formation (Yaylayan, 2006; Vranova and Ciesarova, 2009).

The oxidative pathway involving PUFAs leads to a key intermediate, namely the secondary lipid oxidation product 4-hydroxy-2-butenal, that can cyclise to 2,5-dihydro-2-furfural and subsequently release  $\text{H}_2\text{O}$  to afford furan. The carbohydrate pathways can be driven via the well known Maillard reaction, involving also amino acids. Sugar breakdown without amino acid assistance furnishes smaller fragments (e.g. acetaldehyde) or aldotetrose derivatives, such as 3,4-dihydroxybutanal. Cyclisation affords the tetrahydro-2,4-furandiol, that through loss of water yields furan. Alternatively, dehydration of 3,4-dihydroxybutanal gives rise to 4-hydroxy-2-butenal, that reacts further as described above for the PUFAs. An alternative pathway to furan is furoic acid, that could be formed by a Cannizzaro reaction of 2-furfural. Decarboxylation under thermal conditions is probably rapid, and may be an alternative pathway in certain products (Varelis and Hucker, 2011).

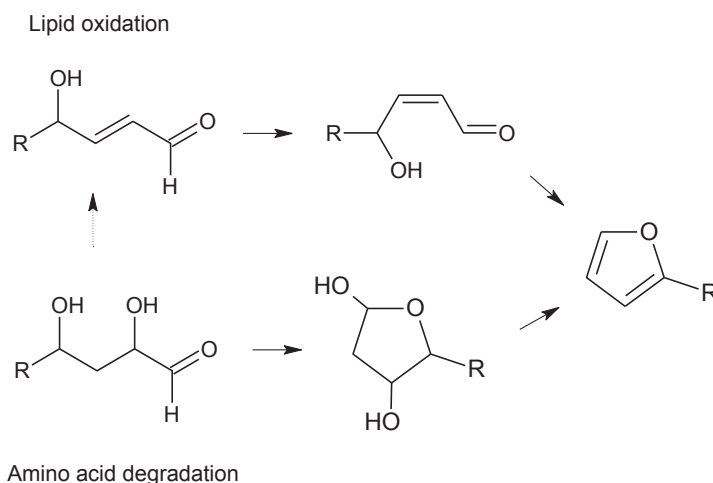
Fewer studies have been conducted on the formation of alkylfurans. Becalski et al. (2010) demonstrated the formation of both 2-MeF and 3-MeF from  $\beta$ -carotene and other isoprenoids. This pathway may be predominant in foods that harbour corn or tomato constituents. Carbonyl- or hydroxycarbonyl degradation products of amino acids (e.g. threonine, alanine) can rearrange and furnish alkylfurans, a pathway of relevance in meat products. Alternatively, lipid oxidation may lead to 4-hydroxyalk-2-enals that can rapidly cyclise under release of  $\text{H}_2\text{O}$  to afford the corresponding alkylfuran (Fig. 1).

### Levels in Foods

#### Furan

Occurrence data for furan are available for thermally processed foods, such as coffee, canned and jarred foods, including baby foods, soups and sauces. Besides the occurrence data from the published literature, national and international health authorities such as the US FDA and the European Food Safety Authority (EFSA) are collecting analytical data made available publicly (US FDA, 2008; EFSA, 2011, 2017). The EFSA (2017) has summarised close to 18,000 analytical data from European member states, non-European governments, from food industry associations and the published literature. Occurrence data from different sources were shown to be generally in agreement. Highest levels of furan were identified in coffee (including beans, roast and ground coffees, instant coffees and brews) followed by certain alcoholic beverages, composite foods, herbs, spices and condiments, and foods for infants and small children (Table 2). In baby foods highest furan levels were found in meat and vegetable-based products, while lower levels were reported in fruit and cereal based baby food products (EFSA, 2011). A large proportion of the analytical data available have been generated on products as taken from the shelf, and not as prepared for consumption.

Losses of furan due to evaporation during final preparation have been investigated in a couple of studies with very variable results (Roberts et al., 2008; Kim et al., 2009). Based on limited information and a several assumptions such losses were quantified for coffee and baby foods in jars (EFSA, 2017), and were taken into account in the exposure assessment (see below). Although practices used in home cooking in theory may similarly lead to furan generation, a few studies indicate that home cooking of freshly prepared foods is not a significant source of furan exposure, neither through inhalation nor through consumption (Crews,



**Figure 1** Simplified reaction pathways from PUFAs and amino acids to alkylfurans.

**Table 2** Furan levels in foods

Food group	Mean MB range ( $\mu\text{g/kg}$ )
All coffee beverages (powder)	126.5–1990.9
All coffee beverages (liquid)	2.3–146.7
Alcoholic beverages	1.1–57.4
Composite food (including frozen products)	11–33.5
Herbs, spices and condiments	3.4–31.6
Food for infants and small children	2.6–31
Grains and grain-based products	7.8–28.7
Snacks, desserts, and other foods	11.1–15.3
Fish and other seafood (including amphibians, reptiles, snails and insects)	14
Starchy roots and tubers (potatoes and potato products)	12.7
Cocoa beans and cocoa products	10.9
Meat and meat products (including edible offal)	8.6
Sugar and confectionary	8
Milk and dairy products	0.8–7.7
Legumes, nuts and oilseeds	6.1
Vegetables and vegetable products (excl. solids for beverage preparation)	6
Fruit and fruit products	3.7–5.5
Fruit and vegetable juices	3.5
Tea and herbs for infusions (solid)	1.5
Animal and vegetable fats and oils	0.8
Tea (infusion, liquid)	0.8

Levels represent the range of mean middle bound (MB) values across the sub-categories. Different sub-categories were combined to present the powder and liquid categories of coffee (across coffee, instant coffees and coffee substitutes). Modified from EFSA (2017).

2009; Fromberg et al., 2014; Roberts et al., 2008), including baby foods freshly cooked at home (Bianchi et al., 2006; Lachenmeier et al., 2009). Home cooking using pressure-cooking devices did not generate measurable amounts of furan (Arisseto et al., 2013).

### Alkylated Furans

Due to their structural similarity with furan, possibly similar formation pathways and indications for co-occurrence, the alkylated furans 2- and 3-MeF and 2,5-dimethylfuran (2,5-diMeF) have been addressed concomitantly with furan (Becalski et al., 2010; Fromberg et al., 2014; Habibi et al., 2013; Palmers et al., 2015). Apart from a few specific examples, furan seems to be the predominant form in various canned and jarred foods. Most notably, concentrations of 2-MeF in coffee were consistently 4 to 5 times higher than those of furan. 2-MeF was detectable in most canned and jarred foods, including baby foods in jars, at levels lower than furan (Becalski et al., 2010; Palmers et al., 2015; Palmers et al., 2016); except for some canned tomato products, and canned meat and seafood products that contained significantly higher 2-MeF levels compared to furan. Specifically canned corn and tomato products seemed to contain higher levels of 3-MeF, otherwise levels of 3-MeF were rather low. In agreement with Becalski et al. (2010), Fromberg et al. (2014) reported 2-MeF levels lower than furan, but about 3 times higher levels of 2,5-diMeF in canned foods (no details available). Habibi et al. (2013) determined high levels of 2-MeF and 2,5-diMeF in infant cereals (low sample number) with levels of 2,5-DiMeF exceeding 2-MeF and furan. Overall, there is a lack of analytical data on alkylated furans, that preclude statistical analysis and a solid exposure assessment in humans.

### Exposure

Dietary furan exposure assessments are available from national surveys on dietary consumption, using deterministic methodology, as reported by international authorities (JECFA, 2011; EFSA, 2017). Exposure ranges determined in these surveys are summarised in Tables 3 and 4.

Infants are exposed to highest levels of furan, with mean exposures of  $0.99 \mu\text{g (kg bw)}^{-1} \text{ day}^{-1}$  (at the upper bound scenario) and high exposures (95th percentile) of  $1.82 \mu\text{g (kg bw)}^{-1} \text{ day}^{-1}$ . The next highest exposure populations are elderly and very elderly people with mean and 95th percentile exposures of  $0.75$  and  $1.27 \mu\text{g (kg bw)}^{-1} \text{ day}^{-1}$ , respectively.

Overall, mean adult exposures are generally below  $1 \mu\text{g (kg bw)}^{-1} \text{ day}^{-1}$ . Estimates from the US are slightly lower, because they were based on quantified analytical results generated on foods as consumed (JECFA, 2011). The 95th percentile exposure ranges generally remain below  $2 \mu\text{g (kg bw)}^{-1} \text{ day}^{-1}$ , including exposures in infants and children. The mean and high exposures determined by international and national authorities are overall very consistent and differences are likely attributed to different survey methodologies applied.

**Table 3** Mean and 95th percentile exposures in non-European countries by age groups

Country	Age group	Mean exposure [ $\mu\text{g (kg bw)}^{-1} \text{ day}^{-1}$ ]	p95 exposure [ $\mu\text{g (kg bw)}^{-1} \text{ day}^{-1}$ ]	References
USA (furan levels 2004)	2+ years	0.25		JECFA (2011)
	2–5 years	0.23		
	15–45 years	0.24		
USA (furan levels 2007)	2+ years	0.26	0.61	JECFA (2011)
	0–1 years	0.41	0.99	
Brazil	6–11 months	0.46–0.82	1.34–2.40 (p99)	Arisseto et al. (2010)
Chile	9 months	0.25		Mariotti et al. (2013)
	10–13 years	0.50–0.51		
	64 years	0.09–0.11		
China	Adults	0.093	1.767 (p90)	Sijia et al. (2014)
	6 months	0.333		
Korea	6 months	0.017		Kim et al. (2009)
Taiwan	6 months	0.470		Liu and Tsai (2010)

**Table 4** Mean and p95 upper bound (UB) exposures across European surveys by age groups (EFSA, 2017)

Age group	Mean exposure in $\mu\text{g (kg bw)}^{-1} \text{ day}^{-1}$ (range)	p95 exposure in $\mu\text{g (kg bw)}^{-1} \text{ day}^{-1}$ (range)
Infants	0.54 (0.21–0.99)	1.14 (0.42–1.82)
Toddlers	0.45 (0.31–0.65)	0.76 (0.46–1.08)
Other children	0.37 (0.27–0.52)	0.61 (0.40–0.86)
Adolescents	0.22 (0.14–0.31)	0.39 (0.28–0.58)
Adults	0.33 (0.14–0.54)	0.69 (0.25–1.22)
Elderly	0.36 (0.15–0.61)	0.70 (0.28–1.27)
Very elderly	0.34 (0.16–0.75)	0.60 (0.32–0.96)

According to EFSA (2017), the biggest contributor to dietary exposure in adults is coffee (40%–80%), other foods contribute less to exposure (above 5%, but less than 20%): cereal products, vegetable products, meat products and dairy. In older children (aged 4–6 years) the biggest contributors were breakfast cereals (40%), and in small children (>6 months) jarred baby foods were the most important contributor to dietary furan exposure.

The assessment of exposure to alkylated furans is currently based on limited data published in the literature. The inclusion of 2- and 3-MeF strongly affected total furan exposure, in particular in adults, elderly and very elderly, via coffee. Total furan exposure can more than triplicate compared to furan alone, depending on the survey. Infants and small children are much less affected due to the low level of coffee consumption.

Exposure assessment of furan (and alkylated furans) is hampered by a number of uncertainties, that will lead to either over- or underestimation of exposure:

- Most of occurrence data are generated on products as purchased from the shelf and not as prepared for consumption. Only for coffee the losses due to the extraction of powder to brew was taken into account (EFSA, 2017), but not for the possible losses of furan during standing of coffee after brewing and before consumption. The same accounts for the preparation of baby foods in jars (heating in open or closed jar, with or without stirring, etc.). These factors are strongly depending on consumer behaviour, are highly variable and, therefore, not quantifiable.
- Information on the consumption of fresh produce vs commercially processed (including canned and jarred) foods is not usually available in dietary consumption surveys. Assuming that, for instance, all vegetables are consumed from canned sources will lead to exposure overestimation (EFSA, 2017).
- Furan formation during cooking in the home may represent an additional source of exposure, such as toasting of bread at home, or cooking using home pressure cooking devices (Crews, 2009; Arisseto et al., 2013). This would potentially lead to underestimation. However, a few quantitative assessments led to the conclusion that this has a minor impact on furan exposure (EFSA, 2017).

## Toxicological Aspects

The primordial target organ of short and long term oral furan exposure conducted in numerous studies in rats and mice is the liver.

## Absorption, Distribution, Metabolism and Elimination

Furan is efficiently absorbed and metabolized after oral administration in rodents. Important routes of elimination are expired air (as unchanged furan or  $\text{CO}_2$ ) and the excretion of polar metabolites in urine, bile and faeces. Excretion is mostly complete after 24



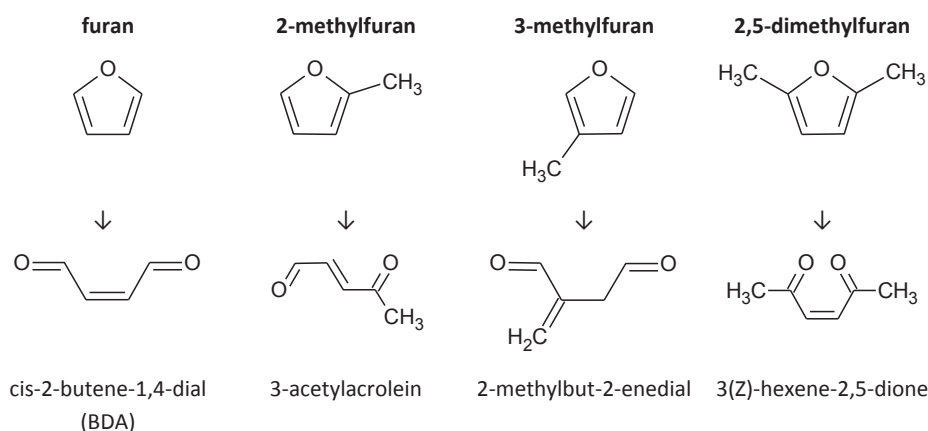
hours. After oral administration of radiolabelled furan, highest concentrations are found in the liver, the organ of extensive metabolism of furan (Burka et al., 1991). Macromolecular binding, mainly to protein, is found in the liver (Burka et al., 1991). A physiologically based pharmacokinetic model was developed from in vitro and inhalation studies that adequately modelled distribution and excretion in rats, including from oral exposure (Kedderis et al., 1993), and was extended to build species specific models for mice and humans (Kedderis and Held, 1996). These models demonstrated that the rate of furan metabolism was shown to be limited by hepatic blood flow, and not by its enzymatic bioactivation. Furan metabolism involves ring opening to the reactive intermediate *cis*-2-butene-1,4-dial (BDA), catalyzed by cytochrome P450 CYP2E1 in the liver (reviewed in Peterson, 2013; Moro et al., 2012). BDA can covalently react with macromolecules like tissue protein and DNA. BDA is metabolized further via glutathione (GSH) conjugation. Multiple GSH conjugates and their degradation products were described in urine, as well as protein and amino acid adducts possibly derived from degraded protein adducts and reaction products with cellular (poly)amines (Peterson et al., 2011; Hamberger et al., 2010).

Trapping experiments in vivo and in vitro, experiments with modulators of liver GSH conjugation and microsomal protein binding studies suggest that 2- and 3-MeF (Ravindranath et al., 1984, 1986; Ravindranath and Boyd, 1985) and 2,5-diMeF (Li et al., 2015; Wang et al., 2014 & 2015) follow similar bioactivation pathways as furan via ring-opened reactive intermediates. The resulting intermediates are shown in Fig. 2.

### Carcinogenicity

In chronic oral studies (2 year cancer bioassays), neoplastic lesions (hepatocellular adenoma and carcinoma) in both male and female mice increased in a dose dependent way, along with a number of non-neoplastic liver lesions including lesions of the biliary tract (NTP, 1993; Moser et al., 2009). Hepatocellular adenoma and carcinoma were similarly increased in rats dosed for 2 years orally with furan (NTP, 1993; Maronpot et al., 1991). In addition, dose related increases of neoplasms of the biliary tract and mononuclear cell leukemia were observed in this species. Markedly, incidences of cholangioadenoma and -carcinoma were very high at the lowest dose levels tested ( $2 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$ ), in both male and female rats. In stop-exposure studies, in which rats were treated orally with furan at  $30 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$  for limited time periods (6 weeks to 13 weeks), and then left for up to 2 years without further treatment, high incidences of cholangiocarcinoma or hepatic tumors were observed as well (Maronpot et al., 1991; Elmore and Sirica, 1993). Because of the high incidences of cholangiocarcinoma observed at the lowest dose of furan ( $2 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$ ) in the NTP cancer bioassay (NTP, 1993), another study was recently completed extending to much lower doses (0, 0.02, 0.044, 0.092, 0.2, 0.44, 0.92 and  $2 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$ ) (Von Tungeln et al., 2017), in order to resolve the dose response at low doses and to be able to perform dose response modeling (see below). Importantly, rats did not develop cholangiocarcinoma at any dose up to the highest dose of  $2 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$ . Liver tumors were not detected (found at higher doses in the previous study, and in mice). Importantly, the classification of hepatobiliary effects was done according to a new and standardized classification scheme for laboratory rodents (Thoolen et al., 2010). Following this scheme, biliary lesions identified were classified as cholangiofibrosis (at  $0.2 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$  and higher), exhibiting a non-linear dose response. Importantly, cholangiofibrosis and cholangioadenoma are considered non-neoplastic lesions (non-cancer, meaning a threshold effect), while only cholangiocarcinoma is defined as a neoplastic effect. In addition, re-classification of samples from the 1993 NTP study confirmed that cholangiocarcinoma was only observed at the highest dose ( $8 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$ ). At the lower doses, cholangiocarcinoma were re-classified as fibrosis or adenoma.

Methylfurans have not been tested in chronic oral studies in rodents. 2-MeF was tested in a 28 day study in rats, exhibiting liver toxicity and increased serum cholesterol and increased levels of thyroid hormones T3 (triiodothyronine) and T4 (thyroxine) at the dose of  $1.5 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$ . A NOAEL of  $0.4 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$  was identified (Gill et al., 2014). In a similar study with 3-MeF, liver toxicity was observed with significant histological changes of the liver at  $1.5 \text{ mg (kg$



**Figure 2** Furan, methylfurans and their ring-opened reactive intermediates. Modified from EFSA (2017).

$\text{bw}^{-1} \text{ day}^{-1}$  and a NOAEL of  $0.3 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$ . Increases in serum T3 and T4 were observed as well (Gill et al., 2015). In a 90 day study with gavage administration of 3-MeF (Gill et al., 2018), increased levels of serum markers of liver injury and histopathological changes in the liver were observed at  $0.25 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$ , a NOAEL was determined at  $0.075 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$ . At higher doses, other organs were affected (kidney, spleen). Benchmark dose modelling resulted in a BMDL<sub>10</sub> (the lower confidence level of the dose that results in an increase of cancer incidence by 10% above background) of  $0.08 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$  in male rats and  $0.05\text{--}0.17 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$  in female rats for increased incidence of liver lesions (Gill et al., 2018). Compared to an earlier, similar study on the parent compound furan (Gill et al., 2010), 3-MeF was more toxic at lower doses and to more organs. Overall, 2- and 3-MeF and furan show similar toxicities to the liver and to the kidney at higher doses after oral dosing, and to the lung (after i.p. dosing). These data also indicate that their toxic potencies are in the same order of magnitude (Gill et al., 2018).

### Genotoxicity and Mutagenicity

Furan was negative in most bacterial mutagenicity tests (Ames mutagenicity test in *Salmonella typhimurium*). Weakly positive results were obtained in strain TA100 on one instance and in TA98 in another instance in the presence of metabolic activation only (reviewed in EFSA, 2017). In most in vitro mammalian test systems furan tested positive, such as for mutagenicity in mouse lymphoma cells, chromosomal aberrations and sister chromatid exchanges in CHO (Chinese hamster ovary) and other cell lines (reviewed in EFSA, 2017). Negative results were obtained in the micronucleus test in lymphocytes. Negative results in bacterial and mammalian in vitro test systems were ascribed to the general deficiency of cell systems in CYP2E1 incapable for metabolic activation of furan to BDA, which is in agreement with positive results obtained directly with the metabolite BDA in bacterial mutation assays (Peterson et al., 2000) and mutations, single strand breaks and crosslinks in mammalian cells (Kellert et al., 2008; Marinari et al., 1984). Similar to furan, 2-MeF and 2,5-diMeF tested mostly negative in bacterial mutation assays, while in mammalian cells positive results were obtained for chromosomal aberrations, in vitro micronucleus and the comet assay (reviewed in EFSA, 2017).

In vivo genotoxicity tests in rats and mice treated short term by gavage gave mixed results. High doses were generally required to obtain genotoxic effects including chromosomal aberrations (oxidative) DNA damage, strand breaks and crosslinks, micronuclei, mutations and adducts (reviewed in EFSA, 2017; Ding et al., 2012; McDaniel et al., 2012).

In vivo test results are unavailable for 2- and 3-MeF. 2,5-diMeF was tested recently for genotoxicity in normal and transgenic mice expressing sulfotransferase. No convincing evidence for genotoxicity in the in vivo SCGE assay was found after single oral doses of 75, 150, or 300  $\text{mg (kg bw)}^{-1}$  (Hoie et al., 2015).

### DNA Adducts

Furan was found to covalently bind to proteins in vitro in liver microsomal extracts, and to tissue proteins in vivo after intragastric administration (Burka et al., 1991; Parmar and Burka, 1993). No DNA binding in vivo was observed in those earlier studies. BDA was found in cell-free systems to react with amino acids, proteins, DNA and individual 2-deoxynucleosides, being able to form crosslinks between amino acids (Peterson, 2006). The formation of crosslinks between amino acids in proteins, between protein and DNA and within DNA double strands was proposed as a mechanism of toxicity of furan (Byrns et al., 2004). Results from in vitro cellular mammalian tests (e.g. the alkaline comet assay in mammalian cells) did not unambiguously demonstrate the formation of DNA crosslinks from BDA (JECFA, 2011).

A more recent study (Neuwirth et al., 2012) examined incorporation of  $^{14}\text{C}$ -isotopically labeled furan into the DNA of liver and kidney of male F344 rats treated orally for 28 days with a low but carcinogenic dose ( $2 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$ ), and a sub-carcinogenic dose ( $0.1 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$ ). DNA isolated from kidney and liver was analyzed. Based on the  $^{14}\text{C}$  incorporation rate into liver DNA, average adduct levels of 1.7 and 33 adducts in  $10^8$  nucleotides were determined, respectively, using sensitive Accelerator Mass Spectrometry.  $^{14}\text{C}$  incorporation into the non-target tissue kidney was about 3 fold lower. Comparing the peak profile of digested liver DNA with reference DNA adducts generated by incubating single 2-deoxyribonucleosides with BDA, however, did not reveal any correspondence. The structures of the peak(s) containing radiolabeled DNA could not be identified. None of the adducts formed after incubation of BDA with individual deoxynucleosides dA, dG, dC or with salmon sperm DNA were identified in liver tissue after treatment of rats with either a single dose of  $0.92$  to  $9.2 \text{ mg (kg bw)}^{-1}$  or after repeated doses of  $4.4 \text{ mg (kg bw)}^{-1} \text{ day}^{-1}$  for 45 to 360 days (Churchwell et al., 2015). In none of the treatment groups levels of liver adducts exceeded the background levels (from vehicle treated animals) of about 1.2–2.4 adducts per  $10^8$  nucleotides, irrespective of dose and treatment duration. The authors concluded this as being consistent with general evidence for the absence of in vivo genotoxicity of furan. Still, uncertainty remains about the possible structurally unidentified adduct (Neuwirth et al., 2012) that might derive from a DNA–DNA or DNA–protein crosslink, which was also suggested from alkaline comet assay results in mouse liver and from results in fetal turkey liver (Cordelli et al., 2010; Jeffery et al., 2012).

### Epigenetic Changes

Apart from the controversial debate on genotoxicity, recent evidence points to persisting epigenetic changes induced by furan, recently reviewed by de Conti et al. (2017). These consist in global DNA demethylation, decreases in acetylation and methylation

of chromatin modifying genes and histones (Conti et al., 2014; de Conti et al., 2015; Tryndyak et al., 2017) and altered expression of microRNAs (Chen et al., 2012; de Conti et al., 2016; Dong et al., 2016) in liver of rats in response to furan treatment, compared to unaffected tissue or tissue from control rats. Recently, histone adducts were also identified with a glutathione-BDA conjugate at a pre-carcinogenic stage after low dose oral furan treatment (Nunes et al., 2016). Although the mechanistic interpretation of the causal implications of specific epigenetic changes is difficult, particularly with respect to the experimental settings of dose and duration of treatment, collectively such changes are thought to support the concept of a non-genotoxic mechanism of action (de Conti et al., 2017).

### Mode of Action

The liver is the primary target organ of furan toxicity. In rodents, furan induces hepatocellular adenoma and carcinoma, and cholangiofibrosis at low doses. Sufficient evidence indicates that the toxicity of furan is mediated through a reactive di-aldehyde intermediate, *cis*-2-butene-1,4-dial (BDA), generated via metabolic activation by the cytochrome P450 CYP 2E1 (Peterson, 2006). BDA is capable to covalently bind to tissue macromolecules (proteins, DNA) (Byrns et al., 2006), and is able to form multiple conjugates with glutathione (GSH) and cellular polyamines (Peterson et al., 2011). The major metabolites and degradation products found in urine (and partially in bile) are derived from GSH-conjugation and degradation of adducted proteins (Kellert et al., 2008; Lu et al., 2009; Lu and Peterson, 2010; Hamberger et al., 2010).

The present thinking is that oxidative stress with associated production of oxidative DNA damage (8-oxodeoxyguanosine) in liver and blood, gene expression alterations and epigenetic changes are likely involved in the mode of action along with an inflammatory and cell proliferative response (EFSA, 2017). In addition, epigenetic effects induced by furan, affecting global DNA methylation and methylation and acetylation of histones for instance, indicate indirect mechanisms of DNA damage and genotoxicity and are considered threshold effects.

### Dose Response Modelling

Based on the rodent toxicities detailed above, food safety authorities considered hepatocellular adenoma and carcinoma, and cholangiofibrosis as the pivotal and most sensitive endpoints for neoplastic and non-neoplastic effects, respectively. Dose response data from several 2-year cancer studies were found suitable for benchmark dose (BMD) modelling. Table 5 summarises the results of the BMD modelling studies, including references to the source data, the mathematical models applied and the resulting BMDL<sub>10</sub> values. These were further used as so-called 'points of departure' (PoD) by food safety authorities to assess the risk using the Margin of Exposure approach (ref to 'Risk Assessment' section).

### Risk Assessment

Since furan carcinogenicity was suspected to involve a genotoxic mechanism of action, and even in the most recent opinion such a mechanism could not finally be ruled out, no safe levels of exposure were derived and the Margin of Exposure (MOE) approach is applied. The MOE consists in calculating the ratio between actual estimated total human exposure and the point of departure (PoD), i.e. the reference dose for the pivotal toxicity endpoint derived from an animal study, usually expressed as the BMDL<sub>10</sub>. In the risk assessment of genotoxic carcinogens, a MOE of 10,000 or higher with the neoplastic endpoint would be considered of low priority for risk management. MOEs for furan with the BMDL<sub>10</sub> for liver cancer were found to be about 10'000 or below depending on the age group and dietary intake surveys. MOEs were below 10,000 in infants fed with baby foods in jars and in the elderly population mainly via intake of coffee. Taking into account additional exposure to methylfurans, the MOEs significantly decreased further in elderly populations. For non-genotoxic compounds, MOEs of more than 100 are usually considered of low health concern, and correspond to the uncertainty factors applied to derive safe exposure levels (Health Based Guidance Levels),

**Table 5** Results of benchmark dose modelling studies

Toxicity endpoint	Species & sex	Cancer bioassay	BMDL <sub>10</sub> [mg (kg bw) <sup>-1</sup> day <sup>-1</sup> ]	Model	References
<b>Neoplastic</b>					
Hepatocellular adenoma & carcinoma	Male rats	NTP (1993)	1.28	average	Carthew et al. (2010)
Hepatocellular adenoma & carcinoma	Female mice	Moser et al. (2009)	0.96	multistage	JECFA (2011)
Hepatocellular adenoma & carcinoma	Female mice	combined from NTP (1993) and Moser et al. (2009)	1.31	average	EFSA (2017)
<b>Non-neoplastic</b>					
Cholangiofibrosiss	Male rats	Von Tungeln et al. (2017)	0.11–0.12	different models	Von Tungeln et al. (2017)
Cholangiofibrosis	Male rats	Von Tungeln et al. (2017)	0.064	average	EFSA (2017)

entailing a factor of 10 for interspecies- and 10 for interindividual variability. Using the relevant non-neoplastic endpoint, cholangiofibrosis, results in MOEs around 100. Addition of methylfurans further decreased the MOEs to below 100, particularly in infants and elderly. Thus, no matter if addressing furan from the angle of genotoxicity or not, including combined exposure with methylfurans or not, margins of exposure are close to or below those considered of low concern in several population groups, including small infants and elderly people. Thus, food safety authorities have come to the conclusion that furan exposure in humans via food may present a health concern (JECFA, 2011; EFSA, 2017), and introduction of mitigation efforts in food are indicated.

## Summary

Furan is a volatile compound formed in food during heating processes including roasting and sterilisation. Human exposure to furan and its methylated derivatives occurs mainly via food, major contributors being canned and jarred foods and roasted foods such as coffee.

Furan and similarly its methylated derivatives are thought to be toxic and carcinogenic via reactive intermediates formed by the metabolism in the liver. The metabolites react with cellular molecules such as proteins, and glutathione, leading to oxidative stress and cell and tissue damage, mainly in the liver. At present the involvement of epigenetic effects is gaining attention. Inflammation and compensatory proliferation are thought to lead to fibrosis, and ultimately to hepatocarcinoma. At present, current evidence strongly points to a non-genotoxic mechanism of action in the carcinogenicity of furan. Cholangiofibrosis is a very sensitive endpoint in rats with a low BMDL<sub>10</sub>, resulting in low margins of exposure for dietary intake in humans. In conclusion, no matter if the human risk is interpreted from the perspective of a genotoxic effect, or if the non-cancer endpoint of cholangiofibrosis is considered, margins of exposure with estimated human dietary intakes are considered low. Authorities have concluded this as an indication for a health concern and mitigation strategies are advised and under development.

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## Processing Contaminants: Furfuryl Alcohol

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### Glossary

**Exposure** Exposure for food risk assessment is typically assessed as daily intake or population-based intake for average and worst-case scenarios.

**Risk** Likelihood or probability that a specific hazardous exposure or dose will produce a toxic effect.

**Risk assessment** The process by which the potential adverse effects arising from exposure are characterized.

### Nomenclature

ADI Acceptable daily intake

EC European Commission

EPA US Environmental Protection Agency

FEMA Flavour and Extract Manufacturers Association

FID Flame ionization detection

GC Gas chromatography

GRAS Generally recognized as safe

HPLC High performance liquid chromatography

IARC International Agency for Research on Cancer

JECFA Joint FAO/WHO Expert Committee on Food Additives

MOE Margin of exposure

MS Mass spectrometry

MS/MS Tandem mass spectrometry

NTP National Toxicology Program

WHO World Health Organization

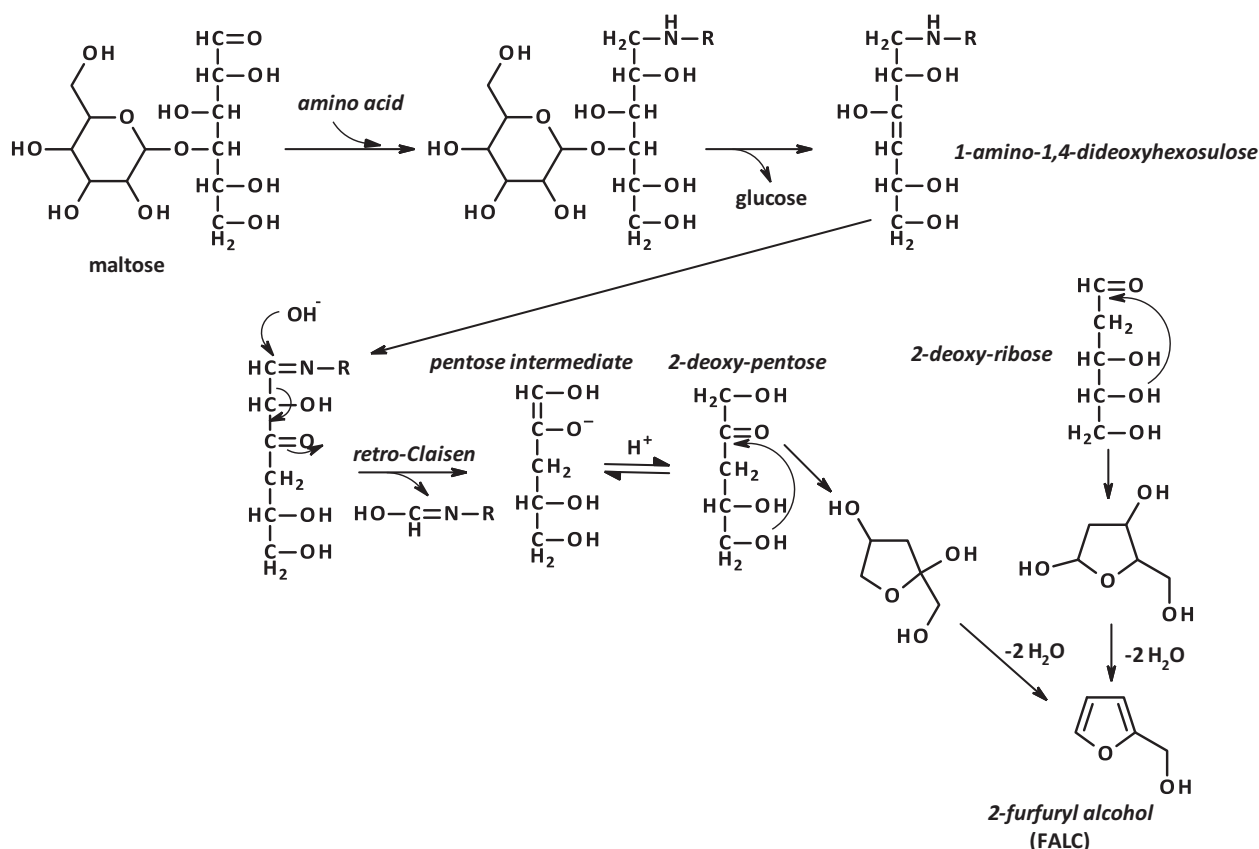
### Overview

Furfuryl alcohol (C<sub>5</sub>H<sub>6</sub>O<sub>2</sub>) (CAS# 98-00-0), also known as 2-furanmethanol, 2-furylcarbinol, 2-furancarbinol,  $\alpha$ -furylcarbinol, furfuralcohol and 2-hydroxymethylfuran, is a colorless to slightly yellowish liquid with a characteristic fainting odor. Selected physical and chemical properties of furfuryl alcohol are described in Ullmann's Encyclopedia of Industrial Chemistry (Hoydonckx et al., 2007). Furfuryl alcohol is a high production-volume chemical with industrial applications, including production of furan resins and wetting agents, and as a solvent (Grosse et al., 2017).

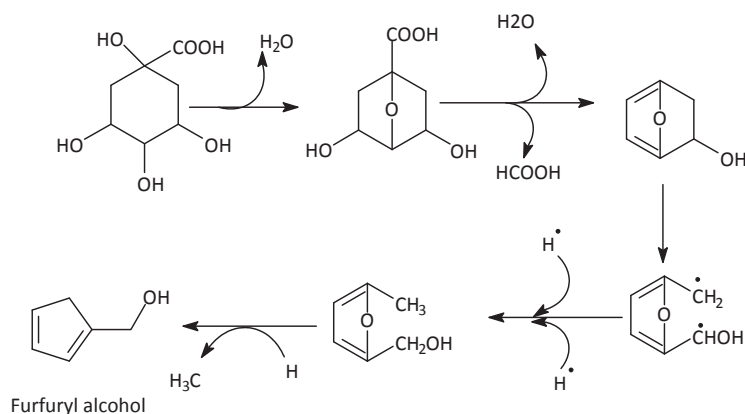
Mechanistically, furfuryl alcohol is a reduction product of furfural, a product obtained from the pentoses of corncobs, roasted coffee meal and sugarcane bagasse (Gandini, 2010). Furfural can be converted to furfuryl alcohol via a number of microbial and chemical pathways. Microbiologically, it is produced following the action of yeast species such as *Saccharomyces cerevisiae*, *Pichia stipitis* (Diaz De Villegas et al., 1992) and *Neospora tetrasperma* (Eilers and Sussman, 1970) on furfural. Fig. 1 illustrates this natural generation of furfuryl alcohol from maltose and 2-deoxyribose while Fig. 2 shows the thermal degradation of quinic acid to furfuryl alcohol. Industrially, the hydrogenation of furfural using catalysts such as palladium (Gandini, 2010), nickel, CuCrO (Jacobson et al., 1958; Taylor et al., 2017) and other metal alloys produces furfuryl alcohol (Huang et al., 2007). Also on a laboratory scale, furfuryl alcohol can be synthesized from furfural via the Cannizzaro reaction, which involves disproportionation of an aldehyde to an alcohol in basic conditions (Swain et al., 1979).

### Uses

Furfuryl alcohol is an important industrial chemical for the polymer industry that is used to produce synthetic fibres, rubbers, resins, e.g., dark thermostatic resins resistant to acids, bases and resins used for strengthening ceramics and wood (Gandini, 2010; Huang et al., 2007). The compound may serve as a solvent for furan resin, pigment, varnish, as rocket fuel and fuel additive (Vorotnikov et al., 2012). Furfuryl alcohol may be also used as a food additive and a mechanical reinforcement for highly porous polymeric matrices (Cui et al., 2016; Gandini, 2010). Furfuryl alcohol is used industrially as a wetting agent, a gel retarder, a liquid propellant,



**Figure 1** Degradation of reducing sugars to generate furfuryl alcohol. Reprinted with permission from Vanderhaegen et al. (2004b). Copyright 2004 American Chemical Society.



**Figure 2** Heat degradation of quinic acid to yield furfuryl alcohol in coffee. Adapted with permission from Moon and Shibamoto (2010). Copyright 2010 American Chemical Society.

a flavouring agent and in foundry cores (Cui et al., 2016). The EU regulation (EC) No 872/2012 lists furfuryl alcohol as a flavouring substance with no limit or restrictions on its use (European Commission (EC), 2012).

## Occurrence and Exposure

Furfuryl alcohol occurs as a natural constituent in a wide variety of foods and food components in varying concentrations and also widely as a food additive. According to a recent review (Okaru and Lachenmeier, 2017), furfuryl alcohol was found in various foods and beverages including coffee beans (>100 mg kg<sup>-1</sup>), fish products (≈ 10 mg kg<sup>-1</sup>), wines (1–10 mg L<sup>-1</sup>) and pineapple juice (8 mg L<sup>-1</sup>) (Okaru and Lachenmeier, 2017). Table 1 summarizes the occurrence of furfuryl alcohol in some foods and beverages.

**Table 1** Furfuryl alcohol content in various foods and beverages

Category [reference]	N	Furfuryl alcohol concentration							Units <sup>a</sup>
		Mean	Median	P90	P95	P97.5	P99	Maximum	
Roasted coffee (Okaru and Lachenmeier, 2017)	30	251	243	342	392	402	406	408	mg kg <sup>-1</sup>
Bread (Okaru and Lachenmeier, 2017)	15	<LOD <sup>b</sup>	—	—	—	—	—	—	mg kg <sup>-1</sup>
Wine (Okaru and Lachenmeier, 2017)	20	<LOD <sup>b</sup>	—	—	—	—	—	—	mg L <sup>-1</sup>
Spirits (Okaru and Lachenmeier, 2017)	50	<LOD <sup>b</sup>	—	—	—	—	—	—	mg L <sup>-1</sup>
Sweet potatoes (Wang and Kays, 2000)	1	0.014	—	—	—	—	—	—	mg kg <sup>-1</sup>
Wine (Spillman et al., 1998)	8	3.4	2.9	7.3	8.5	9.0	9.4	9.6	mg L <sup>-1</sup>
Baked goods (National Research Council (US), 1965) <sup>d</sup>	—	110	—	—	—	—	—	—	ppm
Spirits (National Research Council (US), 1965) <sup>d</sup>	—	10	—	—	—	—	—	—	ppm
Candy (National Research Council (US), 1965) <sup>d</sup>	—	59	—	—	—	—	—	—	ppm
Ice cream/ices (National Research Council (US), 1965) <sup>d</sup>	—	88	—	—	—	—	—	—	ppm
Beverages (National Research Council (US), 1965) <sup>d</sup>	—	19	—	—	—	—	—	—	ppm
Honey (Vázquez et al., 2007)	1	1.6	—	—	—	—	—	—	mg kg <sup>-1</sup>
Popcorns (Park and Maga, 2006)	6	0.064	0.067	0.081	0.081	0.082	0.082	0.082	mg kg <sup>-1</sup>
Fried fish (Park and Maga, 2006)	1	10.5	—	—	—	—	—	—	mg kg <sup>-1</sup>
Breaded fish products (Pérez-Palacios et al., 2013)	4	10.3	8.8	16	18	18	19	19	mg kg <sup>-1</sup>
Wine (Carrillo et al., 2006)	6	1.51	0.89	1.57	1.60	1.62	1.63	1.64	mg L <sup>-1</sup>
Vinegar (Morales et al., 2004) <sup>c</sup>	27	0.35	0.28	0.58	0.59	0.59	0.59	0.59	mg L <sup>-1</sup>
Vinegar (Tesfaye et al., 2004) <sup>c</sup>	9	0.34	0.28	0.58	0.59	0.59	0.59	0.59	mg L <sup>-1</sup>
Coffee (Petisca, 2013)	7	49	49	64	67	68	69	70	mg kg <sup>-1</sup>
Instant coffee (Golubkova, 2011)	1	267	—	—	—	—	—	—	mg kg <sup>-1</sup>
Roasted coffee (Golubkova, 2011)	1	564	—	—	—	—	—	—	mg kg <sup>-1</sup>
Pineapple juice (Golubkova, 2011)	1	8.3	—	—	—	—	—	—	mg L <sup>-1</sup>
Rice cakes (Moon and Shibamoto, 2009)	2	2, 2.3	—	—	—	—	—	2.3	mg kg <sup>-1</sup>
Bread (Jensen et al., 2011)	1	187	—	—	—	—	—	—	mg kg <sup>-1</sup>
Toasted almonds (Vázquez-Araújo et al., 2008)	3	6.4	6.0	8.3	8.6	8.7	8.8	8.9	mg kg <sup>-1</sup>
Non-fat dried milk (Karagül-Yüceer et al., 2002)	1	15	—	—	—	—	—	—	mg kg <sup>-1</sup>
Corn tortilla chips (Buttery and Ling, 1998)	1	0.54	—	—	—	—	—	—	mg kg <sup>-1</sup>
Cocoa powder (Bonvehí, 2005)	1	0.02	—	—	—	—	—	—	mg kg <sup>-1</sup>
Palm sugar (Ho et al., 2007)	1	0.14, 0.52	—	—	—	—	—	—	mg kg <sup>-1</sup>

<sup>a</sup>The ambiguous unit ppm was interpreted as mg L<sup>-1</sup> for liquids/beverages and as mg kg<sup>-1</sup> for solid foods.

<sup>b</sup>All samples evaluated (spirits types whiskey, rum, brandy as well as various wines and breads) were below the limit of detection (LOD; 3.2 mg L<sup>-1</sup>).

<sup>c</sup>Studies from the same research group with probably overlapping data.

<sup>d</sup>Number of samples not provided. The data are suggested as being “usual concentrations” found in these food/beverage types.

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Furfuryl alcohol has been reported to occur naturally in coffee, roasted almonds, cooked apples, apple juice, canned beef, in cognac oil and also it is used to flavour butter, butterscotch, caramel, fruits, bread and brandy among other foods (Burdock, 2004). It has also been found in beverages (19 ppm), ice cream (88 ppm), candy (59 ppm), baked goods (110 ppm) and in spirits (10 ppm) (National Research Council (US), 1965; Okaru and Lachenmeier, 2017). Furfuryl alcohol also contributes to the flavour of foods and beverages such as odor of French fries (Wagner and Grosch, 1998), other deep-fried products such as breaded fish products (IARC, in press) and roasted beef (Cerny and Grosch, 1992).

The occurrence of furfuryl alcohol has also been reported in rice cakes as an odor component (Buttery et al., 1999), non-dry fat milk (Karagül-Yüceer et al., 2002) and also in wines aged in barrels (De Simón et al., 2003) and also in *Boletopsis lucomelas*, a mushroom (Nosaka and Miyazawa, 2014) and also in cooked pine mushrooms and is thought to be formed from the various thermal reactions during cooking and hence could be used as marker for grading cooked pine mushrooms (Cho et al., 2006). Furfuryl alcohol has also been identified as one of the major volatile compounds in the kiwifruit (*Actinidia eriantha*) at concentration of up to 162 µg kg<sup>-1</sup> (García et al., 2012). Lactic acid bacterium affects the production of furfuryl alcohol in soy sauce (Harada et al., 2016). Furfuryl alcohol along with other furanic compounds occurs in smoked-cured bacon (Yu et al., 2008) and paste fish (Gili et al., 2010).

Furfuryl alcohol may also be used as one of the marker compounds to differentiate between *arabica* and *robusta* coffee (Hron et al., 2009). Similarly, the occurrence of furfuryl alcohol in wines is an indicator of microbial guided reduction of furfural during storage in barrels. However, degradation of furfural to furfuryl alcohol may also occur in the absence of bioflora (Pérez-Prieto et al., 2003). Furfuryl ethyl ether, an ageing staling flavor compound in beer is an indicator of the presence of furfuryl alcohol (Vanderhaegen et al., 2004a) which is thought to originate from the Maillard reaction that occurs during wort boiling, malt kilning or as a result of yeast reduction of furfural. However furfural has been shown to inhibit yeast metabolism (Vanderhaegen et al., 2004b).

Apart from diet, exposure to furfuryl alcohol can also be from occupational sources (Ribeiro and Filho, 2006). Furfuryl alcohol in combination with other chemicals such as formaldehyde has been shown to affect lung function in workers handling furan resins

(Ahman et al., 1991). Also it has been detected in gas formed from furan binding resins at a mean concentration of 4.8 ppm with 22% of the determinations exceeding the 5 ppm threshold limit value (TLV) (Virtamo et al., 1976). For review on occupational exposure, see (IARC, in press).

### Analytical Methods

Furfuryl alcohol has been analyzed in various matrices using gas chromatography with flame ionization detection (GC-FID) including in workspaces following active trapping onto suitable adsorbent material and subsequent thermal desorption (International Standard Organization (ISO), 2000; Tschickardt, 2002) and in kiwifruit (Garcia et al., 2012). Also gas chromatography-UV spectrometry (GC-UV), has been used for the determination of volatile organic compounds in settles dust in homes (Nilsson et al., 2005). Gas chromatography-mass spectrometry (GC-MS) has been used to determine furfuryl alcohol in mushrooms (Nosaka and Miyazawa, 2014) and environmental water (Kawata et al., 2001). A headspace solid phase microextraction coupled to gas chromatography-mass spectrometry method for the furanic compounds including furfuryl alcohol was developed and validated for the analysis of deep-fried fish products (Pérez-Palacios et al., 2013, 2012) while high performance liquid chromatography (HPLC) has been used to separate furfuryl alcohol together with other furanic derivatives in transformer/rectifier oil (Lin et al., 2016). Aroma compounds including furfuryl alcohol in Shanxi aged vinegar have been characterized and quantitated using authentic standards by GC-MS/MS operated on selected reaction monitoring mode (Liang et al., 2016). Furfuryl alcohol in coffee and other matrices can also be analyzed using nuclear magnetic resonance (NMR) spectroscopy (Okaru and Lachenmeier, 2017).

### Toxicology

Furfuryl alcohol is well absorbed across biological membranes in humans and animals (Grosse et al., 2017). Furoic acid is the main phase I metabolite of furfuryl alcohol. Furfuryl alcohol is first metabolized by oxidation to furfural before being converted to furoic acid, which is then decarboxylated to CO<sub>2</sub>. The acid may be excreted unchanged or conjugated with glycine (major metabolite) and/or condensed with acetic acid (Nomeir et al., 1992).

Despite the contribution of furfuryl alcohol to the aroma of foods and beverages, some studies have revealed the potential toxicity in animals and humans (Sujatha, 2008; Arts et al., 2004; Goldsworthy et al., 2001; Wilson et al., 1992). The main reported adverse effects of furfuryl alcohol are mainly on the epithelium of the olfactory and respiratory systems. However, pure furfuryl alcohol is harmful when inhaled, if swallowed, and in contact with skin. Currently, furfuryl alcohol does not appear to have genotoxic potential (*in vivo*) with evidence of carcinogenic activity of furfuryl alcohol in rats being thresholded at low doses. In a 2-year inhalation studies, male mice exposed to the highest tested concentration had a significantly increased incidence of renal tubular degeneration and a significantly increased combined incidence of renal tubular adenomas and carcinomas. In a single study on F344 rats and B6C3F1 mice exposed to furfuryl alcohol vapor for 6 hours per day, 5 days per week for 14 days (0, 16, 31, 63, 125, 250 ppm) or 13 weeks (0, 2, 4, 8, 16, 32 ppm) inflammation and/or metaplasia of respiratory epithelium was observed for the 14 day study (Irwin et al., 1997).

The postulated mechanism of carcinogenicity of furfuryl alcohol using reverse mutation assays with *Salmonella typhimurium* TA100-derived strains is by the formation of a 2-sulfo-oxymethylfuran, an electrophile reacting with DNA to form nucleoside adducts of 2'-deoxyadenosine and 2'-deoxyguanosine. Another study indicated that furfuryl alcohol may induce sister chromatid exchange in human lymphocytes *in vitro* and *in vivo* (World Health Organization (WHO), 2001).

According to WHO IARC, the evidence that furfuryl alcohol is metabolically activated via sulphate conjugation to electrophilic 2-sulphoxymethylfuran is judged as "strong". Furfuryl alcohol-specific DNA adducts were found in non-tumour tissue of patients with lung cancer, in mice, and in bacteria expressing human sulfotransferase (Grosse et al., 2017). Based on "sufficient evidence of carcinogenicity in experimental animals" and no data in human beings, the WHO IARC has classified furfuryl alcohol as "possibly carcinogenic to humans" (Group 2B) (Grosse et al., 2017; IARC, in press).

### Risk Assessment and Mitigation

The FEMA reported the typical use levels of furfuryl alcohol (ppm) as a flavouring substance in the following foods/beverages as follows: non-alcoholic beverages (19 ppm), ice cream, ices (88 ppm), candy (59 ppm), baked goods (110 ppm) and alcoholic beverages (10 ppm) (Adams et al., 2011). JECFA sets an acceptable daily intake (ADI) of 0–5 mg kg<sup>-1</sup> body weight and considers it to be of no safety concern when used within the levels permissible for use as a food flavor (Burdock, 2004). According to WHO IARC (IARC, in press), the human intake estimates are well below 0.15 mg kg bw<sup>-1</sup> day from food additives. Consuming one cup of espresso coffee may lead to an intake of 0.03 mg kg bw<sup>-1</sup>. While more detailed exposure assessments are lacking, the current evidence suggests furfuryl alcohol as being a low risk if any.

Nevertheless, since the cooking and handling conditions have a great influence on the residual levels of furanic compounds in heated foods, efforts should be made reduce furanic compounds without affecting the content of volatile compounds that contribute to the aroma and flavor of fried foods (Pérez-Palacios et al., 2013). For instance the addition of sulfite in beers (Vanderhaegen et al., 2004b) and dimethyldicarbonate (Spillman et al., 1998) may inhibit Maillard browning by nucleophilic

reaction with the double bond of Maillard intermediates, such as 3,4-dideoxyhexosulos-3-ene (3-DDH) (Vanderhaegen et al., 2004b).

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# Heterocyclic Aromatic Amines: An Update on the Science

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## Glossary

**Biomarkers** are used for exposure assessment. Some examples of biomarkers are chemicals or their metabolites in urine, plasma, or reactive intermediates covalently bound to DNA- and protein as adducts. These endpoints represent biomarkers of exposure.

**DNA adduct** is a chemical covalently bound to DNA that can lead to a mutation during cell division and initiate the cell to a cancerous state.

**Epigenetics** is the study of biological mechanisms that switch genes on and off. One common mechanism is through the methylation of cytosine to form 5-methylcytosine, resulting in changes in gene expression. In mammals, DNA methylation occurs almost exclusively in CpG dinucleotides. In diseases, such as cancer, CpG islands in the gene promoter regions undergo aberrant hypermethylation, resulting in transcriptional gene silencing.

**Genetic polymorphisms** in carcinogen-metabolizing enzymes are variants in genes encoding for phase I enzymes, such as cytochrome P450 oxidases, or phase II conjugation enzymes, such as glutathione S-transferases. The genetic alterations include single-nucleotide polymorphisms, the insertion or deletions of nucleotides, or changes in gene copy numbers, which can cause an increase, decrease, or abolish the pathways of metabolism and impact the biological effects of carcinogens.

**Signal transduction** is a process by which a chemical or physical signal is transmitted through a cell as a series of molecular events, often by protein phosphorylation catalyzed by protein kinases, which results in a cellular response, such as downstream changes in protein expression and activity.

**The Maillard reaction** is named after the French chemist Louis-Camille Maillard, who initially described the reaction between amino acids and sugars in 1912. The reaction represents a collection of a complex group of chemical processes that can occur between amino acids and reducing sugars that give cooked meats and other browned foods their distinctive flavor and aroma.

**Tumor promotion** is the multistage process of cancer development providing the conditions for a single initiated cell to survive and multiply, and undergo clonal growth.

## Nomenclature

### Heterocyclic aromatic amines

**IQx** 2-amino-3-methylimidazo[4,5-*f*]quinoxaline  
**MeIQx** 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline  
**4,8-DiMeIQx** 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline  
**7,8-DiMeIQx** 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline  
**Trp-P-1** 2-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole  
**Trp-P-2** 2-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole  
**Glu-P-2** 2-aminodiprido[1,2-*a*:3',2'-*d*]imidazole  
**Glu-P-1** 2-amino-6-methyldiprido[1,2-*a*:3',2'-*d*]imidazole  
**MeIQ** 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline  
**IQ** 2-amino-3-methylimidazo[4,5-*f*]quinoline  
**PhIP** 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine  
**MeAαC** 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole  
**AαC** 2-amino-9*H*-pyrido[2,3-*b*]indole  
**dA** 2'-deoxyadenosine  
**dG** 2'-deoxyguanosine

### DNA Adducts

**dG-C8-MeIQx** *N*-(2'-deoxyguanosin-8-yl)-MeIQx  
**dG-N<sup>2</sup>-MeIQx** 5-(2'-deoxyguanosin-N<sup>2</sup>-yl)-MeIQx  
**dA-N<sup>6</sup>-MeIQx** 5-(2'-deoxyadenosin-N<sup>6</sup>-yl)-MeIQx  
**dG-C8-4,8-DiMeIQx** *N*-(2'-deoxyguanosin-8-yl)-4,8-DiMeIQx  
**dG-C8-IQ** *N*-(2'-deoxyguanosin-8-yl)-IQ  
**dG-C8-MeIQ** *N*-(2'-deoxyguanosin-8-yl)-MeIQ  
**dG-N<sup>2</sup>-IQ** 5-(2'-deoxyguanosin-N<sup>2</sup>-yl)-IQ

dA-*N*<sup>6</sup>-IQ 5-(2'-deoxyadenosin-*N*<sup>6</sup>-yl)-IQ  
 dG-C8-A $\alpha$ C *N*-(2'-deoxyguanosin-8-yl)-A $\alpha$ C  
 dG-C8-MeA $\alpha$ C *N*-(2'-deoxyguanosin-8-yl)-MeA $\alpha$ C  
 dG-C8-PhIP *N*-(2'-deoxyguanosin-8-yl)-PhIP

#### Other Abbreviations

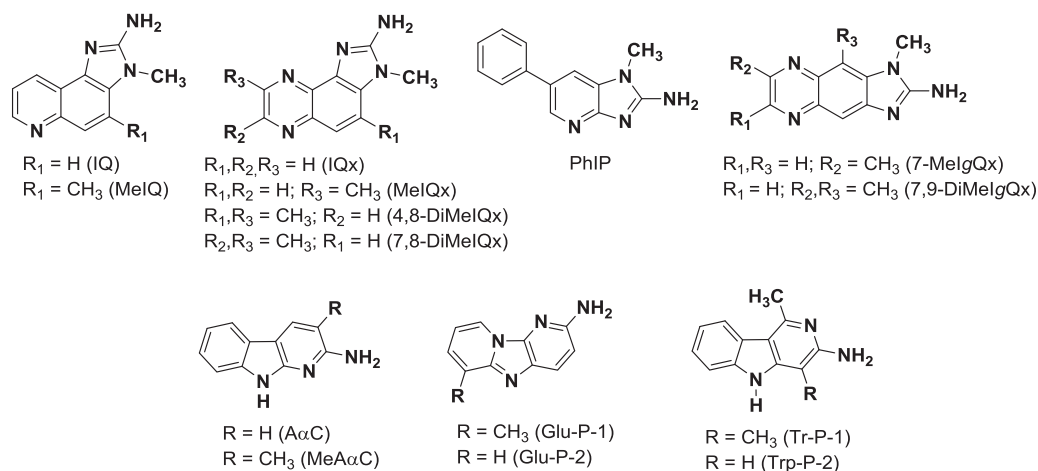
HAA heterocyclic aromatic amines  
 NAT N-acetyltransferase  
 ppb parts per billion  
 SULT sulfotransferase  
 UGT uridine 5'-diphospho-glucuronosyltransferase  
 ER- $\alpha$  estrogen receptor alpha  
 PR progesterone receptor  
 AR androgen receptor  
 MAPK mitogen-activated protein kinase  
 ERK extracellular signal regulated kinase  
 EGFR epidermal growth factor receptor

## Overview

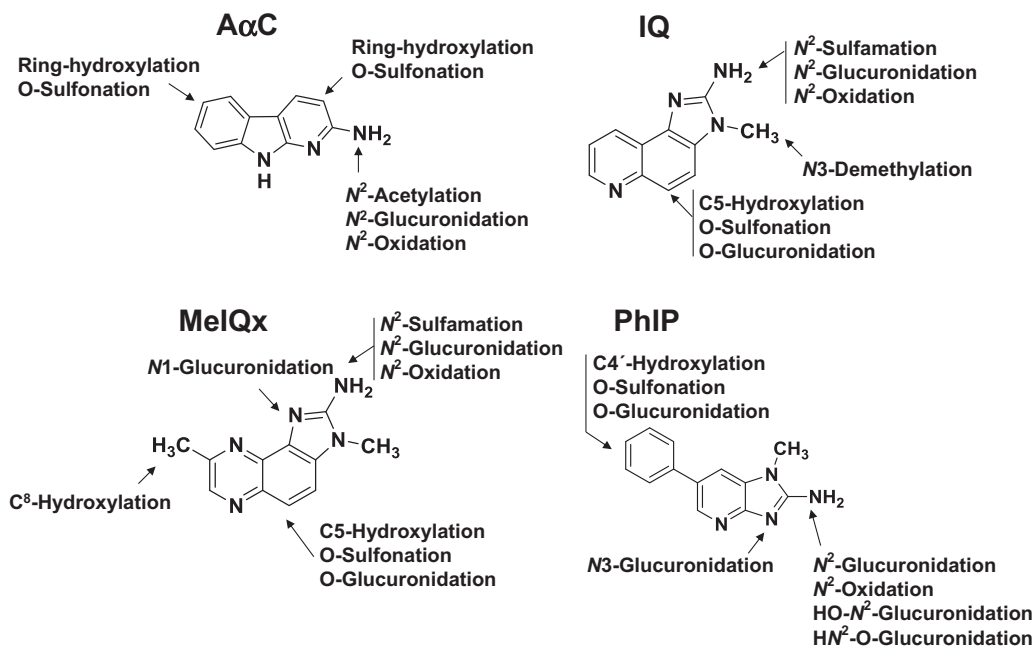
Heterocyclic aromatic amines (HAAs) are formed in well-done grilled meats, poultry, and fish, and several HAAs arise during the combustion of tobacco smoke or emitted in diesel exhaust (Sugimura et al., 2004; Felton et al., 2000). The concentrations of HAAs generally occur in the parts per billion level (ppb) in cooked meats but in some instances the levels can vary by over a 100-fold range. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methylimidazo[4,5-*b*]pyridine (PhIP) are two of the most abundant HAAs formed in grilled ground beef and poultry prepared under common household cooking practices, while 2-amino-9*H*-pyrido[2,3-*b*]indole (A $\alpha$ C) is the most abundant HAA present in tobacco smoke (Manabe et al., 1991; Zhang et al., 2011). The chemical structures of several prevalent HAAs are presented in Fig. 1.

The Maillard reaction is involved in the formation of the amino-imidazo-containing HAAs, where the precursors of HAA formation originate from creatine, amino acids, and hexoses present in the meat (Jagerstad et al., 1991; Murkovic, 2004). The formation of amino-imidazoquinolines and amino-imidazoquinoxalines occurs by reaction of 2-methyl-pyridine or 2,5-dimethyl-pyrazine with acetaldehyde and creatinine (Milic et al., 1993). Other HAAs arise by the high-temperature (>300 °C) heating of amino acids, such as glutamic acid, tryptophan, phenylalanine, or proteins (Matsumoto et al., 1981; Murkovic, 2004).

HAAs are mainly metabolized by hepatic P450 1A2 in rodents and humans, and by P450s 1A1 and 1B1 in extrahepatic tissues (Turesky and Le Marchand, 2011). Cytochrome P450 (P450) enzymes oxidize the heteroaromatic rings of HAAs to form



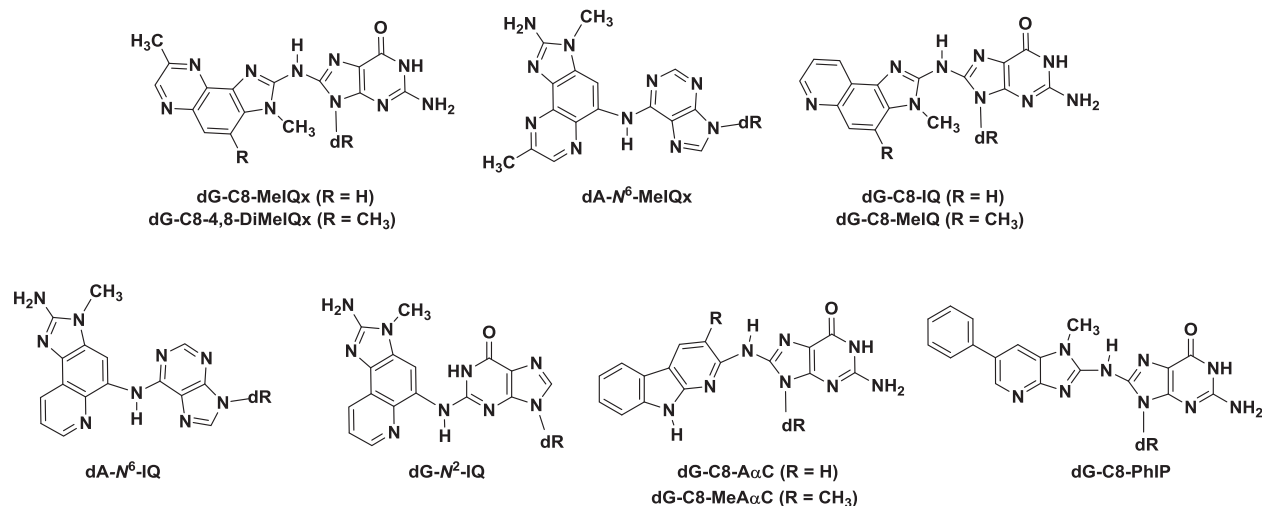
**Figure 1** Chemical structures of representative HAAs formed in cooked meats and tobacco smoke condensate. The top row depicts amino-imidazopyridine, amino-imidazoquinolines and amino-imidazoquinoxalines, and the bottom row represents several HAAs derived from the pyrolysis of amino acids.



**Figure 2** Major pathways of metabolism of several prototypical HAAs by P450s and phase II conjugation enzymes.

detoxicated metabolites, whereas N-oxidation of the exocyclic amine group produces the genotoxic N-hydroxylated-HAA metabolites (Fig. 2) (Turesky and Le Marchand, 2011). These metabolites can undergo conjugation reactions by phase II enzymes such as N-acetyltransferases (NATs) or sulfotransferases (SULTs) (Hein, 2000; Chevereau et al., 2017), to form unstable intermediates that react with DNA to form adducts; some of these adducts may induce mutations during cell division (Fig. 3). The principal HAA-DNA adducts arise from reaction between the C-8 atom of deoxyguanosine (dG) and the electrophilic N-oxidized exocyclic amino groups of the HAAs, to produce dG-C8-HAA adducts. The reactive intermediates of IQ and MeIQx also form adducts between the  $N^2$  group of dG and the C-5 atom of IQ and MeIQx, and minor adduct formation occurs at the  $N^6$  atom of deoxyadenosine (dA) and the C-5 atom of IQ and MeIQx (Fig. 3) (Jamin et al., 2007; Bessette et al., 2009). These adducts are believed to be responsible for the mutagenic effects of HAAs. There are notable interspecies differences in catalytic activity and regioselectivity of the oxidation of HAAs by P450s; such differences can influence the DNA-binding potential and toxicological properties of these genotoxins (Turesky et al., 1999, 2001). Moreover, components in the diet can act as chemopreventive agents and alter the metabolism of HAAs, and thus diminish their biological effects (Higdon et al., 2007).

Some risk assessment studies suggest that HAAs may not pose an important health risk because daily exposure levels to HAAs are 100,000 to  $1 \times 10^6$  of the daily dose required to induce tumors in rodent models (Layton et al., 1995; Lutz and Schlatter, 1992; Gaylor and Kadlubar, 1991). However, humans are exposed to more than 20 HAAs, and interspecies differences in metabolism



**Figure 3** DNA adducts of representative HAAs formed with dG and dA. dR: deoxyribose

and bioactivation of HAAs, and tumor promoting factors may increase the carcinogenic potential of HAAs (Sugimura, 1992; Sugimura et al., 2004; Turesky and Le Marchand, 2011). Some epidemiological studies have observed an association between frequent consumption of cooked red meats containing HAAs and other carcinogens and an increased risk for the development of colorectal cancer (Norat et al., 2005). The working group of the International Agency of Research on Cancer recently concluded that red meat is 'probably carcinogenic to humans' (Group 2A carcinogen) and a risk factor for colorectal cancer (Bouvard et al., 2015). The frequent consumption of red meat was also positively associated with pancreatic and with prostate cancer. PhIP, in particular, has been the focus of much research because it is the most mass-abundant carcinogenic HAA formed in well-done cooked meats and poultry (Sugimura et al., 2004; Felton et al., 2000), and the only HAA reported to induce tumors in the colorectum, pancreas, and prostate of rodents (Shirai et al., 2002; Sugimura et al., 2004). Recent studies have shown that PhIP also exerts epigenetic effects *in vitro* and changes in cellular events that impact cellular growth and tumor promoting properties at dose concentrations approaching human exposures (Laubert et al., 2004; Chen et al., 2017). Thus, further research is required to understand the role of HAAs in the etiology of dietary associated cancers.

### Biological Activity: Genotoxicity and Cancer

The genotoxicity of HAAs varies by over a >1000-fold in bacterial mutagenicity assays (Sugimura et al., 2004); however, these large differences in ranges of potency are generally not observed in mammalian cell assays or in long-term carcinogen bioassays (Sugimura et al., 2004; Glatt, 2006). These discrepancies in biological effects are attributed to differing metabolic enzyme activation systems, differing DNA adduct repair capacities, differing gene locus endpoints for mutagenicity, and different base sequence contexts and neighboring base effects on the HAA-DNA lesions, all of which affect mutation frequencies (Turesky, 2002). HAAs induce tumors in rodents at multiple organs that include: the oral cavity, liver, stomach, lung, colorectum, pancreas, prostate and mammary glands, during long-term feeding studies (Sugimura et al., 2004). Despite the large differences in genotoxic potencies of HAAs in different cell assays, the carcinogenic potency of many HAAs in experimental animals is within approximately 10-fold, depending upon the species or organs affected. 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ) is also a powerful liver carcinogen in non-human primates, and one of the most powerful carcinogens assayed in cynomolgous monkeys (Adamson et al., 1990). Recent studies have reported a rapid induction of colon and prostate-induced carcinogenesis in P450 1A-humanized mice compared to wild-type mice following treatment with PhIP (Cheung et al., 2011; Li et al., 2012), reinforcing the notion that human P450 enzymes more efficiently bioactivate HAAs to reactive intermediates than the rodent counterparts (Turesky et al., 1999; Turesky and Le Marchand, 2011).

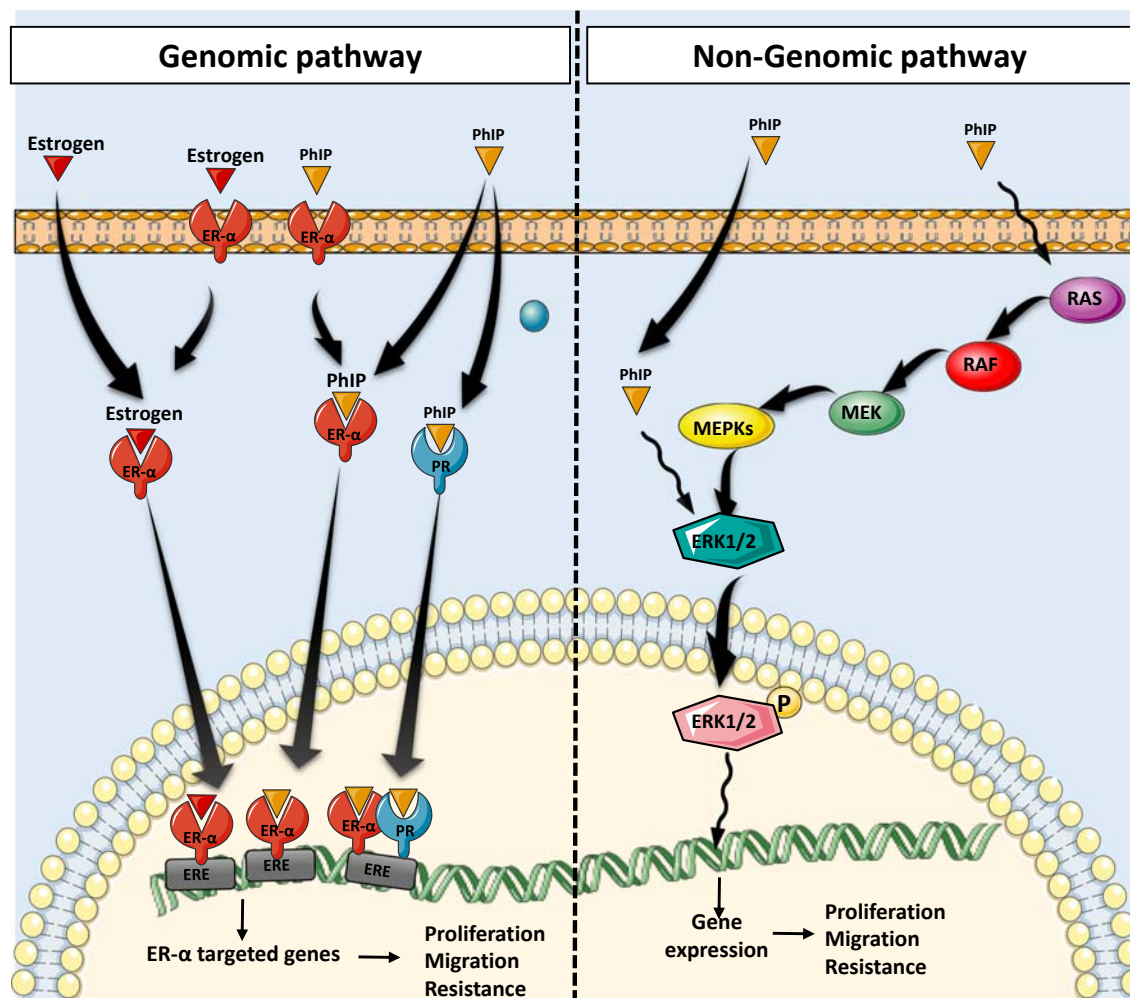
### HAA and Genomic and Non-genomic Regulation

In addition to their DNA-damaging and mutagenic properties, certain HAAs, such as PhIP, are capable of inducing changes in gene expression by different mechanisms (Fig. 4). In human breast cancer cells, PhIP is a potent estrogen and binds to receptor alpha (ER- $\alpha$ ) (Laubert et al., 2004). ER- $\alpha$  is a transcription factor that regulates the expression of targeted genes upon the binding of its ligand, the estrogens. Estrogen signaling through ER- $\alpha$  in breast cancer cells results in cell proliferation and survival signals but also the suppression of anti-proliferative and apoptotic signals (Frasor et al., 2003). In MCF-7 cells, a breast cancer cell line that expresses ER- $\alpha$ , PhIP induces transcriptional activities of ER- $\alpha$  at concentrations ranging between  $10^{-10}$  to  $10^{-6}$  M; the lower concentrations approach the levels of dietary exposure to PhIP in humans (Laubert et al., 2004). Compared to other environmental chemicals, the estrogenic potency of PhIP is strong. For example, butylated hydroxyanisole, a widely used dietary antioxidant and a known stimulator of ER- $\alpha$ , induces the transcriptional activity of ER- $\alpha$  *in vitro* at concentrations starting from  $10^{-5}$  M (Jobling et al., 1995). The activation of ER- $\alpha$  by PhIP leads to the proliferation of MCF-7 cells and an up-regulation of the expression of the proto-oncogene *c-Myc* (Laubert et al., 2004). *c-Myc* is a key factor for cell cycle regulation and progression. Under normal quiescent cell conditions, the expression of *c-Myc* is under tight transcriptional control and its level of expression ranges between undetectable to very low. However, under cellular proliferation conditions, the expression of *c-Myc* is up-regulated (Gabay et al., 2014).

Another genomic mechanism by which PhIP can induce proliferation in breast cancer cells is through the progesterone receptor (PR). Following exposure to PhIP, there is an increase in the expression of PR as a marker of ER- $\alpha$  activation (Laubert et al., 2004). Like the estrogens, progesterone is believed to be major player in breast cancer development. In breast cancer cells, the PR is activated by the binding of its ligands, which include naturally occurring ovarian steroid hormones, progesterone, or synthetic ligands such as progestins. The activation of PR leads to the transcriptional up-regulation of its targeted genes, including epidermal growth factor receptor (EGFR), *c-fos*, *p21*, and *cyclin D1*. Many of these genes are associated with tumor proliferation, resistance to chemotherapies, and tumor aggressiveness (Lange and Yee, 2008).

PhIP also binds to the androgen receptor (AR), leading to downstream AR events in human prostate cancer cells (Glass-Holmes et al., 2015). Like ER- $\alpha$  and PR, AR is a nuclear factor which is activated by interaction with androgenic hormones, such as testosterone and dihydrotestosterone, to express multiple genes involved in normal growth, homeostasis of the prostate. Under pathological conditions, AR and its downstream events are critical factors in prostate carcinogenesis (Lonergan and Tindall, 2011).

PhIP can also modulate the proliferation and the survival of breast cancer cells through cell signaling pathways. For example, the treatment of MCF-7 cells with PhIP leads to the activation of the mitogen-activated protein kinase (MAPK) extracellular signal regulated kinase (ERK) pathway (Laubert et al., 2004; Creton et al., 2007). The ERK pathway is activated



**Figure 4** Mechanisms of gene expression modulation of PhIP, by binding to ER- $\alpha$  (Genomic pathway) and through cell signaling (non-genomic, ERK phosphorylation), in breast cancer cells. The mechanism(s) by which PhIP impacts ERK pathway activation is unknown.

by the phosphorylation of ERK-1 and/or ERK-2, which lead to the modulation of transcription of genes that are involved in the regulation of proliferation, differentiation, and survival of breast cancer cells (Maemura et al., 1999). ERK pathway is activated by PhIP independently from the activation of ER- $\alpha$  since the activation of ERK pathway is also observed in MCF-10A cells, a breast cancer cell line that does not express ER- $\alpha$  (Venugopal et al., 1999). However, the mechanism by which PhIP leads to the activation of the ERK pathway remains unknown (Fig. 4). The activation of ERK pathway by PhIP is associated with an inhibition of apoptosis in MCF-10A under serum and growth factor deprivation conditions. Indeed, under these conditions, PhIP lead to the phosphorylation of ERK-2 with a concomitant increase in the expression of the two anti-apoptotic factors Bcl-2 and Bcl-xl (Venugopal et al., 1999).

## Human Biomonitoring

### Urinary Metabolites

Urine is a useful biological fluid for the measurement of exposure to various classes of carcinogens, since large quantities can be obtained noninvasively. The analysis of HAAs or their metabolites in urine do not shed light on DNA damage; however, such measurements can assess the capacity of an individual to bioactivate or detoxicate HAAs, and assess the impact of genetic polymorphisms in carcinogen metabolism enzymes on health risk (Hecht, 2002). HAAs are rapidly absorbed from the gastrointestinal tract and are eliminated in urine as multiple metabolites, with up to several percent of the dose present as the parent compounds within 24 hours of consuming grilled meats (Boobis et al., 1994; Turesky and Le Marchand, 2011). The percent of the dose of MeIQx and PhIP excreted in urine as genotoxic *N*-oxidized metabolites greatly exceeds that reported in the rodent, showing that human P450 1A2 *in vivo* biotransforms a larger portion of the HAA dose into genotoxic intermediates than rodents (Turesky et al., 1998; Walters et al., 2004; Kulp et al., 2004). Major pathways of detoxication of MeIQx occur by conjugation enzymes



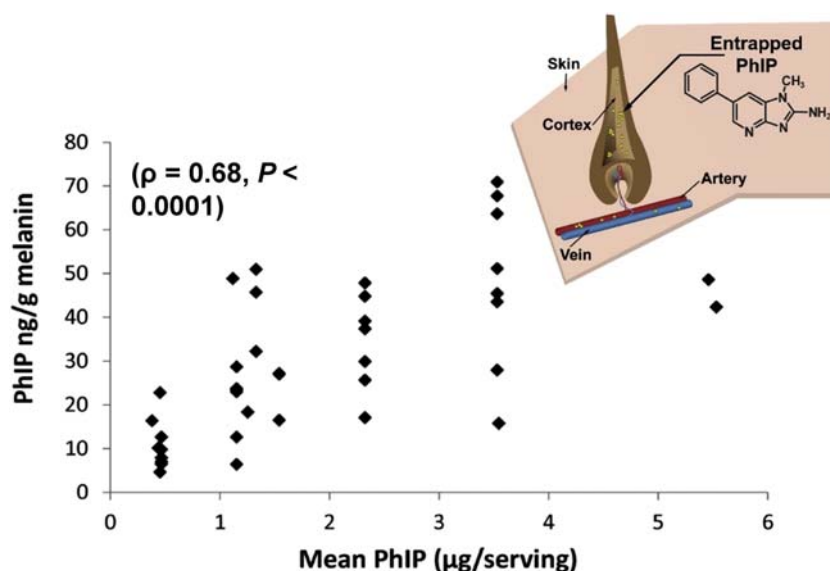
including SULT and uridine 5'-diphospho-glucuronosyltransferases (UGTs), to produce, respectively, the sulfamate and  $N^2$ -glucuronide conjugates of MeIQx (Fig. 2) (Turesky et al., 1998; Gu et al., 2011). UGT isoforms 1A1 and 1A9 strongly contribute to the metabolism of PhIP, and  $N$ - and  $O$ -linked conjugates of the genotoxic metabolite 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5- $b$ ]pyridine (HONH-PhIP) have been identified as the major metabolites of PhIP in urine of carnivores (Walters et al., 2004; Kulp et al., 2004; Turesky et al., 2015; Cai et al., 2016). A $\alpha$ C undergoes P450-mediated ring oxidation at the 3- and 6-positions of the heteroaromatic rings as major pathways of metabolism and detoxication, by human liver microsomes, human hepatocytes, and in vivo in humans (Fig. 2) (Bellamri et al., 2017; Konorev et al., 2015).

### Hair Biomarker

Urinary biomarkers of HAAs may be at undetectable levels for individuals who chronically but intermittently consume cooked meats, and these individuals can be misclassified. There is a need to establish stable, long-term biomarkers of HAAs. Human hair has served as a matrix to biomonitor various drugs and contaminants, such as nicotine, narcotics, and hormones (Alexander et al., 2002; Appenzeller and Tsatsakis, 2012). PhIP strongly binds to pigments in hair, and the levels of PhIP accrued in hair in a dose-dependent manner (Fig. 5). However, the binding of other HAAs to hair is considerably less. The levels of PhIP accrued in hair may be superior to inferring estimates of dietary intake of PhIP through food frequency questionnaires (Le Marchand et al., 2016; Turesky et al., 2013). However, long-term, non-invasive biomarkers of the biologically effective internal dose of HAA, such as DNA or protein adducts are still sought for molecular epidemiology studies.

### DNA Adduct Formation

Early reports on HAA-DNA adducts formation in rodents was done by  $^{32}$ P-postlabeling techniques (Schut and Snyderwine, 1999). The level of adduct formation in tissues does not necessarily correlate with tumorigenesis, indicating that tumor promoting factors, such as cell proliferation, are important in the tumorigenesis process (Sugimura, 1992). HAA-DNA adducts of  $^{14}$ C-radiolabeled-MeIQx and  $^{14}$ C-PhIP measured by accelerator mass spectrometry were reported to form at higher levels in human than formed in colon of rats given the same size proportional dose of chemical (Dingley et al., 1998).  $^{14}$ C-PhIP-DNA adduct formation in white blood cells of human subjects given an oral dose of PhIP (70  $\mu$ g/person) reached levels of modification up to 1.5 total adducts/ $10^9$  DNA bases 4–6 h post-treatment, but rapidly declined at 24 h post-treatment (Dingley et al., 1999). A gas chromatography/mass spectrometry method, based upon alkaline hydrolysis of DNA, revealed the presence of PhIP in colorectal mucosa of several individuals at levels of up to several adducts per  $10^8$  DNA bases (Friesen et al., 1994). Another study detected a labile adduct of PhIP at levels of several adducts per  $10^8$  DNA bases in long-lived lymphocytes in about 30% colorectal cancer subjects (Magagnotti et al., 2003). The levels of adduct varied 10-fold between the lowest and highest levels, suggesting differences in the intake of PhIP or interindividual variation in PhIP metabolism and bioactivation. The PhIP-DNA adduct (dG-C8-PhIP) was detected in exfoliated epithelial cells from milk of lactating mothers in 30 of the 64 samples analyzed, with a mean value of 4.7 adducts/ $10^7$  nucleotides, by the  $^{32}$ P-postlabeling method (Gorlewska-Roberts et al., 2002). In the other study, PhIP adducts, presumably dG-C8-PhIP, were detected in human breast tissues at levels of  $\sim 1$  adduct per  $10^7$  bases, by means of an immunohistochemistry method, in 82% and 71% of the normal breast tissue sections from the cancer and control patients respectively (Zhu et al.,



**Figure 5** The accrual of PhIP in newly grown hair in volunteers on a four week semi-controlled feeding study of well-done cooked meat (Le Marchand et al., 2016).

2003). Other studies employing immunohistochemistry reported frequent detection of dG-C8-PhIP in the DNA of pancreas and prostate of cancer patients (Zhu et al., 2006; Tang et al., 2007). The MeIQx-DNA adduct (dG-C8-MeIQx) adduct was detected by the  $^{32}\text{P}$ -postlabeling assay in colon and kidney DNA of several individuals at levels estimated at several adducts per  $10^9$  DNA bases (Totsuka et al., 1996). A recent pilot study employing liquid chromatography with high resolution accurate tandem mass spectrometry identified dG-C8-PhIP, but not DNA adducts of other HAAs, in prostate DNA from about 25% of the prostate cancer patients (Xiao et al., 2016). Adduct levels ranged from 2–120 adducts per  $10^9$  DNA bases. Collectively, these findings reveal that HAA-DNA adduct formation occurs in human tissues, even though the concentrations of HAAs in the diet are generally at low ppb levels. However, the biological significance of HAA-DNA adducts at these low levels of DNA modification remains to be elucidated (Nagao et al., 1996).

## Future Trends

The major pathways of metabolism of HAAs reveal interspecies similarities as well as important differences in the enzymology of metabolism which can influence the toxicological properties of HAAs. Recent advances in mass spectrometry instrumentation, particularly high resolution accurate mass spectrometers and improved sensitivity permit the detection of multiple DNA adducts at extremely low levels, or permit the use of less DNA for measurements of DNA adducts (Xiao et al., 2016; Balbo et al., 2014; Guo et al., 2017). With the identification of such biomarkers, the interactive effects of genetic polymorphisms of enzymes involved in metabolism (both activation and detoxication) and DNA repair enzymes may be correlated to the levels of DNA adduction products of HAAs and clarify the role of these chemicals in dietary associated cancers.

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## Relevant Websites

- NIH/National Cancer Institute: Chemicals in Meat Cooked at High Temperatures and Cancer Risk: <https://www.cancer.gov/about-cancer/causes-prevention/risk/diet/cooked-meats-fact-sheet>.
- National Toxicology Program: Report on Carcinogens, fourteenth ed.: <https://ntp.niehs.nih.gov/pubhealth/roc/index->.
- Heterocyclic amine from Wikipedia, the free encyclopedia: [https://en.wikipedia.org/wiki/Heterocyclic\\_amine](https://en.wikipedia.org/wiki/Heterocyclic_amine).

# Managing Acrylamide at the Agricultural Stage: Variety Selection, Crop Management, and the Prospects for Solving the Acrylamide Problem Through Plant Breeding and Biotechnology

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## Glossary

**Margins of exposure (MOE)** A term used in toxicology, the MOE is defined as the ratio of the level at which a small but measurable effect is observed to the estimated exposure dose; it is also sometimes defined as the ratio of the maximum no-adverse-effect-level to the estimated exposure dose.

**Maillard reaction** This reaction, which was named after the French chemist, Louis Camille Maillard, who first described it in 1912, comprises a series of non-enzymatic reactions between reducing sugars and amino groups, principally those of amino acids. The Maillard reaction is promoted by high temperature and low moisture content and, therefore, occurs mainly in cooked foods prepared by frying, baking and roasting.

**Reducing sugar** A reducing sugar is one that is capable of acting as a reducing agent because it has a reactive double bond in a free aldehyde group (e.g. glucose and maltose) or a free ketone group (e.g. fructose).

**RNA interference (RNAi)** A genetic modification technique that results in gene silencing.

**Genome editing** A term given to several techniques that use specially-designed oligonucleotides or targeted nucleases to generate mutations in specific target genes within an organism's genome.

**TALENs** Transcription activator-like effector nucleases, used in genome editing.

**CRISPR-Cas** Clustered, regularly-interspaced, short palindromic repeats (CRISPR) and the Cas9 nuclease, used in genome editing.

## Introduction

Processing contaminants may be defined as substances that are produced in a food when it is cooked or processed, are not present or are present at much lower concentrations in the raw, unprocessed food, and are undesirable either because they have an adverse effect on product quality or because they are potentially harmful (Curtis et al., 2014b). A processing contaminant that is proving particularly difficult for the food industry is acrylamide ( $\text{CH}_2=\text{CH}-\text{CONH}_2$ ). Acrylamide is classed as a probable (Group 2a) human carcinogen by the International Agency for Research on Cancer (1994) based on its action in rodents, and also has reproductive and neurotoxicological effects at high doses (Friedman, 2003). It forms in food as a result of the Maillard reaction; a series of non-enzymic reactions between free (soluble, non-protein) amino acids and reducing sugars, such as glucose, fructose and maltose, that occurs during high-temperature ( $>120^\circ\text{C}$ ) cooking and processing. Acrylamide is produced when the free amino acid that participates in the later stages of the reaction is asparagine (Mottram et al., 2002; Stadler et al., 2002). Fried, baked and roasted potato, cereal and coffee products are the major sources of dietary intake. The issue is not easy to solve because acrylamide forms from naturally-occurring precursors (free asparagine and reducing sugars), and because the Maillard reaction also gives rise to the colors, flavors and aromas that consumers expect of fried, baked and roasted foods, and that define food types and brands. Indeed, acrylamide formation correlates closely with color formation in products such as potato chips and color can be used as a quality control measure. This was the basis of the UK Food Standards Agency's 'Go for Gold' campaign of 2017, which encouraged consumers to cook foods to a light brown color (Food Standards Agency, 2017).

Acrylamide is present in food at levels typically ranging from tens of parts per billion (ppb) ( $\mu\text{g kg}^{-1}$ ) in soft bread to several hundred ppb in biscuits, French fries and potato chips (UK crisps). The concentrations used in rodent toxicology studies may be two to three orders of magnitude higher than that, and the risk posed by dietary exposure is therefore difficult to gauge, requiring extrapolation from the effects of high doses for relatively short periods of time in rodents to lower doses for much longer periods of time in humans. Nevertheless, the European Food Safety Authority (EFSA) Expert Panel on Contaminants in the Food Chain (CONTAM) stated in its 2015 report that the margins of exposure for acrylamide indicate a concern for neoplastic (tumour-inducing) effects (EFSA CONTAM Panel, 2015). This forced the hand of the European Commission with respect to reviewing its regulations on the presence of acrylamide in food. The Commission had already issued 'Indicative Values' for the presence of acrylamide in food in 2011 and revised them downwards for many products in 2013 (European Commission, 2013). These values were not meant to be maximum limits or safety thresholds; rather they were set at levels that the Commission believed should be achievable, based on the results of its monitoring programme (EFSA CONTAM Panel, 2015). If a product is found to exceed the Indicative Value the relevant food safety authority should take action to ensure that the manufacturer addresses the problem. As a result of the CONTAM report, the Commission has drawn up strengthened risk management measures including compulsory Codes of Practice and



**Table 1** Indicative values and benchmark levels for acrylamide in food, set by the European Commission (2013, 2017)

<i>Food</i>	<i>Indicative value 2013 (<math>\mu\text{g kg}^{-1}</math>) (ppb)</i>	<i>Benchmark level 2017 (<math>\mu\text{g kg}^{-1}</math>) (ppb)</i>
French fries	600	500
Potato chips (UK crisps)	1000	750
Soft bread (wheat)	80	50
Soft bread (other)	150	100
Breakfast cereals	400	300
Biscuits	500	350
Crackers	500	400
Crispbread	450	350
Gingerbread	1000	800
Cereal-based baby foods	50	40
Roast coffee	450	400
Instant coffee	900	850

the renaming of Indicative Values as Benchmark Levels, with reduced Benchmark Levels for most products (European Commission, 2017) (Table 1). These regulations could be in force by mid-2018. The proposals also state that the setting of Maximum Levels with regulatory enforcement for acrylamide in certain foods should be considered as soon as the new framework is in place.

### The Need for Agricultural and Crop Management Solutions to the Acrylamide Problem

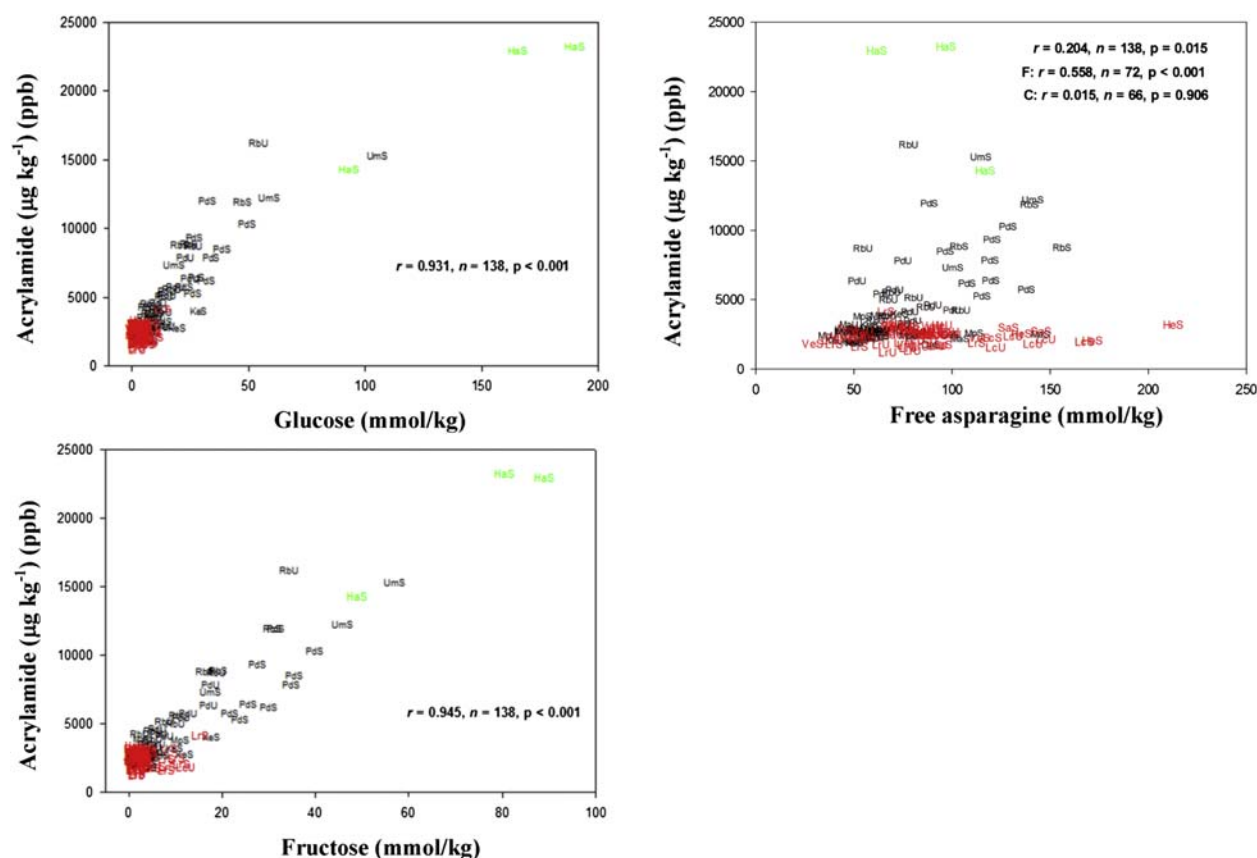
Acrylamide was first discovered in food in 2002 (Tareke et al., 2002). Since then, the food industry has worked hard to reduce the levels of acrylamide in its products, and the methods that have been developed have been compiled in a 'Toolbox' produced by FoodDrinkEurope, the latest update of which was published in 2014 (Food Drink Europe, 2014). The measures that have been implemented for potato chips, for example, include improved control of cooking temperature and duration, monitoring of moisture levels in the finished product, post-frying quality control based on color, switching to very low-sugar potato (*Solanum tuberosum*) varieties, used only within their optimum storage window, and the careful control of storage temperature and conditions. In potato products, the concentration of reducing sugars has more impact on acrylamide formation than free asparagine, although free asparagine concentration does contribute to the variance in some datasets, particularly for French fry as opposed to chipping varieties, due to the generally higher concentrations of reducing sugars in the former (Fig. 1). Manufacturers have therefore also introduced checks on potato reducing sugar concentration at time of harvest, during storage and at the factory gate. Potato tubers contain very little maltose, so glucose and fructose are the predominant reducing sugars.

Analyses of European manufacturers' data on acrylamide in potato chips showed a 53% reduction in mean acrylamide levels from 763 ppb in 2002 to 358 ppb in 2011 (Powers et al., 2013). However, analysis of data up to 2016 showed that since 2011 there has been a levelling off, with the mean level for 2016 being 412 ppb; still a 46% reduction from 2002 but higher than the 2011 figure (Powers et al., 2017). This suggests that the most effective acrylamide reduction measures had been devised and implemented by 2011. Acrylamide levels also showed marked seasonal variability, being highest in the first half of the year when potatoes were being used from storage, and lowest from July to September when potatoes were being harvested (Fig. 2). This was consistent with the results of several studies showing an effect of storage on the concentration of reducing sugars and potential for acrylamide formation in potato (De Wilde et al., 2005; Elmore et al., 2015; Halford et al., 2012b; Muttucumaru et al., 2014). These studies led to advice being issued that potatoes should only be used for chipping, frying and roasting within their recommended storage window (Halford et al., 2012a), and highlighted the challenge faced by the food industry in processing such a variable raw material to give a level of acrylamide in the end-product that consistently complies with Indicative Values or Benchmark Levels. Even with additional processing measures, further substantial reductions may not be achievable without a step change in the acrylamide-forming potential of the raw material.

The study by Powers et al. (2017) also showed a marked geographical variation in acrylamide levels in chips, with the levels in chips produced in the north of Europe (Denmark, Finland, Lithuania, Latvia, Norway and Sweden) considerably higher than the rest of Europe. This, coupled with the seasonality of acrylamide levels, means that if the new European Benchmark level is confirmed at 750 ppb, the 'failure' rate in this region is likely to be > 30% during the first half of the year (Fig. 3). Even in the other regions of Europe, the 'failure' rate will exceed 10% in some months.

Given these findings, and the fact that the potato supply and processing chain must be prepared for medium- and long-term changes in the regulatory framework on acrylamide, there is an urgent need for the breeding of potatoes with much less potential for acrylamide formation than are available now. In other words, potato varieties with much lower concentrations of reducing sugars and/or free asparagine, together with improved stability of sugar concentrations during storage.



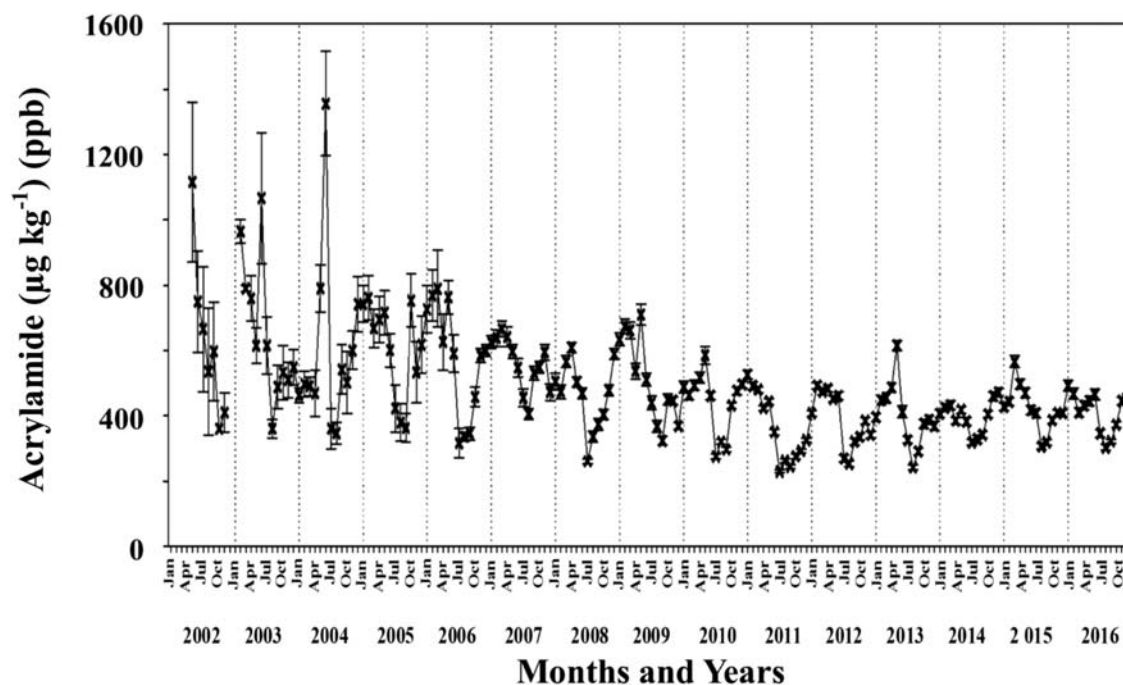


**Figure 1** Graphs showing correlations between glucose, fructose and free asparagine concentration and acrylamide formation in potato flour heated to 160 °C for 20 min. Points on the graphs from French fry varieties are denoted by F in black, while those for crisping varieties are denoted by C in red and the boiling variety, Harmony, in green. The results for correlation ( $r$ ) are given for all three types overall and in the case of free asparagine also for French fry and crisping types separately. The points are codes for the varieties Maris Piper (Mp), Pentland Dell (Pd), King Edward (Ke), Daisy (D), Markies (Ma), Russet Burbank (Rb), Umatilla Russet (Ur), (Lady Claire (Lc), Lady Rosetta (Lr), Saturna (Sa), Hermes (He), Verdi (Ve) and Harmony (Ha), followed by un-stored (U) or stored (S). Reproduced from Muttucumaru, N., Powers, S.J., Bridson, A., Elmore, J.S., Mottram, D.S., Halford, N.G., 2014. Evidence for the complex relationship between the concentrations of free amino acids, sugars and acrylamide-forming potential in potato. *Ann. Appl. Biol.* 164, 286–300.

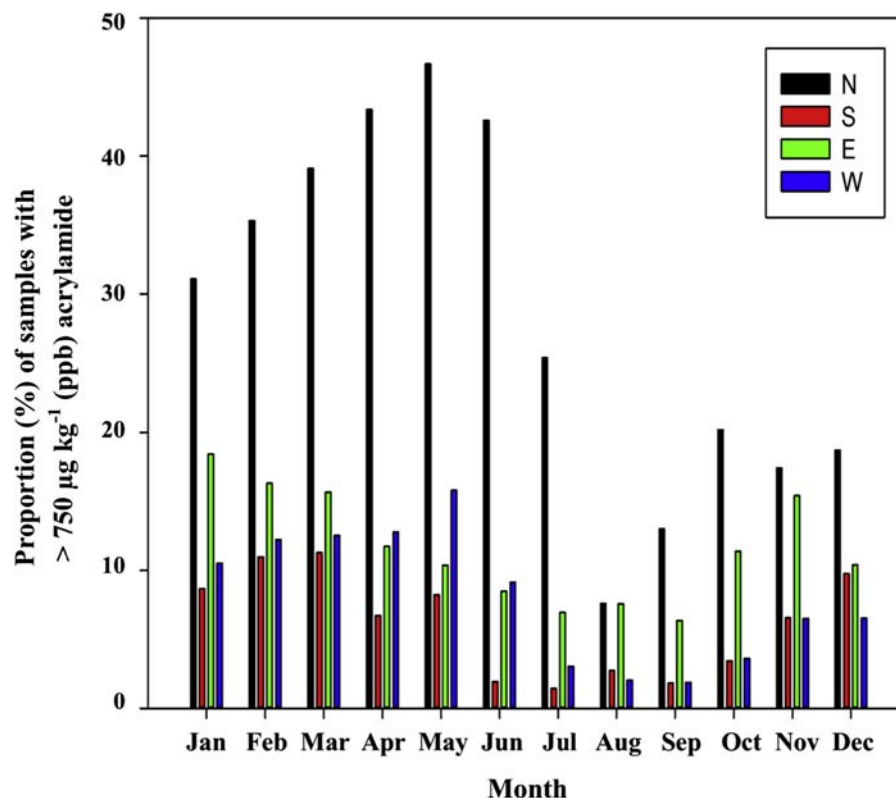
Similar data is not available for cereal products, but there is no reason to believe that the situation is any different, with the need for low acrylamide-forming potential varieties just as urgent. Cereal grains generally have lower concentrations of free asparagine than potato tubers, and higher concentrations of reducing sugars, with maltose being a major reducing sugar in addition to glucose and fructose. The different ratio of free asparagine to reducing sugars means that it is free asparagine rather than reducing sugar concentration that determines acrylamide-forming potential (reviewed by Curtis et al., 2014b) (Fig. 4).

### Genetic and Agronomic Approaches to Reducing the Acrylamide-Forming Potential of Potato

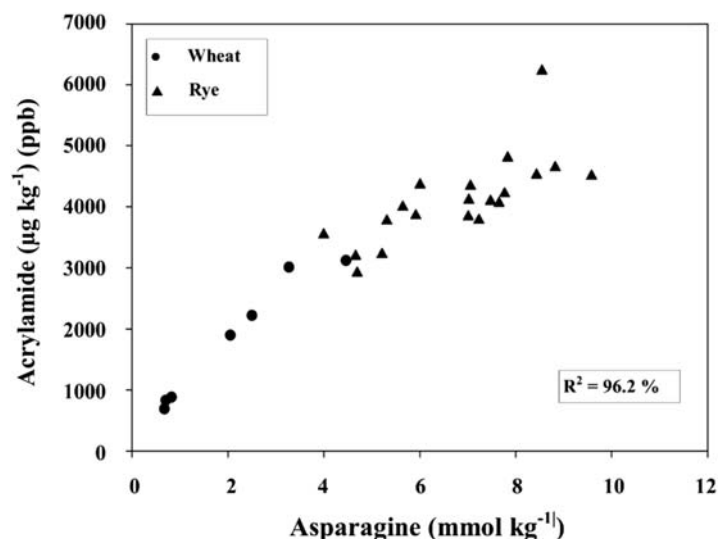
Most potato chip manufacturers favor a light brown chip color, with any intrinsic flavor incidental compared with the strong, artificial flavors that are added to the product. This means that low reducing sugar concentration has been a target for breeders developing potato varieties for that market since long before the acrylamide issue arose. Varieties used for French fry production and home cooking tend to have higher reducing sugar concentrations because consumers expect French fries to have color, and fresh potatoes to develop color and flavor as they fry and roast them. Indeed, most consumers will use color development as an indicator that the potatoes are cooked. However, there is considerable variation between varieties even within the different types, both in their composition at harvest and the stability of their sugar concentrations during storage (Elmore et al., 2015; Halford et al., 2012b; Muttucumaru et al., 2014, 2017). Fig. 5 contrasts the acrylamide levels in chips made from two UK chipping varieties, Lady Claire and Lady Rosetta, and two French fry varieties, Pentland Dell and Markies, through nine months of cold storage after their harvest in October 2011 (Halford et al., 2012b). The first striking aspect of this graph is the range in acrylamide levels, from approximately 100 ppb for lady Claire throughout the storage period to over 2500 ppb for Pentland Dell by the end of the storage period. The varieties also show contrasting responses to storage, with Lady Rosetta comparable with Lady Claire during early storage but already



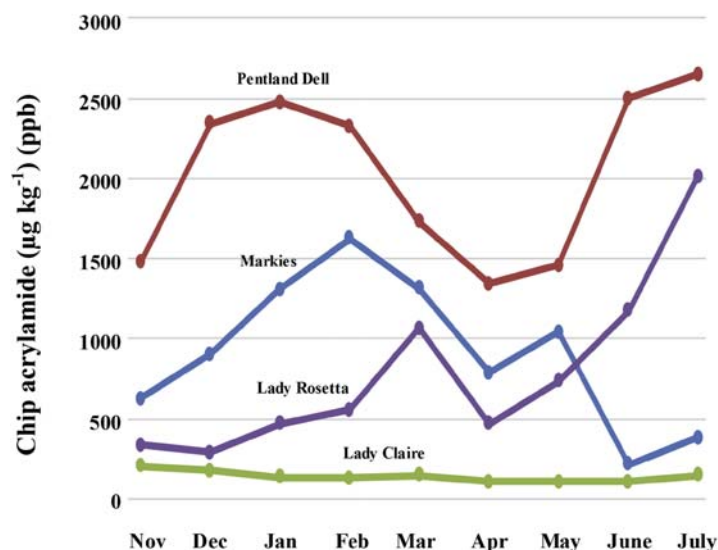
**Figure 2** Mean acrylamide levels over time (2002–2016) in samples of potato chips produced in Europe from 2002–2016, with standard errors, plotted monthly, showing the seasonality in acrylamide levels. Reproduced from Powers, S.J., Mottram, D.S., Curtis, A., Halford, N.G., 2017. Acrylamide levels in potato crisps in Europe from 2002 to 2016. Food Addit. Contam. Part A 34, 2085–2100.



**Figure 3** Proportion (%) of samples of potato chips produced in Europe from 2002–2016 with more than  $750 \mu\text{g g}^{-1}$  (ppb) acrylamide for each month over the period 2011–2016 for geographic regions, North, South, East and West. Plotted by Stephen Powers, Rothamsted Research, from data provided by Powers, S.J., Mottram, D.S., Curtis, A., Halford, N.G., 2017. Acrylamide levels in potato crisps in Europe from 2002 to 2016. Food Addit. Contam. Part A 34, 2085–2100.



**Figure 4** Relationship between free asparagine concentration and acrylamide formation in wheat and rye flour heated for 20 min at 180 °C. Replotted from Curtis, T.Y., Postles, J., Halford, N.G., 2014. Reducing the potential for processing contaminant formation in cereal products. *J. Cereal Sci.* 59, 382–392.



**Figure 5** Acrylamide formation ( $\mu\text{g kg}^{-1}$ ) (ppb) in chips made from four contrasting potato varieties through nine months of storage at 8.5–9.5 °C. Plotted from data provided by Halford, N.G., Muttucumaru, N., Powers, S.J., Gillatt, P.N., Hartley, L., Elmore, J.S., Mottram, D.S., 2012. Concentrations of free amino acids and sugars in nine potato varieties: effects of storage and relationship with acrylamide formation. *J. Agric. Food Chem.* 60, 12044–12055.

coming out of its optimal storage window by January. Markies on the other hand showed the highly unusual characteristic of decreasing levels of acrylamide formation during late storage.

This and other studies (Amrein et al., 2003, 2004; Becalski et al., 2004; De Wilde et al., 2005; Elmore et al., 2015; Muttucumaru et al., 2014, 2017) showed that the use of appropriate varieties for specific end uses, for example switching to very low-sugar varieties for chip production, was an important strategy for reducing acrylamide formation in potato products. It also led to the advice that potatoes should be stored under carefully-controlled temperature (typically 8.5–9.5 °C) and conditions, and used only within their optimum storage window, which differs between varieties (Halford et al., 2012a). The increases in acrylamide formation in chips made from potatoes that have been stored for a long time arise from increases in reducing sugar concentration, which in turn are caused by cold and senescent sweetening. Cold sweetening occurs at storage temperatures around and just above 4 °C, while senescent sweetening occurs as potato tubers break dormancy. The onset of senescent sweetening defines the end of the optimum storage window for a particular variety. The storage temperature of 8.5–9.5 °C is a compromise because colder temperatures would promote cold sweetening while warmer temperatures would allow the potatoes to sprout. Both cold and senescent

sweetening are associated with vacuolar invertase activity, which converts sucrose to glucose and fructose (Clasen et al., 2016; Zhu et al., 2014; Wiberley-Bradford and Bethke, 2018).

As well as identifying potato varieties with low acrylamide-forming potential, attempts have been made to define optimum crop management regimes. Nitrogen application, for example, can increase acrylamide-forming potential of potatoes but the effect is type-dependent, with French fry varieties showing an increase in response to nitrogen that is not apparent in chipping varieties (Muttucumaru et al., 2013). Even within type, different varieties exhibit different responses, making the situation even more complicated. Sulfur application has no significant effect on its own, although application at 15 kg per hectare does mitigate the effect of high nitrogen application on the acrylamide-forming potential of some varieties.

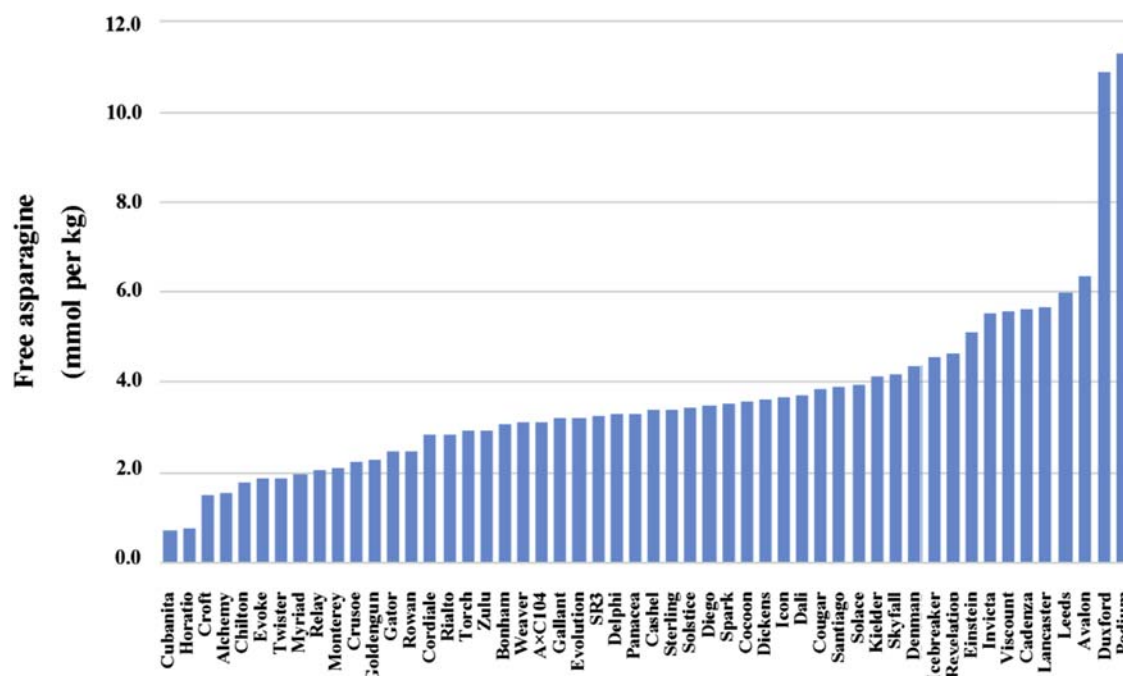
The effect of water supply has also been investigated, and even mild drought stress (e.g. lack of irrigation in the field in the UK) has been shown to cause significant changes in tuber composition (Muttucumaru et al., 2015). However, these differ from those caused by more extreme drought stress, with free proline accumulating in some varieties under mild drought stress and free asparagine in some varieties under severe drought stress. Notably, in that study, potatoes grown commercially in the UK with irrigation had more acrylamide-forming potential than potatoes grown without irrigation (Muttucumaru et al., 2015), but different genotypes responded differently to the same treatment, indicating that there is no single, unifying potato tuber drought stress response.

The complexity and inconsistency between varieties of these findings mean that no advice has been given on crop management of potato for acrylamide mitigation, except to fertilise with nitrogen and irrigate sufficiently only to ensure the health and yield of the crop, and no more. Given the lack of a crop management strategy, reductions in the acrylamide-forming potential of potatoes will require breeding of new, improved varieties. A key question for breeders is what targets should they aim for: low reducing sugar concentration and sugar stability during storage, or reduced free asparagine concentration, or both. Decreasing the concentration of reducing sugars may seem the obvious target, but chipping varieties in particular have been bred for low sugar concentrations for many years, and the lowest may already be close to the minimum to be 'fryable' to give an acceptable color and texture, as well as flavor and aroma for those products with subtle or no added flavoring. The concentration of reducing sugars affects all products of the Maillard reaction, whereas free asparagine concentration specifically affects acrylamide formation, making the latter arguably the better target. Extensive genetic resources are now available for breeders to enable them to reduce the free asparagine concentration of potatoes. The trait has been shown to segregate in a breeding population and quantitative trait loci have been identified (Shepherd et al., 2010, 2013), while potato genome data is now available (Potato Genome Sequencing Consortium, 2011) and genes encoding asparagine synthetases and other important enzymes can be mapped.

The problem of reducing the acrylamide-forming potential of potatoes also lends itself to biotechnological approaches. Indeed, genetically modified (GM) potato varieties with greatly decreased concentrations of free asparagine and/or reducing sugars, together with improved stability of sugar concentrations during cold storage, have already been developed and marketed in the USA by the Simplot Company of Boise, Idaho. The varieties are called Innate<sup>®</sup> and Innate<sup>®</sup> Generation 2. Both have reduced expression in the tuber of asparagine synthetase gene, *ASN1*, as well as reduced expression of two genes encoding enzymes of starch breakdown (phosphorylase L (*PhL*) and starch-associated R1 (*R1*)), and a gene (*PPO5*) encoding polyphenol oxidase, an enzyme involved in bruising, all as a result of RNA interference (RNAi). Innate<sup>®</sup> Generation 2 also has reduced expression of vacuolar invertase (*VInv*), and increased resistance to late blight through incorporation of a resistance gene from *Solanum venturii*. The low concentration of free asparagine and reducing sugars in the tubers of Innate<sup>®</sup> Generation 2 is claimed to reduce acrylamide-forming potential by 90% compared with conventional potatoes, effectively solving the problem. There is currently no prospect of these or any other genetically modified varieties being grown in Europe, highlighting how far behind crop biotechnology is in the UK and Europe compared with the USA, and how this is beginning to compromise efforts to improve food safety. Nevertheless, the new biotechnological techniques of genome editing are also applicable to acrylamide mitigation and being essentially mutagenesis techniques may escape the restrictive regulations covering GM crops in Europe. Indeed, the *VInv* gene has already been targeted using transcription activator-like effector nucleases (TALENs) (Clasen et al., 2016). Lines with full *VInv* knockouts were produced, and the tubers from these plants had extremely low levels of reducing sugars and were used to make chips with much lower levels of acrylamide than chips produced from control tubers.

## Genetic and Agronomic Approaches to Reducing the Acrylamide-Forming Potential of Cereal Grains

Free asparagine concentration determines the acrylamide-forming potential of cereal grain, and both wheat (*Triticum aestivum*) and rye (*Secale cereale*) show large differences between varieties (Curtis et al., 2009, 2018, 2010; Postles et al., 2013). Fig. 6, for example, shows the free asparagine concentration in the grain of commercial wheat varieties grown in a field trial in the UK in 2012–2013. Free asparagine concentration in wheat grain can vary from location to location and year to year (Curtis et al., 2009), but eight varieties, Claire, Cocoon, Croft, Delphi, Monterey, Horatio, Myriad and Cordiale, have been shown to be consistently low in free asparagine concentration, relative to other varieties, across multiple field trials (Curtis et al., 2018). Of these, Claire, Cocoon, Croft, Delphi and Monterey are all soft, biscuit types (classified as Group 3 in the UK), while Horatio and Myriad are soft Group 4 (used mainly for animal feed and bioethanol production). Only Cordiale is a hard (Group 2) wheat considered appropriate for breadmaking.



**Figure 6** Free asparagine concentration in grain of winter wheat varieties grown in a field trial in the UK in 2012–2013. Reproduced with modification from Curtis, T.Y., Powers, S.J., Wang, R., Halford, N.G., 2018. Effects of variety, year of cultivation and sulphur supply on the accumulation of free asparagine in the grain of commercial wheat varieties. *Food Chem.* 239, 304–313.

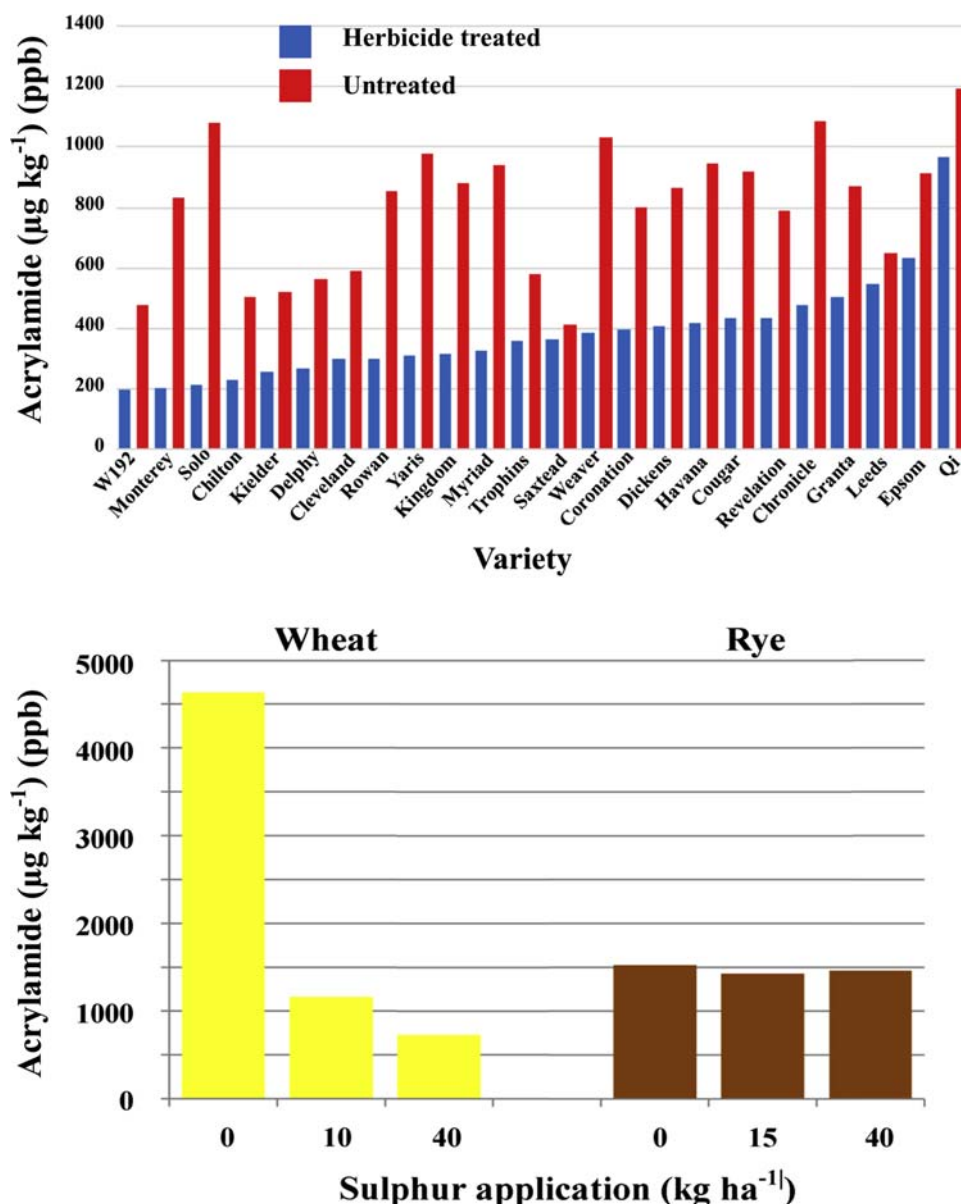
Most of the 'low' varieties are therefore soft wheats, but selecting varieties for low free asparagine simply on the basis that they are soft would be simplistic and potentially counter-productive because some soft varieties are high in free asparagine. Note also that the list of 'low' varieties may not be exhaustive because there is insufficient data available for some varieties to make an assessment.

As robust data on free asparagine concentrations becomes available it would be appropriate to include it in variety descriptions. Currently there may still be too much uncertainty for end users to trust variety rankings, partly because free asparagine concentration is very sensitive to environmental and crop management factors. However, excluding varieties in the 'high' group could be a sensible course of action in the short term. Another problem that food producers face is that the turnover in varieties in the UK is so rapid that by the time enough is known about asparagine concentrations the variety may no longer be available. The only solution to this is for testing to be carried out by breeders during variety development, and this is currently not done. Food producers may also find that the availability of low free asparagine, soft wheat varieties is limited: soft, biscuit wheat cultivation in the UK has declined dramatically in recent years, partly because Claire, a stalwart of that market, is susceptible to new strains of rust. Nevertheless, wheat users in the UK are relatively well off compared with producers who are reliant on other grains, or any cereal grain produced outside the UK: while there is some data available on free asparagine concentration in commercial rye varieties grown in the UK (Postles et al., 2013), data for wheat or rye grain in other countries is very limited, and there is no data that I am aware of for other cereals, at least in the public domain.

The fact that lower free asparagine concentrations would reduce the acrylamide-forming potential of cereal grains has reinvigorated interest in the genetic control of asparagine accumulation. Asparagine is of interest anyway, because it is an important nitrogen storage and transport molecule in many plant species, due to its relatively high nitrogen to carbon ratio (2:4, compared with 2:5 for glutamine, 1:5 for glutamic acid and 1:4 for aspartic acid, for example) and its relative chemical inertia (Lea et al., 2007). It accumulates to high concentrations during processes such as seed germination and in response to a range of abiotic and biotic stresses (Lea et al., 2007; Lea and Azevedo, 2007; Halford et al., 2015), including poor disease control (Curtis et al., 2016) (Fig. 7). Indeed, it can become the predominant free amino acid in cereal grains under some stress conditions, an example of how stress can have profound effects on crop composition (Halford et al., 2015).

Free asparagine concentration also correlates positively with nitrogen availability in the grain of barley (*Hordeum vulgare*) (Winkler and Schön, 1980), wheat (Martinek et al., 2009) and rye (Postles et al., 2013, 2016), while deficiencies in other minerals become important when there is a plentiful supply of nitrogen (reviewed by Lea et al., 2007). Sulfur deficiency in particular can cause a massive (up to 30-fold) increase in the accumulation of free asparagine in wheat, barley and maize (*Zea mays*) (Baudet et al., 1986; Curtis et al., 2009, 2014a; Granvogl et al., 2007; Muttucumaru et al., 2006; Shewry et al., 1983) (Fig. 7). Rye responds in similar fashion in response to severe sulfur deficiency in pot experiments (Postles et al., 2016) but is less responsive under field conditions (Postles et al., 2013) (Fig. 7). Consistent with this, asparagine synthetase gene expression in wheat and rye has been shown to increase under sulfur-limited growth conditions (Byrne et al., 2012; Gao et al., 2016; Postles et al., 2016), a response that appears to involve the protein kinase, TaGCN2 (Byrne et al., 2012).



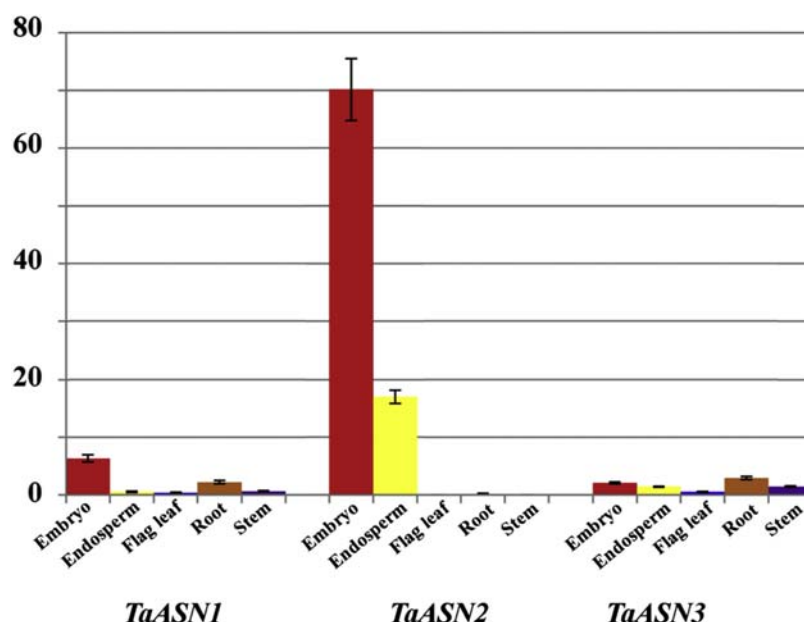


**Figure 7** Top. Acrylamide formation in flour from wheat grown with or without fungicide treatment. Bottom. Effects of sulfur availability on acrylamide-forming potential in wheat and rye. Reproduced from Top: Curtis, T.Y., Powers, S.J., Halford, N.G., 2016. Effects of fungicide treatment on free amino acid concentration and acrylamide-forming potential in wheat. *J. Agric. Food Chem.* 64, 9689–9696; Bottom: Curtis, T.Y., Postles, J., Halford, N.G., 2014. Reducing the potential for processing contaminant formation in cereal products. *J. Cereal Sci.* 59, 382–392.

The responses of wheat shown in Fig. 7 mean that the practice of ensuring sulfur sufficiency (an application rate of 20 kg sulfur per hectare is recommended; Curtis et al., 2014a) and effective disease control should be adopted to mitigate the problem of acrylamide formation in wheat products. In addition, late application of nitrogen should be avoided. In particular, if nitrogen is applied when sulfur is limiting it will accumulate as free asparagine, increasing acrylamide risk without achieving the desired result of improving protein quality. These crop management practices have been included in the draft Codes of Practice published by the European Commission (European Commission, 2017). It would also be helpful for regulatory authorities to take the consequences for acrylamide-forming potential into account when considering the risks and benefits of mineral fertiliser and fungicide usage.

A key enzyme in asparagine synthesis is asparagine synthetase, which catalyses the ATP-dependent transfer of the amino group of glutamine to a molecule of aspartate to generate glutamate and asparagine. Wheat has four asparagine synthetase genes, called *TaASN1-4* (Gao et al., 2016). *TaASN4* has not been characterised in any detail. Of the others, the expression of *TaASN2* in the embryo (wholegrain fraction) and endosperm (white flour fraction) during mid to late grain development is the highest of any of the genes in any tissue (Fig. 8). This makes *TaASN2* a logical target for genetic interventions, including genome editing.





**Figure 8** Differential expression of asparagine synthetase genes in different tissues of wheat. Reproduced from Gao, R., Curtis, T.Y., Powers, S.J., Xu, H., Huang, J., Halford, N.G., 2016. Food safety: structure and expression of the asparagine synthetase gene family of wheat. *J. Cereal Sci.* 68, 122–131.

## Concluding Remarks

Genetic and agronomic approaches to solving the acrylamide problem could eventually lead to massive savings for the food industry, dwarfing the cost of the research involved. Step reductions in the acrylamide-forming potential of potatoes have already been achieved using GM techniques, and may also be possible in all the affected crops using other modern breeding methods, such as mutagenesis and genome editing, effectively solving the problem. Success in this area will ensure that consumers continue to enjoy the variety and health benefits of potato and cereal products while food safety is improved. It could also ensure the continued use of grains that are known for their health benefits but which currently have a relatively high acrylamide-forming potential, such as wheat, barley and rye. These are also the major grains produced in northern and western Europe and other temperate regions.

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- <http://www.efsa.europa.eu/en/topics/topic/acrylamide> – EFSA acrylamide page with links to EFSA documents.
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# MCPD Esters and Glycidyl Esters: A Review of Analytical Methods

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## Nomenclature

2-MCPD 2-monochloropropanediol

3-MCPD 3-monochloropropanediol

3-MBPD 3-monobromopropanediol

AOCS American Oil Chemists' Society

EFSA European Food Safety Authority

ESI Electrospray Ionization

GC-MS Gas chromatography-mass spectrometry

GPC Gel permeation chromatography

IARC International Agency for Research on Cancer

JECFA Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives

LC-MS Liquid chromatography-mass spectrometry

LC-MS/MS Liquid Chromatography Tandem Mass Spectrometry

LC-TOF-MS Liquid Chromatography Time of Flight Mass Spectrometry

PBA phenyl boronic acid

SPE Solid-phase extraction

WHO World Health Organization

## Introduction

Fatty acid esters of 3-monochloropropanediol (3-MCPD), 2-monochloropropanediol (2-MCPD), and glycidol are toxicologically relevant contaminants produced during the deodorization of edible oils. They are not found in virgin, unrefined oils, and are generated during the high temperatures (>200 °C) applied during deodorization (Svejkovska et al., 2006; Destailats et al., 2012a, 2012b; Shimizu et al., 2012; Freudenstein et al., 2013; Ermacora and Hrnčířík, 2014a). While there are still questions regarding their specific mechanism of formation, there is evidence that 3-MCPD esters are formed from organochlorines naturally present in palm oil prior to processing (Nagy et al., 2011). In addition, while they do form in all edible oils during deodorization, they tend to form at the highest concentrations in palm oil (Kuhlmann, 2011; MacMahon et al., 2013c).

Fatty acid esters of glycidol, 3-MCPD, and 2-MCPD all pose potential food safety concerns. The risk is not from the fatty acid esters themselves, but from the free forms of glycidol, 3-MCPD, and 2-MCPD, which are produced when the esters are hydrolysed during digestion. Glycidol is classified by the IARC as a "likely human carcinogen" and some authors have suggested it should be kept at concentrations as low as is reasonably achievable in food (WHO, 2000; EFSA, 2016). Free 3-MCPD has been shown to be carcinogenic in rats, and the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives recommended a maximum tolerable daily intake for 3-MCPD from its esters of 4 µg/kg body weight per day (FAO, 2016). While 2-MCPD is not nearly as well studied from a toxicological perspective, it has been shown to have heart and muscle affects (Schilter et al., 2011; Andres et al., 2013). While these risks are associated with the free versions of these compounds, animal studies have shown that free 3-MCPD is produced from 3-MCPD esters during digestion with 86% efficiency (Abraham et al., 2013), with the digestion efficiency of glycidyl esters even higher (Appel et al., 2013). As the risk from these contaminants is from the free compounds, and not the intact esters, results are often reported as "bound MCPD" and "bound glycidol." This refers to the total concentration of MCPD or glycidol that would be released if all the fatty acid esters present in a sample were quantitatively hydrolysed.

While the potential toxicological risk is from the free compounds produced during digestion, the fact that the compounds exist in oils as fatty acid esters significantly complicates their analysis. Vegetable oils consist primarily of 5 fatty acids, palmitic acid, linolenic acid, linoleic acid, oleic acid, and stearic acid. Palm kernel and coconut oil also contain significant quantities of lauric acid and myristic acid. Those 7 fatty acids occurring in combination in an edible oil produce a possibility of 7 different glycidyl esters. The situation is far more complicated in MCPD esters, as they have 2 –OH groups that can form fatty acid esters. There are 7 possible 2-MCPD mono-esters and 14 possible 3-MCPD mono-esters (because in 3-MCPD esters, the two –OH groups are not equivalent). In addition, there are 28 possible di-esters for 2-MCPD and 3-MCPD (Dubois et al., 2012). As a result, analytical methods cannot simply target 3 contaminants, but theoretically must target 84 different compounds to ensure the detection of all possible fatty acid combinations.

To address the complexity of detecting so many target compounds, some researchers employ so-called “indirect” methodology. Instead of targeting the intact fatty acid esters, the methods use a hydrolysis step to release free 3-MCPD, 2-MCPD and glycidol. In general, this is followed by a derivatization step and analysis by gas chromatography-mass spectrometry (GC–MS). Early methods developed to target these contaminants exclusively utilized this approach, and were critical in bringing attention to the presence of MCPD and glycidyl esters in oils (Weißhaar, 2008). However, the most widely used method at the time was later shown to produce inaccurate results (Haines et al., 2011; Hrnčič et al., 2011; Kaze et al., 2011). Partially in response to this issue, direct methods were developed that were capable of detecting the intact fatty acid esters without the need for hydrolysis.

The accuracy of indirect methodology has improved, and now there are several which have been collaboratively studied (AOCS official method Cd 29a-13, 2017; AOCS official method Cd 29b-13, 2017; AOCS official method Cd 29c-13, 2017). Indirect approaches present many advantages. The methods release and target the free compounds, which are the source of risk from a food safety perspective. Because only the 3 compounds are targeted, only 3 analytical standards (potentially along with 3 internal standards) are required. The indirect methods utilize GC–MS, which are generally readily available and easy to operate, where direct methods require liquid chromatography-mass spectrometry (LC-MS). However, unlike direct methods which do not involve any conversion of the target analytes, indirect methods present the possibility of the formation of artefacts. In addition, information about the esters as they occur in oils and foods is lost. As some studies have indicated potential differences in the toxicology of MCPD mono- and di-esters, that information could be useful in assessing potential risk (Buhrke et al., 2011). In addition, measuring the fatty acid esters directly can be useful in determining the impact of industrial efforts to mitigate the formation of these esters during deodorization, as it may be more straightforward to reduce the formation of glycidyl and MCPD mono-esters relative to MCPD di-esters (MacMahon et al., 2013c). In addition, as these methods are extended to complex foods, which incorporate an extraction component, the direct methods are able to demonstrate extraction performance across all the fatty acid esters, which significantly differ from one another in polarity.

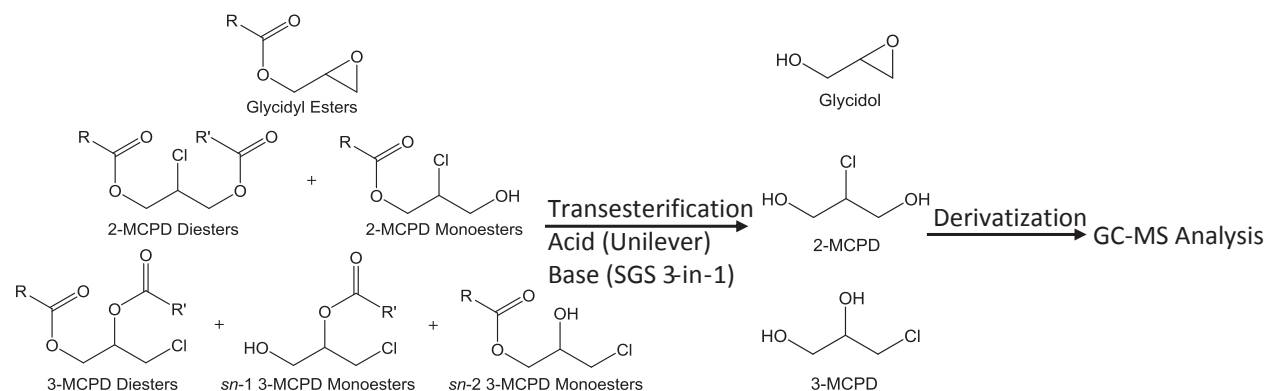
This chapter will review the various indirect and direct methods that have been published for the detection of MCPD and glycidyl esters in oils and foods. Their relative strengths and weaknesses will also be discussed, as appropriate. The methods will be presented chronologically, with an emphasis on methods that represented major advances in the field and are still widely used.

## Discussion

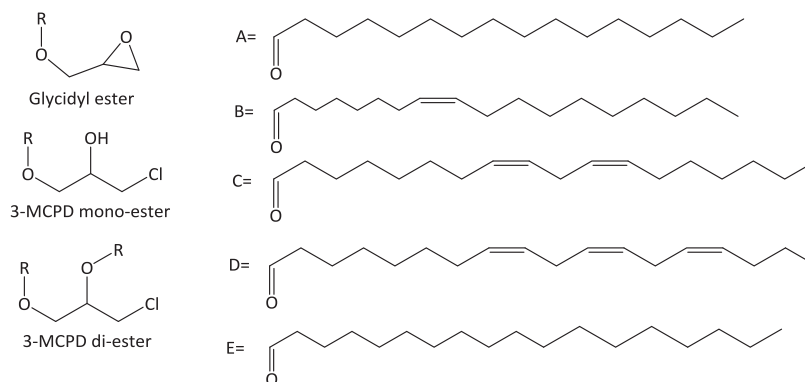
Analytical methods that were first proposed for the detection of MCPD and glycidyl esters used an indirect approach (see Fig. 1). By hydrolysing the wide variety of fatty acid esters, releasing free 2-MCPD, 3-MCPD, and glycidol, the analysis is greatly simplified, essentially only requiring 3 standards. While the methods first proposed were critical for bringing attention to this issue, one of the most widely used early methods (Weißhaar, 2008) was later shown to provide inaccurate results (Hrnčič et al., 2011; Kaze et al., 2011). The sodium chloride used in the procedure reacts with non-MCPD compounds in the oil to produce MCPD, leading to artificially high detected concentrations (Haines et al., 2011).

It was a direct method developed by researchers at Archer Daniels Midland (ADM) (Haines et al., 2011) that provided perhaps the most compelling evidence of the issues with early indirect methodology. The ADM method requires no sample clean-up; a droplet of oil is weighed, combined with internal standard, and analysed by high-performance liquid chromatography with time of flight mass spectrometry (LC-TOF-MS). The method uses low concentrations of sodium acetate in the mobile phase and employs electrospray ionization (ESI) to monitor the sodium adducts of the MCPD mono- and di-esters of palmitic, linolenic, linoleic, oleic and stearic acids. Interferences are minimized by taking advantage of the high resolution of the TOF-MS system. The method also detects glycidyl esters of palmitic, linolenic, linoleic, oleic, and stearic acids (see Fig. 2).

Comparisons between the ADM approach and the indirect methods of the time helped lead to the development of accurate indirect methods for the detection of MCPD and glycidyl esters. However, the use of non-volatile salts requires extensive cleaning of the TOF-MS system and replacement of the ESI needle on a weekly basis. In addition, the chromatographic conditions do not



**Figure 1** Approach for indirect analytical methods.



Compound Name	R =	R =
<b>3-MCPD Di-esters</b>		
1,2-Bis-linolenoyl -3-chloropropanediol	D	D
Linoleoyl-linolenoyl -3-chloropropanediol	C	D
<b>1,2-Bis-linoleoyl -3-chloropropanediol</b>	<b>C</b>	<b>C</b>
Palmitoyl-linolenoyl -3-chloropropanediol	A	D
<b>Oleoyl-linolenoyl -3-chloropropanediol</b>	<b>B</b>	<b>D</b>
<b>Palmitoyl-linoleoyl -3-chloropropanediol</b>	<b>A</b>	<b>C</b>
<b>Oleoyl-linoleoyl -3-chloropropanediol</b>	<b>B</b>	<b>C</b>
Stearoyl-linolenoyl -3-chloropropanediol	E	D
<b>1,2-Bis-palmitoyl -3-chloropropanediol</b>	<b>A</b>	<b>A</b>
<b>Palmitoyl-oleoyl -3-chloropropanediol</b>	<b>A</b>	<b>B</b>
<b>1,2-Bis-oleoyl -3-chloropropanediol</b>	<b>B</b>	<b>B</b>
<b>Stearoyl-linoleoyl -3-chloropropanediol</b>	<b>E</b>	<b>C</b>
<b>Palmitoyl-stearoyl -3-chloropropanediol</b>	<b>A</b>	<b>E</b>
<b>Oleoyl-stearoyl -3-chloropropanediol</b>	<b>B</b>	<b>E</b>
1,2-Bis-stearoyl -3-chloropropanediol	E	E
<b>3-MCPD Mono-esters</b>		
1-Palmitoyl -3-chloropropanediol	A	
1-Oleoyl -3-chloropropanediol	B	
1-Linoleoyl -3-chloropropanediol	C	
1-Linolenoyl -3-chloropropanediol	D	
1-Stearoyl -3-chloropropanediol	E	
<b>Glycidyl Esters</b>		
Glycidyl Palmitate	A	
Glycidyl Oleate	B	
Glycidyl Linoleate	C	
Glycidyl Linolenate	D	
Glycidyl Stearate	E	

**Figure 2** Structure of esters targeted by ADM Direct Method.

separate 3-MCPD esters from isomeric 2-MCPD esters. As they both have identical precursor ion masses, it is impossible to accurately quantify 2-MCPD esters and 3-MCPD esters without separating them chromatographically.

As mentioned previously, the ADM method did help lead to the development of greatly improved indirect approaches. One such approach, the SGS 3-in-1 method (Kuhlmann, 2011), utilizes a low temperature (below  $-22^{\circ}\text{C}$ ) base-catalyzed hydrolysis to indirectly detect fatty acid esters of 3-MCPD, 2-MCPD and glycidol (See Fig. 1). These conditions avoid the undesirable conversions resulting from previous indirect methodologies. The hydrolysis is followed by reaction with a derivatizing agent, phenyl boronic acid (PBA), to produce an analyte volatile enough for analysis by GC-MS. PBA reacts with both 2-MCPD and 3-MCPD, producing derivatives that can be separated by GC and analysed independently. The analysis of glycidol is accomplished by its conversion to 3-monobromopropanediol (3-MBPD), and the application of a matrix dependent correction factor to account for the partial formation of 2- and 3-MCPD from glycidol during the alkaline transesterification.

This method produces accurate results, has been collaboratively studied, and is an official method of the American Oil Chemists' Society (AOCS Cd 29-13b) and the German Center for Fat Analysis (DGF). The ability to detect 2-MCPD, 3-MCPD and glycidyl esters in a single analysis is a major advantage, although a second injection of the oil sample is needed to determine the matrix dependent correction factor for glycidol. One challenge is the maintenance of the low temperatures applied during hydrolysis



( $-22^{\circ}\text{C}$ ), which are critical for minimizing undesirable side reactions. In addition, the hydrolysis requires 16 hours, so the analysis cannot be completed in a single day.

Researchers at Unilever proposed an acid-catalyzed transesterification which produced no degradation of either 3-MCPD (Hrnčirík et al., 2011) or 2-MCPD (Ermacora and Hrnčirík, 2012), allowing for simultaneous analysis of both isomers (see Fig. 1). While acid catalysis does create the possibility of side reactions with chlorides, the authors eliminated this possibility by including a liquid-liquid extraction step (Ermacora and Hrnčirík, 2012) to remove chloride ions. The acid hydrolysis process does lead to the conversion of glycidol to a glycerol ether (Hrnčirík et al., 2011). While this ensures glycidol will not interfere with the analysis of MCPD esters, it would seem to eliminate the possibility of a method which can simultaneously detect 2-MCPD, 3-MCPD and glycidyl esters, as glycerol ethers are also produced from the hydrolysis of triglycerides. To allow for the analysis of glycidyl esters, prior to acid hydrolysis, the sample is reacted with sodium bromide under mildly acidic conditions. This converts the glycidyl esters to 3-monobromopropanediol (3-MBPD) mono-esters. As 3-MBPD esters are chemically similar to MCPD esters, they can all be transesterified, derivatized, and analysed by GC-MS simultaneously. The formation of 3-MBPD from glycidol was employed by Kuhlmann, as well, but by reacting glycidol with sodium bromide after the alkaline hydrolysis step was complete.

Beyond the use of a small number of standards and relatively simple analytical equipment, characteristics of all indirect methods, the Unilever approach offers a number of unique advantages. By producing 3-MBPD mono-esters from glycidyl esters prior to hydrolysis, the sensitivity of the method to detect bound glycidol is increased. The approach also allows for the analysis of 2-MCPD, 3-MCPD, and glycidyl esters in a single quantitative analysis. Most other indirect approaches require two separate analyses, either to account for conversions that take place during alkaline hydrolysis or to determine the concentrations of glycidyl esters via the difference between two assays. And while the method employs a 16 hour hydrolysis, it is conducted at  $40^{\circ}\text{C}$ , an easier temperature to maintain than the  $-22^{\circ}\text{C}$  required by the SGS 3-in-1 method. The method has also been successfully collaboratively studied and is an official method of the American Oil Chemists' Society (AOCS Official Method Cd 29a-13, 2017).

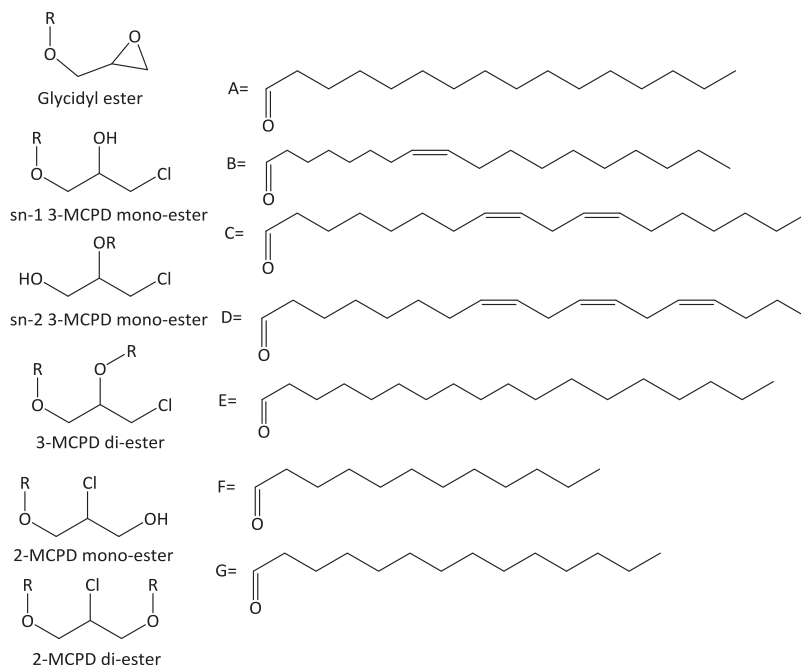
Due to the lengthy hydrolysis times required in indirect methods, combined with the resulting loss of structural information of the esters as they occur in oils, there have been continued efforts in the development of direct methods. The Nestlé Research Center has extensively researched MCPD and glycidyl esters, including formation mechanisms, mitigation strategies, analytical methods, and toxicology. One major contribution was the demonstration that only 10 3-MCPD di-ester analytical standards (shown in bold in Fig. 2) are required to target more than 90% of the bound 3-MCPD in the di-ester form in most commonly consumed vegetable oils (the only exceptions are palm kernel and coconut) (Dubois et al., 2012).

Nestlé's approach for the direct analysis of MCPD and glycidyl esters incorporates a solid-phase extraction (SPE) clean-up to remove more polar material from the oil matrix prior to analysis using ESI LC-TOF-MS (Dubois et al., 2012). While this greatly improved ruggedness compared to the ADM approach, the researchers also acknowledged that the method's inability to separate isomeric 2-MCPD and 3-MCPD esters impacts the accuracy of the quantitation, with the method only capable of roughly estimating the combined concentrations of the two analytes. As the two compounds have different toxicological properties, they need to be accurately quantified separately for risk assessment purposes. In addition, Nestlé researchers demonstrated that they have vastly different ionization efficiencies using ESI, so even accurately estimating the combined concentrations of the analytes in a single chromatographic peak is impossible. The method also targets glycidyl esters, but does so with recoveries ranging from 44%–87%. Accurate analysis of glycidyl esters required the use of a gel permeation chromatography (GPC) extraction procedure, followed by additional clean-up by SPE and LC-MS analysis (Dubois et al., 2012). While this did increase the effort required to analyze an individual oil sample, this approach improved recoveries for glycidyl esters to 68%–111%.

Researchers at the U.S. Food and Drug Administration published the most extensive direct method, using a two-step SPE clean-up and liquid chromatography-tandem mass spectrometry (LC-MS/MS) using ESI to target the 2-MCPD, 3-MCPD, and glycidyl esters containing lauric, myristic, palmitic, linolenic, linoleic, oleic and stearic acid (see Fig. 3) (MacMahon et al., 2013a, 2013b, 2014). This method was the first direct method to chromatographically separate isomeric MCPD mono- and di-esters, allowing it to provide accurate quantitation for both sets of analytes. As they constitute 90+% of vegetable oil matrices, removal of triglycerides is critical for accurate and reproducible LC-MS method performance. As triglycerides fall between glycidyl esters/MCPD mono-esters and MCPD di-esters in polarity, two separate SPE clean-ups to purify those two groups of target analytes were required.

This approach offers a number of advantages. At the time of publication, all other direct methods analysed isomeric 2-MCPD and 3-MCPD esters as a single chromatographic peak, making accurate quantitation impossible. The two-step SPE clean-ups ensure reliable instrument performance even after hundreds of injections. While the clean-up does take several hours to complete, it is much faster than the hydrolysis step required for indirect methods, and allows the complete analysis of oil samples within a single working day. The method employs several isotopically-labelled internal standards, avoiding the need for time consuming and laborious standard addition quantitation. It was used in a survey of 116 vegetable oil samples purchased in the United States (MacMahon et al., 2013c). However, compared to indirect methods, the requirement of a high-end LC-MS/MS system is costly in terms of purchasing the equipment and requires a high level of expertise to operate. The cost for the large number of standards required is also significant. The method also requires two separate two-step SPE clean-ups, as well as two injections, for the analysis of all MCPD and glycidyl esters in a given sample. In addition, while they have identical ionization efficiencies using ESI, the method cannot chromatographically separate isomeric sn-2 3-MCPD esters from 2-MCPD mono-esters; only the sum of the two can be accurately quantified.





3-MCPD di-esters		
Compound Name	R =	R =
1,2-Bis-lauroyl-3-chloropropanediol	F	F
Lauroyl-linolenoyl-3-chloropropanediol	F	D
Lauroyl-myristoyl-3-chloropropanediol	F	G
Lauroyl-linoleoyl-3-chloropropanediol	F	C
1,2-Bis-linolenoyl-3-chloropropanediol	D	D
Myristoyl-linolenoyl-3-chloropropanediol	G	D
1,2-Bis-myristoyl-3-chloropropanediol	G	G
Lauroyl-Palmitoyl-3-chloropropanediol	F	A
Lauroyl-Oleoyl-3-chloropropanediol	F	B
Linoleoyl-linolenoyl-3-chloropropanediol	C	D
Myristoyl-linoleoyl-3-chloropropanediol	G	C
1,2-Bis-linoleoyl-3-chloropropanediol	C	C
Palmitoyl-linolenoyl-3-chloropropanediol	A	D
Oleoyl-linolenoyl-3-chloropropanediol	B	D
Myristoyl-palmitoyl-3-chloropropanediol	G	A
Lauroyl-stearoyl-3-chloropropanediol	F	E
Myristoyl-oleoyl-3-chloropropanediol	G	B
Palmitoyl-linoleoyl-3-chloropropanediol	A	C
Oleoyl-linoleoyl-3-chloropropanediol	B	C
Stearoyl-linolenoyl-3-chloropropanediol	E	D
Myristoyl-stearoyl-3-chloropropanediol	G	E
1,2-Bis-palmitoyl-3-chloropropanediol	A	A
Palmitoyl-oleoyl-3-chloropropanediol	A	B
1,2-Bis-oleoyl-3-chloropropanediol	B	B
Stearoyl-linoleoyl-3-chloropropanediol	E	C
Palmitoyl-stearoyl-3-chloropropanediol	A	E
Oleoyl-stearoyl-3-chloropropanediol	B	E
1,2-Bis-stearoyl-3-chloropropanediol	E	E
sn-1 3-MCPD Mono-esters		
1-Lauroyl-3-chloropropanediol	F	
1-Myristoyl-3-chloropropanediol	G	
1-Palmitoyl-3-chloropropanediol	A	
1-Oleoyl-3-chloropropanediol	B	
1-Linoleoyl-3-chloropropanediol	C	
1-Linolenoyl-3-chloropropanediol	D	
1-Stearoyl-3-chloropropanediol	E	

**Figure 3** Structure of esters targeted by FDA Direct Method.

sn-2 3-MCPD mono-esters		
1-Palmitoyl-3-chloropropanediol	A	
1-Oleoyl-3-chloropropanediol	B	
2-MCPD di-esters		
Linoleoyl-linolenoyl-2-chloropropanediol	C	D
Dilinoeoyl-2-chloropropanediol	C	C
Palmitoyl-linoleoyl-2-chloropropanediol	A	C
Oleoyl-linoleoyl-2-chloropropanediol	B	C
Dipalmitoyl-2-chloropropanediol	A	A
Palmitoyl-oleoyl-2-chloropropanediol	A	B
Dioleoyl-2-chloropropanediol	B	B
Stearoyl-linoleoyl-2-chloropropanediol	E	C
Palmitoyl-stearoyl-2-chloropropanediol	A	E
Oleoyl-stearoyl-2-chloropropanediol	B	E
Distearoyl-2-chloropropanediol	E	E
2-MCPD mono-esters		
Palmitoyl-2-chloropropanediol	A	
Oleoyl-2-chloropropanediol	B	
Linoleoyl-2-chloropropanediol	C	
Glycidyl esters		
Glycidyl Laurate	F	
Glycidyl Myristate	G	
Glycidyl Palmitate	A	
Glycidyl Oleate	B	
Glycidyl Linoleate	C	
Glycidyl Linolenate	D	
Glycidyl Stearate	E	

Figure 3 (continued).

Expanding methods to target MCPD and glycidyl esters in foods presents a new challenge. The analysis of oils involves dissolving the entire sample in a non-polar solvent prior to analysis; there is no need for an extraction step. This is not the case with complex foods, where the contaminants are found in a matrix environment made up not only of fat, but polar elements such as proteins, sugars, water, and other natural components of a given food. An extraction procedure must be used that is stringent enough to quantitatively extract all of the MCPD and glycidyl esters, but is still mild enough to ensure that no structural conversions take place. Many traditional methods of fat extraction from foods involve harsh procedures, such as the use of hydrochloric acid or ammonia, which would be likely to cause conversion or destruction of the target analytes. Complex foods also may contain interferences not found in oils, potentially requiring adjustments to established clean-up or quantitation procedures.

Another critical point relates to the demonstration of accuracy for detecting these contaminants in a complex food. In oils, spiking MCPD and glycidyl esters represents a reasonable approach to demonstrate method accuracy, as the analytes will readily disperse homogeneously throughout the sample. For complex foods, this is not the case, and it is nearly certain that a MCPD/glycidyl ester spike applied to the surface of a food matrix will be more easily extracted than if the esters were contained in the vegetable oil ingredient and brought through the entire production process. The current lack of available food reference materials with known concentrations of MCPD and glycidyl esters further complicates matters, as method authors must find another approach beyond simple spiking to properly demonstrate the method's accuracy.

Unilever researchers published the first validated approach for the detection of MCPD and glycidyl esters in oil-based food products, specifically targeting fat-based spreads, creams, margarine, and mayonnaise (Ermacor and Hrnčirik, 2014b). While these products contain a high percentage of fat, extending the analytical methodology to these matrices proved quite challenging. A number of different solvent systems were compared, with the addition of water and 1:2 heptane: methyl *tert*-butyl ether providing the highest extraction yields. Extraction solvent volumes also required extensive optimization, with a 2:3 (v/v) ratio of organic to aqueous extraction solvent producing 30%–40% higher yields than a 2:1 (v/v) ratio. A number of temperatures were also examined, with 60 °C sufficient to enhance the break-up of emulsions without leading to the formation or decomposition of target analytes.

As discussed previously, the lack of available reference materials or existing reference methods makes conducting a proper validation challenging. The authors took advantage of an industrial production facility by producing pilot scale (100 kg batch per production) samples incurred with known amounts of MCPD and glycidyl esters. The authors produced 13 formulations with different levels of fats, compositions, and ester concentration.

The method represents the first thoroughly validated method for the detection of MCPD and glycidyl esters in complex food products. The use of pilot scale production of reference materials ensured the extraction was applicable to actual samples. And the method targets highly consumed, high fat products that have the potential to be significant sources of exposure to MCPD and glycidyl esters. It was successfully collaboratively studied, and is an official method of the American Oil Chemists' Society (AOCS Official Method Cd 30-15). That said, it does target a fairly narrow group of foods, as all these products are oil-in-water

or water-in-oil emulsions. While it's possible the method would be applicable to other food matrices, the extraction yields were lower in products containing milk proteins, with recoveries improved by the addition of a small amount of isopropanol to the aqueous extraction solvent. The authors specifically state that methods to target MCPD and glycidyl esters in food products should be dedicated and validated for specific food types.

U.S. Food and Drug Administration research focused on the development of a direct method to detect MCPD and glycidyl esters in infant formula (Leigh and MacMahon, 2016). Due to infants' low body weights and formula commonly being used as the sole source of infant nutrition, there is a need for methodology to detect MCPD and glycidyl esters in formula to determine infant exposure and to assess risk. Extraction methods for infant formula pose a particular challenge given the complexity of the matrix, consisting of dried, thoroughly homogenized fats, carbohydrates and proteins.

Attempts to use the extraction system developed by Unilever to extract oil-based products were unsuccessful, as emulsions formed with the formula samples that led to very low extraction yields. A number of extraction solvent systems were employed, and while nearly all worked for soy-based formula, very few provided high fat extraction yields in milk-based formula. More polar organic solvents proved more effective at extracting the fat from milk-based formula samples. This is likely due to the relatively hydrophobic character of milk proteins, with the more polar solvent minimizing the interactions between the extraction solvent and the proteins, allowing for a more distinct separation between aqueous and organic layers. While 1-butanol produced the highest extraction yields of all the solvents tested, it is not very volatile and took hours to evaporate. Therefore, ethyl acetate was selected as the optimal practical extraction system. The incorporation of a high-speed centrifugation (14,500 g) and the addition of sodium sulfate led to acceptable fat recoveries (>90%) and reproducibilities.

While the extraction was effective at removing the fat from all types of infant formula, it was critical to confirm that the ethyl acetate was also effective at recovering the entire range of MCPD and glycidyl esters. As their polarities vary, with MCPD di-esters less polar than triglycerides and MCPD mono-esters and glycidyl esters significantly more polar, it's conceivable that the extraction system could be biased towards extracting one group or the other. As discussed previously, spiking is not suitable to demonstrate extraction efficiencies in complex foods, so the authors produced an in-house infant formula reference material using ingredients typically found in formula. A combination of oils with known concentrations of MCPD and glycidyl esters were used to produce a liquid infant formula, which was then freeze dried. The final product was visually indistinguishable from commercial formula and, since it was milk-based, would present a challenging matrix to measure extraction yields. Recoveries ranged from 82%–109% for the entire range of 3-MCPD and glycidyl esters that occur in vegetable oils, helping confirm the method was effective for the analysis of infant formula.

The FDA method involves a simple, liquid–liquid extraction procedure that did not involve the use of complex instrumentation or expensive equipment. In addition, method performance was confirmed beyond simple spiked samples by analyzing the recoveries of the intact esters in a homemade reference material. Based on ester recovery data from this reference material, as well as validation data obtained from a variety of spiked commercial infant formula samples, the performance of the extraction procedure is rugged and reliable, yielding highly reproducible results. Using the previously published LC-MS/MS methodology for detection and quantitation, this procedure is suitable for producing MCPD and glycidyl ester occurrence data in all varieties of commercially available infant formulas, and was used in the most extensive survey of infant formula to date (Leigh and MacMahon, 2017). In addition, it's the only extraction method in food that has demonstrated accurate recoveries across all the individual 3-MCPD and glycidyl esters that commonly occur in vegetable oils. The method has since been extended to a wide range of complex foods (Leigh et al., 2017).

Researchers at the Joint Research Center also have published a method for the analysis of bakery and potato products, smoked/fried fish and meat, and cereal products (Zelinkova et al., 2017). The authors employed a pressurized extraction with methyl *tert*-butyl ether as the solvent. The fat extract was then analysed using the Unilever method (AOCS standard Cd29-13a). The authors took care to ensure that this indirect method was applicable to the fat extracts from complex foods. However, while method performance was highly reproducible, there were no reference materials included in the study to ensure the method's accuracy. The method is also not applicable to infant formula (Wenzl et al., 2015), a significant drawback given the importance of monitoring MCPD and glycidyl ester concentrations in formula to determine infant exposure and to assess potential risk. This is consistent with observations from FDA researchers who identified infant formula as the most analytically challenging food matrix they encountered.

While great progress has been made on the analysis of MCPD and glycidyl esters in oils over the past few years, with a number of methods producing comparable and accurate results, the analysis of complex foods is still developing. The challenges of extracting the wide variety of fat-containing foods combined with the lack of commercial reference materials slows the progress of development. However, there are a number of published methods which have recently been validated in complex foods. Moving forward, comparing their performance to one another and to standard reference materials is necessary to ensure accurate data is being produced for exposure and risk assessment purposes.

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## MCPDE and GE: An Update on Mitigation Measures

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### List of Acronyms

3-MCPD(E) 3-Monochloropropane-1,2-diol and its esters  
3-MCPD 3-Monochloropropane-1,2-diol  
3-MCPDE 3-Monochloropropane-1,2-diol fatty acid esters  
BMDL10 Lowest benchmark dose for a 10% response  
DAG diacylglycerols  
EFSA European Food Safety Authority  
FFA free fatty acids  
G(E) glycidol and glycidyl esters  
GE glycidyl fatty acid esters  
HCl Hydrochloric acid  
JECFA Joint FAO/WHO Expert Committee on Food Additives  
KCl Potassium Chloride  
MAG monoacylglycerols  
NH<sub>4</sub>Cl Ammonium Chloride  
PMTDI provisional maximum tolerable daily intake  
T25 dose dose at which is observed a 25% increase in incidence of a tumour above background incidence in the lifespan of the species  
TAG triacylglycerols  
TDI tolerable daily intake  
TRV toxicological reference value  
NTP United States National Toxicology Program

## Background

3-monochloropropane-1,2-diol and glycidol and their esters (so called 3-MCPD(E) and G(E) in the text) are processing -induced contaminants primarily found in refined fats and oils, and foods containing fats and oils.

These compounds have been previously evaluated by JECFA (1993, 2002, 2007, 2017) and EFSA (2013, 2016, 2018).

For free 3-MCPD, the European Commission Regulation (EC) No 1881/2006 has set maximum levels of 3-MCPD of 0.02 mg/kg for hydrolysed vegetable protein and soy sauce (European Commission, 2006). Provisions for methods of sampling and analysis for the official control of 3-MCPD were laid down in Commission Regulation (EC) No 333/2007 (European Commission, 2007). Recently, the European Commission has set limits of GE in vegetable oils and fats, in vegetable oils and fats intended for baby foods and process cereals intended for infants and young children, infant formula, follow-on formula and foods for special medical purposes intended for infants and young children (European Commission, 2018).

## Safety Assessment of 3-MCPD(E) and G(E)

Both 3-MCPD(E) and G(E) are efficiently absorbed following ingestion, the esters being efficiently hydrolyzed into 3-MCPD and glycidol in the digestive tract. Their metabolism has been reviewed in depth by EFSA and JECFA (as cited above).

GE was shown to be genotoxic. Toxicity studies on glycidol showed a low acute toxicity, renal toxicity, temporary/reversible infertility, immunotoxicity and neurotoxicity. In oral long-term studies in mice and rats, glycidol induced tumours in various tissues in both sexes. The Lowest Adverse effect Level was set at 17.9 and 26.8 mg/kg b.w. per day for mice and rats, respectively.

The United States National Toxicology Program (NTP, 1990, 2007) concluded that there was “clear evidence for carcinogenic activity”. The International Agency for Research on Cancer (IARC, 2000) classified glycidol as probably carcinogenic to humans (Group 2A).



Both JECFA and EFSA considered the carcinogenesis as the pivotal effect for risk assessment. JECFA calculated as toxicological reference value (VTR), a benchmark dose for a 10% response (BMDL10) of 2.4 mg/kg bw per day.

EFSA considered that a BMDL10 cannot be derived because of inadequacy of the data for modelling, and set a T25 dose (25% increase in incidence of a specific tumour above background incidence in the lifespan of the species) of 10.2 mg/kg b.w. per day.

3-MCPD(E) was found positive for genotoxicity *in vitro* but not *in vivo*. Short term studies on 3-MCPD showed lethal effects due to acute kidney failure. Two-year long oral toxicity studies at non-lethal doses demonstrated dose-related effects on kidney in rats (Sunahara et al., 1993; Cho et al., 2008a) but not in mice (Jeong et al., 2010; Cho et al., 2008b). Effects occurring at the highest dose were shown on hematology, urine analyses, liver functions, male infertility and neurotoxicity.

Both EFSA and JECFA concluded that the main target organs in rodents are kidneys and male reproductive organs, renal tubular hyperplasia being the most sensitive effect that might also lead to cancer by a non-genotoxic mode of action. JECFA derived a PMTDI of 4 µg/kg bw per day and EFSA set a TDI of 2.0 µg/kg bw per day.

Using different dataset and food consumption surveys, EFSA and JECFA found comparable dietary intakes for 3-MCPD(E) and G(E). For all class of age except infants, G(E) intakes ranged from 0.1 to 2.1 µg/kg bw per day and 3-MCPD(E) dietary intakes were comprised between 0.2 and 3.8 µg/kg bw per day.

Results on infants exclusively consuming infant formula showed similar estimates for glycidol (up to 4.9 µg/kg bw per day). But on 3-MCPD some discrepancies appear: JECFA found dietary intakes up to 25 µg/kg bw per day when the maximum level for EFSA was only 3.2 µg/kg bw per day.

Risks were characterized using the Margin of Exposure (MoE) approach by both JECFA and EFSA. MoEs were calculated by dividing toxicological reference values by exposure levels.

Even considering different toxicological reference values and different ways of dietary intake estimation, both committees reached similar conclusions for G(E). MoEs were too little mainly for high consumers and particularly for infants receiving formula only. EFSA and JECFA then concluded on a health concern linked to G(E) dietary exposures.

Concerning 3-MCPD(E), both EFSA and JECFA concluded on the absence of safety concern for most classes of age including high consumers. However, they expressed some concerns on infants, with some emphasis on infants fed only on formulas, as the PMTDI is exceeded in some countries (JECFA, 2017) and as a slight exceedance of the TDI was observed by the EFSA (2018).

EFSA and JECFA agreed that the dataset of levels of G(E) and 3-MCPD(E) in foods have to be completed to refine the dietary exposure calculations, since food categories such as ice-cream and chocolate were not included in the previous exposure assessment due to limited amount of data. They also pointed out that recent analyses have to be considered because of analytical method improvements and because of current industrial efforts to mitigate these compounds. These analytical efforts need to quantify separately free and esters chemical forms.

Concurrently, additional toxicological testing is also requested to complete the hazard profile (i.e. reprotoxicity on 3-MCPD(E); metabolism and carcinogenicity of G(E)).

Both EFSA and JECFA also evaluated available information on 2-monochloropropane-1,2-diol and its esters (2-MCPD(E)). But, even exposure were estimated by the EFSA, the limited toxicological information available did not allow to undertake a risk characterization (EFSA, 2016; JECFA, 2017). However, mitigation of 3-MCPD(E) as described in the following chapters will have similar effects on 2-MCPD(E) and other chloropropanols.

## Strategies to Mitigate Monochloropropane-1,2-Diol and Glycidyl Esters in Refined Edible Fats and Oils

### Introduction

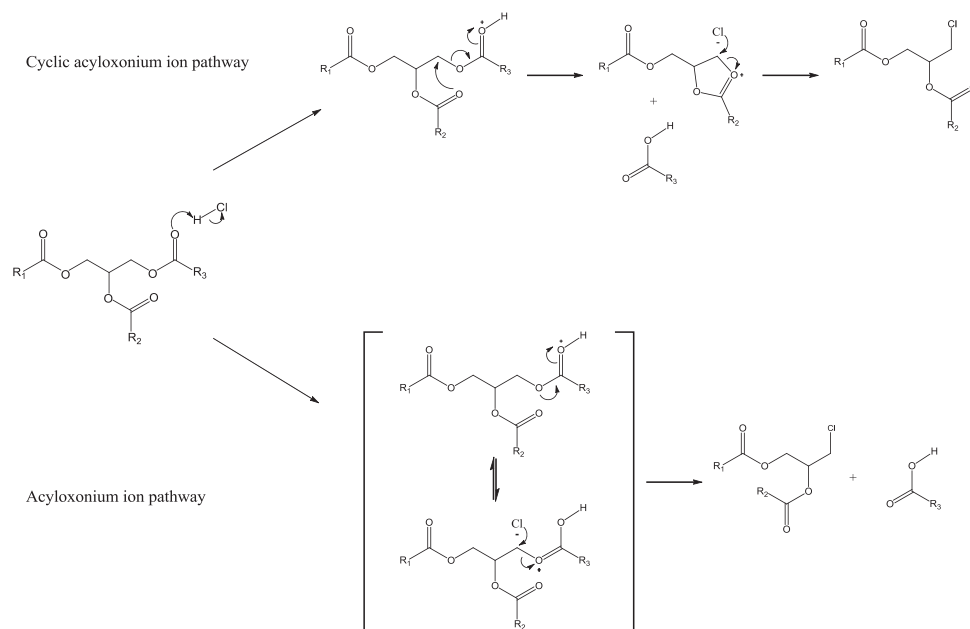
Following a decade of research, it has become clear that the formation of monochloropropane-1,2-diol and glycidyl esters (MCPDE and GE) during edible oil refining is strongly dependent on the quality of the oil at each point in the supply chain. This implies that reduction of these process contaminants can be achieved in several ways at various steps in the supply chain. The applied measures can enhance each other when implemented properly. Focus could be on the removal of the reaction precursors, adapting the reaction conditions and the removal of the reaction products. These strategies are more or less aligned with the oil supply chain, differentiating between crude oil production, refining process, and post-treatment steps.

During the last decade, industry has been actively working on mitigation tools, the results of which have been summarized before in guidance documents (FEDIOL, 2015; BLL, 2016). Also the Codex Alimentarius initiated efforts to develop a Code of Practice (Codex Alimentarius, 2018).

### Proposed Mechanism of 3-MCPDE and GE Formation

Since their discovery, the potential mechanism of 3-MCPDE and GE formation has been studied and reviewed in the literature (Hamlet et al., 2011; Rahn and Yaylayan, 2011; Cheng et al., 2017).

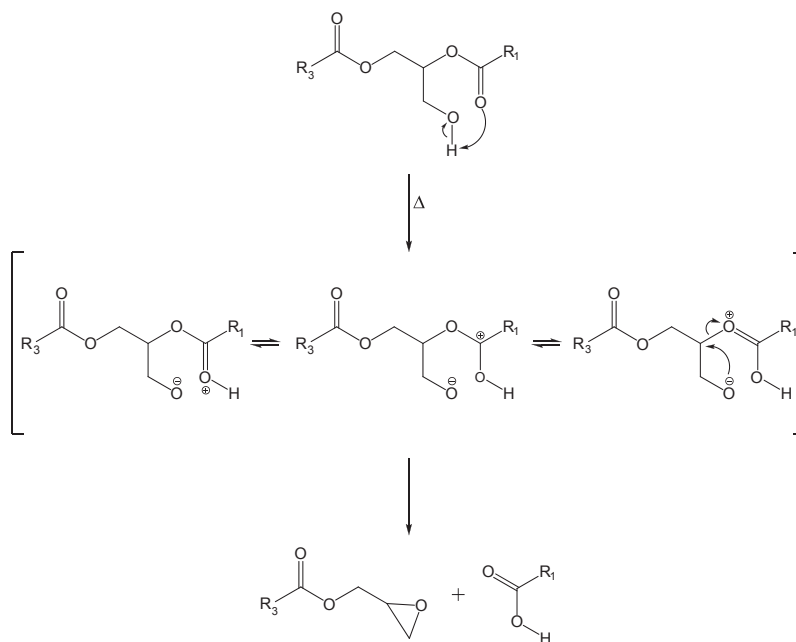
As shown in Fig. 1, formation of 3-MCPDE involves predominantly triacylglycerols (TAG) and a chlorine donor. Destailats et al. (2012a) proposed two formation pathways of MCPD diesters in palm oil. In general, organochlorines in crude and semi refined oils react via thermal decomposition at deodorization temperature, resulting in the release of hydrogen-chloride (HCl). This HCl initiates reaction with acylglycerols, resulting in generation of MCPD diesters and elimination of free fatty acids. The first pathway to



**Figure 1** Proposed mechanisms for the formation of 3-MCPDE at high temperatures ( $T > 200\text{ }^{\circ}\text{C}$ ) in the presence of HCl evolved from the thermal decomposition of trace amounts of organochlorines (Destailats et al., 2012a).

form 3MCPDE involves the formation of a reactive cyclic acyloxonium ion intermediate. The other route is a direct nucleophilic substitution reaction of a chloride ion on the open acyloxonium ion.

As shown in Fig. 2, the precursors of GE formation were identified as diacylglycerols (DAG) (Destailats et al., 2012b). Also monoacylglycerols (MAG) have been proposed as potential precursors for the GE formation (Cheng et al., 2017). The mechanisms described, include intramolecular rearrangement through charge migration and differ from each other in the nature of intermediate (acyloxonium ion or oxonium ion) and the leaving group (free fatty acid or water).



**Figure 2** Proposed mechanisms for the formation of glycidyl ester (GE) from diacylglycerol at high temperatures (Destailats et al., 2012b).

## Refining Principles

Refining is done to obtain stable, clear oils with neutral taste, neutral odor and to improve the shelf-life. It is also imperative that potential contaminants, if present in the crude oil, are efficiently removed. Although - concerning the refining process - 3-MCPDE and GE are formed only during deodorization, the prior steps greatly influence the formation capacity. Various process versions are available to choose for different oil types and qualities. In general, two basic processes are applied, in which the main difference is the way the free fatty acids are removed from the oil (Fig. 3).

In chemical refining, free fatty acids (FFA) are removed by saponification with sodium hydroxide in the neutralization step. In contrast, in physical refining process (Kővári et al., 2000; Stage, 1985) free fatty acids (FFA) are evaporated in the deodorization stage, together with other volatile substances.

In chemical refining the basic process stages are neutralization, washing, bleaching and deodorization (Anderson, 2005; van Duijn and den Dekker, 2013; Gupta, 2017). As process variants, degumming can be applied prior to neutralization, while dry washing (absorption on silica or cellulose) can replace the water washing step. For wax containing oils like sunflower, dewaxing or winterization steps are included in the refining process.

The main steps of physical refining are degumming, bleaching and the so-called deodorization, or the stripping of free fatty acids (Hamm, 2013; van Duijn and den Dekker, 2013). Different degumming and washing techniques as well as dewaxing or winterization can be added to the total process.

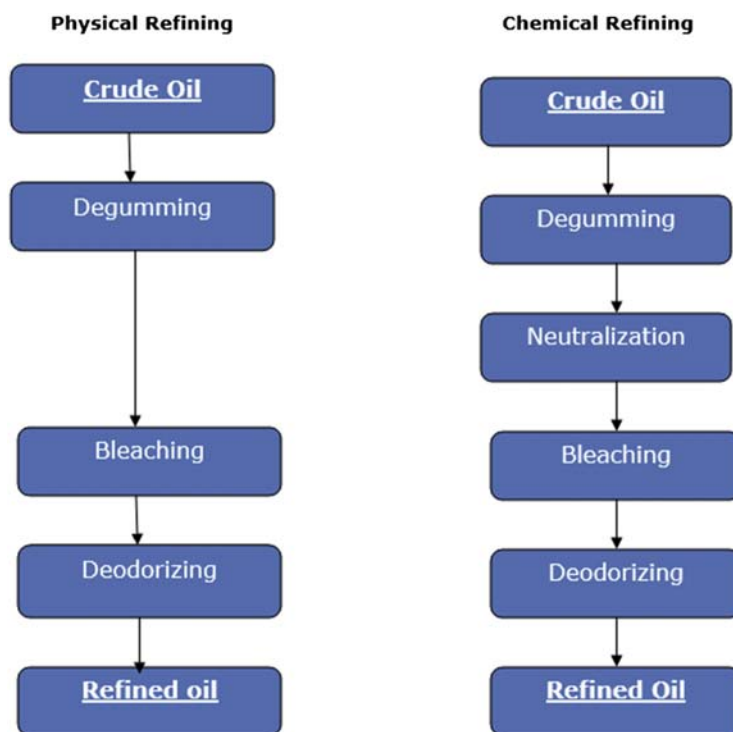
Crude oil with a phosphor content 10–20 mg/kg (like palm oil) can be degummed by the so-called dry-degumming process. Acids (e.g. phosphoric acid) are applied to facilitate hydration of the phosphatides and removal in the bleaching step. The phosphatides are actually removed together with the spent bleaching earth during filtration (Hamm, 2013; Gupta, 2017).

Crude oils with high phosphor content (200–800 mg/kg, like seed oils) require a wet degumming step, which includes again hydration of phosphatides, followed by separation of the formed gums by centrifugation. The wet processes can comprise simple water degumming or acid degumming (enhanced phosphor removal assisted by citric- or phosphoric acid) or enzymatic degumming.

## Reduction of Precursors in Crude Oils

Since the formation of MCPDE and GE depends on the various precursors to form this compounds, effective measures would target the removal of this precursors.

It has been shown that the precursors for MCPDE formation can be derived from a wide range of organochlorine components or inorganic chloride compounds, present in the oil. The chlorine seems to originate from fertilizers, e.g. KCl and NH<sub>4</sub>Cl, and chloride-containing pesticides used in palm oil cultivation or FeCl<sub>3</sub> used as flocculant in waste water treatment (Destailats et al., 2012a; Craft



**Figure 3** Simplified scheme of a vegetable oil refining processes (FEDIOL, 2015).

et al., 2012a). Upon heating the oil during the final processing step of deodorization, the chlorine is released through decomposition yielding Cl<sup>-</sup> ion. This in turn will react with the oil itself, i.e. the tri-, diglycerides to form 2- and 3-MCPDE (Destailats et al., 2012a). Monoglyceride levels in oil are generally very low, typically 0.3% (Goh and Timms, 1985), hence their contribution to MCPDE formation is very low (Destailats et al., 2012b). As the type and amount of pesticides and fertilizer applied will vary for each crop, it can be well conceived that there will be differences in the MCPDE forming potential between different oils, even though the underlying chemistry is the same.

The knowledge on the behavior of organochlorine precursors, even though they have only partially been identified, has led to potentially important approach for MCPDE mitigation. It has been shown that removal of the chlorine precursors by washing can improve the quality of the crude oil, in terms of a lower tendency to form MCPDE as well as GE (Matthäus, 2015; Ramli et al., 2011; Zulkurnain et al., 2013). Moreover, it has been shown that the earlier mitigation is done in the supply chain, the better and the more efficient it works (Craft et al., 2012b; Nagy et al., 2011). Nowadays, water washing of palm oil, generally with 1–5 % w/w of water, is increasingly being tested and implemented at full scale in palm oil mills (Zulkurnain et al., 2013; Ramli et al., 2011).

The other reactant, important for the formation of MCPDE and GE, are obviously the acylglycerols that make the oil themselves. Vegetable oils generally consist of TAGs (86%–96%), DAGs (4%–12%) and MAGs (~0.3%) and free fatty acids (1%–5%) (Goh and Timms, 1985). The partial acylglycerols DAG and MAG are to the larger extent the result of enzymatic hydrolysis, activated during seed or fruit processing. Palm oil processed in the regular way can contain free fatty acid (FFA) levels up to 5% with concomitant diglyceride levels as high as 10% (average around 6.5%) (Goh and Timms, 1985). In seed oils FFA levels are generally 0.5%–1.5% with DAG levels < 2.5% (Gupta, 2017).

In the case of MCPDE, it has been shown that the mechanism of formation is mostly through TAGs reacting with the HCl with consequent release of free fatty acids. However, also DAGs can react in this way albeit that they are less reactive (Destailats et al., 2012b).

The formation of GE was found to be correlated to diglyceride levels (Matthäus, 2011) as most important precursor, but can be formed also from monoglycerides (Destailats et al., 2012a). Contribution of monoglycerides in the formation of GE is small as compared to the role of diglycerides (Goh and Timms, 1985). This relation to DAG is one of the reasons why palm oil and likely other high FFA-oils (e.g. lampante olive oil), contain significantly higher levels of GE as compared to seed oils (EFSA, 2016).

Reducing the level of partial glycerides therefore is another important mitigation tool, especially to mitigate GE. As palm oil is a fruit oil, active enzymes will start to hydrolyze the oil within the fruits itself, the moment the bunch is cut from the tree. Thus minimizing the time delay between harvesting and milling can significantly enhance the oil quality regarding the FFA content. Premium quality palm oil, processed as quickly as possible after harvesting, can contain less than 2% free fatty acid (Craft et al., 2012a).

## Individual Steps in Oil Refining

### Degumming, Neutralization and Washing

Degumming, neutralization and washing are the refining steps, which are common in using water and centrifugal separators (except dry degumming), that have significant impact on MCPDE and GE formation during deodorization. In general, application of water helps removing chlorine containing precursors (Pudel et al., 2011). Changing the acidity of the oil environment can play a crucial role in the MCPDE- and GE forming-potential of an oil. It has been shown that acidic conditions promote the formation of MCPDE (Destailats et al., 2012a), whereas GE is in fact broken down under such conditions (Smidrkal et al., 2011). Conversely MCPDE are rapidly decomposed under alkaline conditions (Smidrkal et al., 2011; Velišek et al., 2002; Hamlet et al., 2003). Even an equilibrium has been suggested between the formation of MCPDE and GE, depending on the reaction conditions (Freudenstein et al., 2013), especially hydrochloric acid level (Destailats et al., 2012a).

Wet degumming processes are specifically used for seed oils because of their high phosphatide content as discussed in Refining Principles section. Apart from water, citric acid or phosphoric acid can also be used to facilitate non-hydratable phospholipid removal. The percentage of hydration water typically equals with the percentage of phosphatides in the crude oil. Under these conditions chlorine and chlorinated compounds may also be removed to the gum phase, which was observed with reduction of 3-MCPDE formation capacity (Ramli et al., 2011; Matthäus, 2011; Craft et al., 2012a).

Oils can also be degummed using phospholipase A or C enzymes.

For palm oil, often dry degumming is applied, in which concentrated acid is added to the oil, removing the phosphatides in the subsequent bleaching process. It means that the added acid is not removed by centrifuging, but in the bleaching step. Applying acid in dry degumming is a significant factor of MCPDE formation as the acid can increase formation of 3-MCPDE (Schurz, 2010). The impact depends on the acid dosage and the amount of bleaching earth added for decolorization and removing the acid (Ramli et al., 2011).

In the neutralization process, first phosphoric acid is added to the oil to help removing the non-hydratable phosphatides and then this acid and the free fatty acids are neutralized with caustic solution. The caustic can optionally be applied in excess. Turning to basic conditions in neutralization contributes to lower MCPDE in the refined oil. This has also been suggested by Papastergiadis et al. (2016) and Pudel et al. (2011), describing that alkaline washing is more effective than regular water washing. The effect was described to be more pronounced on fresh crude oil. This is in agreement with the consequence from the need to reduce and remove the acidity in the oil prior to high temperature heating, i.e. the final deodorization step and a potentially more efficient chlorine-precursor removal.

Washing with water is routinely applied for seed oils after neutralization or post-degumming. Concerning washing of crude palm oil, [Matthäus \(2015\)](#) demonstrated the positive impact of increasing temperature (60–100 °C) and washing water amounts (5%–20%).

### Bleaching

Bleaching has important influence on MCPDE through the type and amount of bleaching earth. It is important to realize, that the bleaching step actually shows two effect, being on one hand the precursor removal by adsorption and on the other hand a potential addition of acidity towards the deodorization step. As shown, the higher the pH of the clay, the lower the MCPDE content of the refined oil is ([Ramli et al., 2011](#); [Ibrahim et al., 2016](#)). Because there are actually different effects taking place in bleaching, the overall effect on 3-MCPDE and GE of bleaching clay dosage on MCPDE and GE is not obvious. [Pudel et al. \(2011\)](#) did not find significant influence of increasing clay use (0.7% to 1.5%), [Matthäus et al. \(2011b\)](#) showed positive impact of excess dosing (3% versus 1%) and [Brüse \(2015\)](#) showed various relations depending BC type and refining steps applied (chemical vs. physical refining). Concerning hydrochloric acid activated bleaching earth, [Ibrahim et al. \(2016\)](#) and [De Greyt \(2014\)](#) concluded that with increasing chlorine content in the clay, increasing levels of MCPDE were found in the oil after deodorization. Whereas mostly hydrochloric acid-activated clays have been used in the past, today there is a clear shift towards phosphoric acid-activated, or physically activated (non-acid treated) bleaching clays, reducing the acidity of the oil as well as the chlorine input into the process. The effect of reduced acidity is discussed in more depth in the following chapter.

A more extreme example of adsorption was shown in ([Ermacora and Hrnčirik, 2014](#)). Isolation of polar compounds with silica treatment impacted significantly the MCPDE and GE formation. Similar adsorption but far less effective was also observed earlier by ([Strijowski et al., 2011](#)). However, both methods make use of high amounts of adsorbents lowering the oil processing yield and cost and further post-refining would be required to achieve normal quality oil.

### Acidity Control Towards Deodorization Step

Changing the acidity of the oil environment can play a crucial role in the MCPDE- and GE forming-potential. It has been shown that acidic conditions promote the formation of MCPDE ([Destailats et al., 2012a](#)). Conversely MCPDE are rapidly decomposed under alkaline conditions ([Smidrkal et al., 2011](#); [Velišek et al., 2002](#); [Hamlet et al., 2003](#)) and even an equilibrium has been suggested between the formation of MCPDE on the one hand, and GE on the other, depending on the reaction conditions ([Freudenstein et al., 2013](#)), especially the hydrochloric acid level ([Destailats et al., 2012a](#)). This pH character of the oil is especially important in the deodorization step.

All process steps can actually influence the pH of the oil before the deodorization step. Obviously, a dosing alkaline component directly to the deodorization can be more efficient than an alkali treatment like neutralization which is followed by a bleaching step with acid activated bleaching clay. For this reason and further reasons on practicality and economics, various means have been studied to neutralize acidity towards deodorization. Good results have been shown in below examples mostly in small scale experiments, now requiring transfer and implementation to full factory scale.

Sodium carbonate and sodium bicarbonate were shown to lead to low levels of MCPDE when added to oil prior to heating or deodorization ([Smidrkal et al., 2011](#)). Also common bases like sodium hydroxide, potassium hydroxide have been proposed for neutralizing the oil and thereby avoiding high MCPDE levels in the refined oil. Whether this is the result of the complete inhibition of the formation reaction or the degradation of MCPDE formed is unclear at this point.

Next to the bases mentioned before, bases commonly used as interesterification catalyst have shown to lead to low MCPDE levels when added to the oil before high temperature treatment. The use of sodium methoxylate and ethoxylate is claimed to reduce MCPDE level, independent on the question whether the interesterification process takes place or not ([Brüse and Kruidenberg, 2012](#); [Hed et al., 2014](#); [Bhaggan and Werleman, 2012](#)).

Finally, the addition of small traces of soap for this purpose has been described ([Ronglone and Heydinger-Galante, 2012](#)). This option is actually not so surprising if one considers the fact that adding bases will always lead to lowering acidity but also to soap formation. As this is an equilibrium reaction, adding soap will also lead to a lower acidity. After above processes soap still need to be removed by any adequate post treatment step.

Interestingly, it is known since long that soaps can also induce interesterification during high temperature deodorization ([Willems and Padley, 1985](#)). As both interesterification and MCPDE- and GE-formation follow similar mechanisms, both revolving around the formation and removal of a cyclic acyloxonium ion pathway, this may explain why soaps are involved in both processes ([Rozendaal and Macrae, 1997](#); [Destailats et al., 2012a,b](#)).

### Deodorisation

This process step is where the actual formation of MCPDE and GE formation takes place. Oils that are refined chemically, are generally deodorized at 220–240 °C depending on the oil quality and presence of volatile contaminants. In the physical refining process, where FFA is removed, higher temperatures in the range of 235–250 °C, are applied for seed oils, whereas in the case of palm oil 250–270 °C is used to remove the free fatty acid and to achieve the low color targets for the refined oil as well.

Based on experiments in a gastight system it was observed that the reaction formation of MCPDE is practically complete within half hour after heating ([Shimizu et al., 2012](#)). Other experiments also indicate that the deodorization temperature and time have a limited influence on the MCPDE content ([De Greyt, 2014](#); [Matthäus, 2015](#)).



GE formation during deodorization particularly depends on the DAG level (Craft et al., 2012a) and the temperature. Its formation will start at temperatures  $>200^{\circ}\text{C}$  (Destailats et al., 2012a). In heating and deodorization experiments at  $235^{\circ}\text{C}$  temperature, and threshold DAG content of 4% (corresponding to 1.9%–2.5% FFA in the crude oil) was observed, above which GE content increased exponentially. The lower free fatty acid content in crude oil and lower deodorization temperature could explain the lower sensitivity of seed oils to GE formation, compared to palm oil. Obviously, time and temperature are always of the essence as in any chemical reaction. This is clearly illustrated - next to positive effect of lower pressure - by the results from experiments comparing deodorization of corn oil at 235 and  $250^{\circ}\text{C}$  (Papastergiadis et al., 2016).

Reduced GE formation and the same time efficient FFA removal can be obtained applying dual temperature deodorization. This includes short time stripping of volatile compounds at  $240\text{--}260^{\circ}\text{C}$  followed by deodorization at low temperature, 200 or  $220\text{--}230^{\circ}\text{C}$  (Papastergiadis et al., 2016).

In palm oil, a high temperature during deodorization is required not only to remove free fatty acids but to induce 'heat bleaching', i.e. degradation of carotenoids as well, GE will form under these conditions. Therefore such processing requires a second treatment to remove GE through bleaching to meet the low GE levels required nowadays ( $<1$  ppm in the EU), followed by a second low-temperature deodorization step in a 'two-stage' refining process (Brüse and Kruidenberg, 2012). This will be explained further in Removal of Contaminants From Refined Oil section.

Next to temperature and time, also other conditions like pressure and steam will impact the 3-MCPDE and especially GE results. This is for a significant part due to removal of the GE and will therefore be explained further in the next chapter. However, enhancing the GE stripping and reducing formation through high vacuum short path distillation was shown to yield oils with no detectable levels of MCPDE and  $\text{GE} < 0.7$  ppm at a deodorization temperature of  $150\text{--}210^{\circ}\text{C}$  (Pudel et al., 2011). Conventional deodorization of the same oil sample at  $260^{\circ}\text{C}$  resulted in much higher levels of GE (6.4 ppm) and 3MCPDE (3.0 ppm). In the same study poor sensory quality of the refined oil samples was observed, while high color was obtained due to lack of heat bleaching. The insufficient sensory quality was restored applying an additional post-deodorization at a significantly lower temperature ( $180^{\circ}\text{C}$ ) (Pudel et al., 2016). Similar results were also obtained in a dual temperature deodorization process where heat bleaching was obtained at  $250\text{--}270^{\circ}\text{C}$  with a short-residence time, followed by a longer second deodorization step at  $200^{\circ}\text{C}$  to reduce off-flavors without formation of GE (Pudel et al., 2011, 2016).

### Removal of Contaminants From Refined Oil

Glycidyl esters have a molecular weight low enough to be stripped off during regular deodorization conditions, similar to monoglycerides (Goh and Timms, 1985). The stripping of GE was indeed shown to occur, comparing levels obtained in oil after heat treatment in closed ampoules at lab-scale, and samples from regular open-steam stripping. In closed system significantly higher levels of GE were observed (Craft et al., 2012a). Like for other volatiles, the efficiency of removal is dependent on the temperature and stripping steam rate applied (Özdikicierler et al., 2016). This means that during deodorization formation and removal happens simultaneously and GE content of the deodorized oil is determined by the rate difference.

Similar principles can also be applied on finished products. One example is the stripping of GE in packed bed column under low vacuum ( $<1$  mbar) as described by (Hogan, 2017). The high surface area, combined with an enhanced steam stripping efficiency at the low vacuum would result in a refined oil with good quality and low GE.

Another example is the polishing or post-refining of fully refined oils after long distance transport. Such oils and fats show some off-flavors and to get such oils back into the accepted specifications for application in food, they are often re-deodorized upon arrival. This refreshment usually does not impact the 3-MCPDE content, because their formation already took place in the deodorization step applied in the first refining process. However, the glycidyl ester content may be influenced in this deodorization step. The new "equilibrium" depends on GE precursor (DAG) levels and the conditions in the deodorization (temperature, pressure, and steam). This means, that post-refining of oils and fats, if done at mild conditions and on oils containing significant amount of GE, will reduce the GE level. However, if the oil to be re-processed already has a low level of GE, the polishing step itself could contribute to a further increase in the level of GE.

Alternative to polishing by post-deodorisation is a full post-refining step including bleaching and deodorization. This process is applied if metal (e.g. Fe) content is increased, or if lower color is desired, or on purpose to reduce glycidyl ester (GE) levels. During the bleaching step the glycidyl esters are reacted to monoglycerides. The efficiency of this step depends on temperature, bleaching clay type, the amount and the moisture content. Because the reduction of GE is a catalytic process, acid activated clays are more efficient than neutral clays, but both types are capable in removing the glycidyl esters. Because bleaching has negative impact on flavor and taste, after bleaching a post-deodorization step is required. If this deodorization is done at low temperatures ( $130\text{--}190^{\circ}\text{C}$ ) with retention time, steam and pressure at standard conditions, glycidyl ester (GE) levels can be kept at very low levels (Brüse and Kruidenberg, 2012).

A large range of materials was investigated on the removal capacities for 3-MCPDE and GE mainly based on silica, e.g. zeolites, alumina-, magnesium and calcium silicates (Strijowski et al., 2011). Only some of these materials were shown to lead to up to 40% reduced levels of GE. Also cellulose, Kieselguhr, and activated carbon were tested and especially acid-washed active carbon was found to reduce GE levels in palm oil by some 95% (Cheng et al., 2017).

Using higher volatility of 3-MCPDE and also GE compared to Triglycerides (3-MCPDE have similar volatility like Diglycerides) short path evaporation at elevated temperatures of  $200\text{--}280^{\circ}\text{C}$  can be applied (Brüse, 2014, 2015; Mellerup, 2014). Significant 3-MCPDE reduction will only be possible with high yield losses.



## Impact of Oil and Fat Modification Techniques

Next to the standard refining, there are also oil modification steps applied to oils and fats, depending on the required properties and end application. For most of these modification techniques fully refined oils and fats are applied and these processes are common part of oil refineries. The most important technologies in this field fractionation, interesterification and hydrogenation. The following sections will describe the impact of these modification steps on the levels of 3MCPDE and GE.

### Fractionation

The most common way of fractionating fats is the so-called 'dry fractionation' technique, especially used to fractionate palm oil. During fractionation crystals are formed upon cooling the oil. The oil is then separated into a liquid fraction (olein) and a more solid fraction (stearin) by filtration. The 3-MCPDE and GE in this process concentrate in the liquid fraction like also other minor compounds (e.g. colour compounds or DAG). For the stearin fraction, this means that reduction of 3-MCPDE and GE up to 40% can be observed, while for the olein fraction an increase of about 10% can be found. Further fractionation of the olein can lead to an increase in 3-MCPDE and GE in the superolein of about 30%.

### Hydrogenation

The hydrogenation of fats and oils is a metal (normally Nickel) catalyzed process adding hydrogen to the double bonds in unsaturated fatty acids in the oil. The hydrogenation process is applied to increase the melting point by turning these unsaturated fatty acids in less-unsaturated-, and fully saturated ones. This broadens the options for application of these hydrogenated oils and fats. Until now little research was done on the impact of hydrogenation on the levels of 3-MCPDE and GE. However, observations at full factory scale revealed, that there is only limited impact on the levels of 3-MCPDE, while glycidyl esters are normally removed completely. When to be used in food applications, after hydrogenation the oil needs to be re-bleached to guarantee sufficiently low nickel levels. Finally, the oil will undergo another deodorization step during which again GE can be formed.

### Interesterification

Interesterification is a process of rearranging the fatty acids on the glycerol backbone. This rearrangement of the fatty acids results in a new triglyceride composition and therewith new crystallization- and melting properties, which is required for application in many final products.

Two different interesterification processes are applied in the oil and fat industry, chemical interesterification and enzymatic interesterification. Chemical process usually applies an alkaline catalyst such as sodium methoxide, while in enzymatic interesterification a lipase is applied (usually immobilized on a carrier). The enzymatic interesterification is a process under normal conditions and does not show an effect on 3-MCPDE or GE. However, because the process can include a post bleaching step, and will also include deodorization there can again be an overall impact on final glycidyl ester levels depending on the execution of those two steps.

The chemical interesterification process can significantly impact the 3-MCPDE and also the GE level. The interesterification process can be split into the following process steps: 1) contacting the oil or fat blend with the catalyst, 2) neutralization of the catalyst to stop the reaction, 3) post-bleaching, and 4) post-deodorisation. During the first step 3-MCPDE is reduced. At the same time, glycidyl ester levels increase during this step. After the subsequent neutralization of the catalyst and the bleaching, these glycidyl esters are removed. Again some GE could be formed in the final deodorization step required after the interesterification bleaching.

## Conclusions

Science showed health concerns of 3MCPDE and GE, process contaminants formed during the refining of oils and fats. Since about a decade, research and development work did improve understanding on toxicology and bioavailability of these compounds, did improve analytical methods, did lead to better understanding of the formation of these compounds, and helped to develop mitigation measures that could partly be implemented. The developed mitigation methods are dealing with avoidance of precursors in the supply chain, reduction of precursors during refining, suppression of formation during the deodorization step and removal of 3-MCPDE and especially GE from the finished products. Because the formation mechanism of both compounds is different, and because single mitigation methods are often not sufficient, in many cases combinations of mitigation methods are required. The selection of methods to be implemented need also to take into account the type of oil involved, its quality, the available technologies in a plant, and possible environmental barriers.

Mitigation methods needed to be scaled up from small lab scale to industrial scale, which first was done focusing on special products and now is also applied more for bulk products. During implementation of mitigation measures no compromises can be made on food-safety or quality aspects, which in many cases will require extra steps or even double refining.

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# Mineral Oils in Food: An Update

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## Glossary

GCxGC comprehensive two-dimensional gas chromatography

MOSH mineral oil saturated hydrocarbons

MOAH mineral oil aromatic hydrocarbons

POSH polymer oligomeric saturated hydrocarbons

White mineral oil mineral oil product low in MOAH

## Definition of Mineral Oil

What is referred to as “mineral oil” in foods are products predominantly derived from crude mineral oil through various raffination procedures. However, analytically they cannot be distinguished from Fischer-Tropsch oils made of natural gas or even from methane obtained from wastes. For toxicological reasons, the main components were separated into mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH) (Biedermann et al., 2009). Rather crude products may also include heterocyclic components, such as thiophenes (sometimes used as markers).

Mineral oil products vary widely, primarily according to the raffination process. They differ according to molecular mass range (distillation), MOAH content (extraction of MOAH, hydrogenation) and viscosity (ranging from low viscosity oils to waxes; dewaxing).

## Mineral Oil in Food: A Historical View

Most of the early findings are from the Kantonales Labor Zürich, Switzerland, a small official food control authority, which meant that, whenever possible, measures were taken to stop the contamination. Much of the history was determined by the difficulty to analyze mineral oils as contaminants. In the 1980s, the Kantonales Labor Zürich developed on-line coupled HPLC-GC, i.e. the pre-separation of the sample or extract by normal phase HPLC and the automated transfer of the complete HPLC fraction containing the components of interest into GC. This was the technique of choice, but few other laboratories dealt with the subject up to probably 2008, since on-line HPLC-GC was considered too complicated. This caused progress to be slow. The method used up to 2008 only determined the MOSH; the MOAH content was estimated considering the detected mineral oil product.

In 1986, during the analysis of the fatty acid composition, more than 1% MOSH were detected in a biscuit, which was from spraying the baking mold with a white mineral oil used as release agent. In 1989, it was incidentally noted that the hazelnuts on the market (but not those from the own garden) contained typically 20–50 mg kg<sup>-1</sup> MOSH. It turned out that the hazelnuts (from Turkey, some from Italy) were imported in jute bags of around 50 kg. These bags, most manufactured in Bangladesh, were made of fibers batched with around 7% of a mineral oil to render them suitable for spinning. Already the next harvest largely arrived in jute bags batched with vegetable oils. In 1991, a first article was published on hazelnuts, coffee, cocoa beans/chocolate and rice contaminated from jute and sisal bags at up to many 100 mg kg<sup>-1</sup> of mineral oil (Grob et al., 1991a).

The analysis of the MOAH in a batching oil revealed polyaromatic hydrocarbons up to the benzopyrenes, but largely alkylated, i.e. different from the polycyclic aromatic hydrocarbons (PAH) formed at high temperatures (Grob et al., 1991b). Only after it had also been shown that such MOAH were present in food (Moret et al., 1997), industry took measures to switch to bags free of mineral batching oil for imports to Europe, but the bags used outside Europe are still mostly the conventional ones.

Subsequently many more sources of food contamination with mineral oil hydrocarbons were identified. White oils were used to get rice shiny after raffination (typically 1000–3000 mg kg<sup>-1</sup> rice), as release agents (in bakery products typically 600–2000 mg kg<sup>-1</sup>), lubricating oils, hydraulic oils, cleaning agents used in food manufacturing (e.g. vegetable oils), defoamers, additives in plastics, binders for fines in animal feeds or dust binders for wheat and rice. Waxes were used in paper for cheese and processed meat or to improve water-resistance of paperboard, but also as coatings and glazing agents for confectionery. At public collection sites, used motor oils and similar wastes ended up in used edible oils and in this way entered feeds for hens and pigs. Finally, diesel oil and lubricants from harvesting machinery, as well as aerosols from environmental pollution, mainly from diesel oil, lubricants and debris from tires and road bitumen, were shown to contaminate foods. A summary was given in the opinion of the European Food Safety Authority (EFSA) from 2012 (EFSA CONTAM Panel, 2012) and a review by Purcaro et al. (2016), but the list of sources is probably still incomplete. Many of these sources of contamination were reduced or stopped, largely without legal measures or enforcement outside Switzerland.

In 2008, mineral oil was detected in Ukrainian sunflower oil, which drew the attention to this type of contamination and triggered numerous laboratories to analyse mineral hydrocarbons. EFSA evaluated the related health risk for crude and refined sunflower oils with up to 7300 and 2000 mg kg<sup>-1</sup> mineral oil, respectively, and concluded that “although being undesirable for human consumption, [it] would not be of public health concerns” (EFSA, 2008). However, it was neglected that the mineral oil contained 17%–37% MOAH (Biedermann and Grob, 2009). This prompted the further development of the analytical method to enable the determination of the MOAH fraction (Biedermann et al., 2009).

Since the year 2000, recycling of polyethylene terephthalate (PET) for food contact materials (FCMs) became widely discussed, and EU Regulation 282/2008 provided strict rules on the materials acceptable for recycling and the cleaning process. This created a striking contrast to recycling of paper and board, since most recycled fiber material is not made for food contact and usually no cleaning is applied. As mineral oils were a timely subject, they seemed suited to bring not only the recycling of paper and board to the attention of authorities, but also the use of mineral oil solvents in off-set printing inks used for FCMs (Biedermann and Grob, 2010), in particular to the German Federal Institute for Risk Assessment (BfR), widely recognized as the leading reference for paper and board in food contact. This initiative triggered an avalanche of activities.

Printers rapidly switched to mineral-oil-free inks. After a statement of the BfR in December 2009 (German Federal Institute, 2009), the German Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) presented a first draft of a regulation on the migration of MOSH and MOAH from recycled paper and board. It struggled with numerous difficulties and, for a number of reasons, was still not implemented in 2017: lacking assessment to derive risk-based legal limits, the contamination not being limited to migration from recycled paper and board (a more general regulation would be preferable), mineral oil hydrocarbons just being an example of a far wider range of contaminants (Biedermann and Grob, 2013) and the subject not limited to Germany (an EU regulation would be more adequate). This placed the German authorities under pressure, primarily by NGOs (which seemed unfair, as the German authorities took the initiative).

In early 2017, the European Commission initiated a project on a broad monitoring of mineral oil hydrocarbons in foods (Commission Recommendation (EU), 2017/84), intended as a database for an evaluation by EFSA and possible measures.

## Chemical Analysis

MOSH and MOAH are complex and widely varying mixtures; their determination as contaminants is demanding. Flame ionization detection (FID) is the method of choice for quantitation, since all hydrocarbons give virtually the same response per mass. Gas chromatography is best suited, not only because of FID, but also for the separation from other hydrocarbons. However, MOSH and MOAH mainly form broad humps of unresolved chemical species, which presupposes their isolation from all other constituents in the extracts and causes sensitivity to be relatively low.

Pre-separation can be achieved by liquid chromatography on small columns packed with silica gel modified with a small amount of silver nitrate (e.g. Fiselier et al., 2013), but most routine laboratories use HPLC on silica gel on-line and in automated manner coupled to GC-FID. Coupling to GC requires a technique enabling to transfer whole HPLC fractions, usually comprising 300–500 µL of eluent. In the case of mineral oil analysis, this is done by eluent evaporation in a 7–10 m × 0.53 mm i.d. uncoated precolumn partially concurrently with the transfer, exploiting solvent trapping and the retention gap technique. The solvent vapors are largely discharged through a vapor exit installed at the exit of the precolumn (Biedermann and Grob, 2012a).

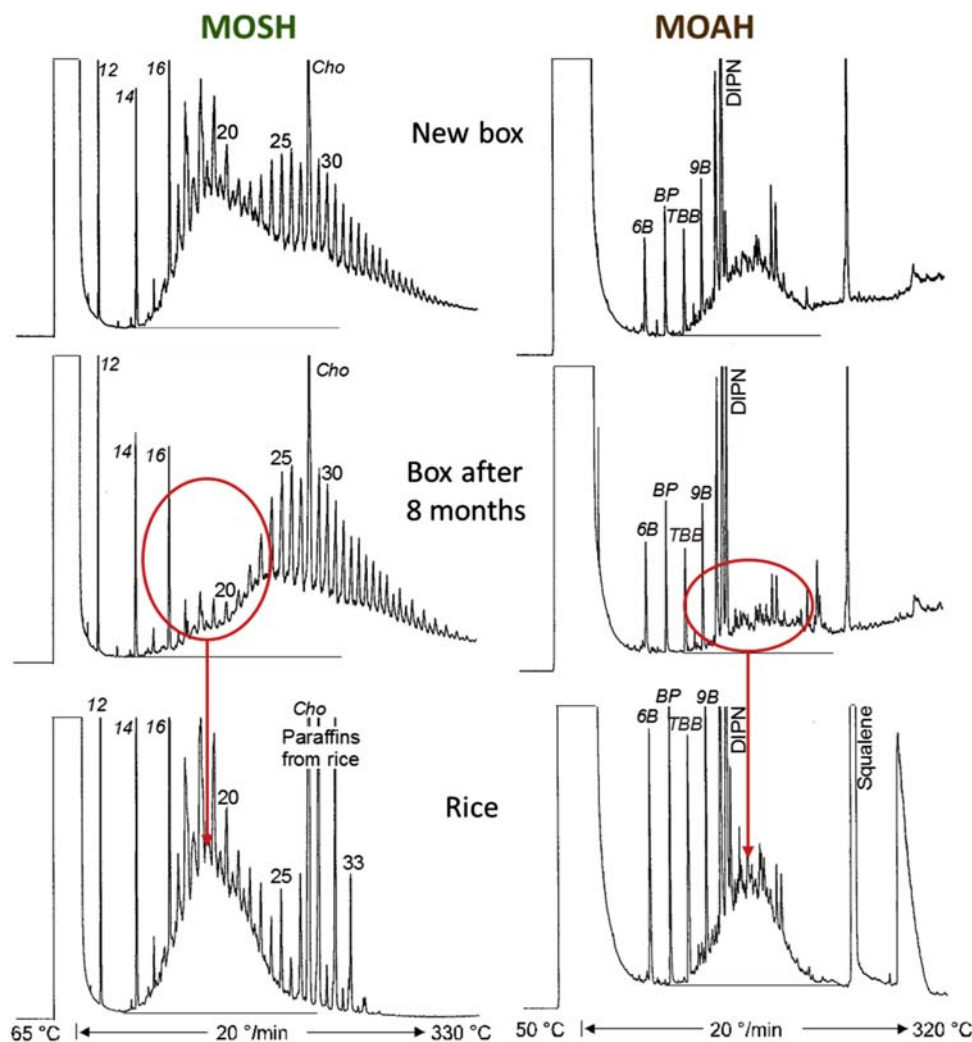
The most critical step is the interpretation of the chromatograms, particularly drawing the baseline above which the hump is integrated and the recognition of interfering hydrocarbons of other origins, such as *n*-alkanes of plant waxes, squalene and other terpenes as well as synthetic hydrocarbons (such as POSH or oligomers of polyolefins used in paperboard coatings). This presupposes experience on how such interferences are presenting themselves in chromatograms (Biedermann and Grob, 2012b), but often also auxiliary techniques, either to remove the interferences, e.g. of long chain *n*-alkanes by activated aluminum oxide or epoxidation of olefins (Biedermann and Grob, 2012a). A more detailed analysis, e.g. better distinguishing between MOSH and POSH or characterizing the composition of the MOAH, can be obtained by comprehensive two-dimensional gas chromatography (GCxGC) (Biedermann and Grob, 2015).

Fig. 1 shows HPLC-GC-FID chromatograms of MOSH and MOAH related to a sample of rice packed in a box of recycled board (Biedermann and Grob, 2010). The top chromatograms (MOSH and MOAH) are from an extract of the new box. The MOSH formed a hump ranging from *n*-C<sub>14</sub> to about *n*-C<sub>50</sub>, with some *n*-alkanes on top. The major part (from *n*-C<sub>14</sub> to about *n*-C<sub>25</sub>) was from printing inks used in the recycled material, primarily newspapers. The later eluted *n*-alkanes are from waxes, probably used to improve the water resistance of some recycled paper and board. They are located on a part of the hump that could have originated from defoaming agents used in pulping.

After eight months of storage, most of the early eluted MOSH were transferred to the rice (see circle and arrow; 19 mg kg<sup>-1</sup> rice). Since transfer was through the gas phase (evaporation and recondensation), the migration was limited to hydrocarbons up to about *n*-C<sub>24</sub>.

The MOAH formed a hump, in terms of volatility or GC retention times corresponding to the early eluted MOSH (probably part of the ink solvent). This hump was topped by diisopropyl naphthalene (DIPN) from recycled carbonless copy paper (the most specific marker for mineral hydrocarbons from recycled paper and board) and a later eluted pair of peaks probably from the coating (butylene styrene). Again, the more volatile compounds largely migrated into the rice (5 mg kg<sup>-1</sup> rice).





**Figure 1** HPLC-GC-FID chromatograms of extracts from a new box of recycled paperboard, the box after storing rice in it during eight months and of the rice into which the volatile hydrocarbons had migrated. DIPN, diisopropyl naphthalene; numbers, n-alkanes of given carbon number. 12, 14, 16, Cho (cholestane), 5B (n-pentyl benzene), BP (biphenyl), TBB (tri-tert. butyl benzene) and 9B (n-nonyl benzene), internal standards. Adapted from Biedermann and Grob (2010).

## Toxicity

The earlier risk assessments were mostly limited to MOSH, i.e. “white” (largely MOAH-free) products. In the 1990s, a number of subchronic 90-days studies with rats were reported using a range of mineral oil products characterized by viscosity and range of molecular mass. The focal end point was granuloma formation in liver, spleen and mesenteric lymph nodes. Mainly based on the results of Smith et al. (1996), the Joint FAO/WHO Expert Committee on Food Additives (JECFA) established a classification in 2002 that is widely respected up to today. High acceptable daily intakes (ADIs) were allocated to microcrystalline waxes (0–20 mg kg<sup>−1</sup> body weight; bw; specified by an average relative molecular mass ≥500 Da and a carbon number at the 5% distillation point ≥25), to high viscosity mineral oils P100 (0–20 mg kg<sup>−1</sup> bw; average mass ≥500 Da and a carbon number at the 5% distillation point ≥28), and to medium and low viscosity class I oils P70 (0–10 mg kg<sup>−1</sup> bw; average mass ≥480 Da and a carbon number at the 5% distillation point ≥25). To waxes or oils of lower mass range, no ADI or a temporary ADI of 0.01 mg kg<sup>−1</sup> bw were allocated, respectively, the low ADI being withdrawn in 2012 owing to insufficient data. Hence, products of a relatively high molecular mass were strongly privileged and considered acceptable up to very high intake.

This evaluation did not take into account that before 1990 numerous papers reported frequent occurrence of granulomas in human tissues that had been associated to mineral oil (e.g. Boitnott and Margolis, 1970; Dincsoy et al., 1982; Cruickshank, 1984).

Early doubts about the JECFA classification were cast by Scotter et al. (2003), showing that the strongest MOSH accumulation in Fischer 344 rats fell into the range of the oils considered of least concern. Also in human adipose tissue (obtained from Caesarean sections), much of the strongly accumulated MOSH was in this range (Concin et al., 2008).



In 2012, EFSA published an opinion on mineral hydrocarbons in food (EFSA, 2012). No health-based guidance values were derived for MOSH because of insufficient data, particularly with regard to accumulation, for MOAH because of potential presence of genotoxic constituents. Dietary exposure to MOSH in the European population, estimated as 0.03–0.3 mg kg<sup>-1</sup> bw/day, was considered of “potential concern”. MOAH with three or more rings were also considered of “potential concern”, as they may include mutagenic and carcinogenic species.

In 2014, human tissues were analyzed for mineral hydrocarbons. No MOAH were detectable. The mean MOSH concentration in the mesenteric lymph nodes (MLN) was 223 mg kg<sup>-1</sup>, in liver 131 mg kg<sup>-1</sup>, in adipose tissue 130 mg kg<sup>-1</sup>, in spleen 93 mg kg<sup>-1</sup> and in lung 12 mg kg<sup>-1</sup>. The maxima, found in MLN and spleen, were 1390 and 1400 mg kg<sup>-1</sup>, respectively. For a quarter of the subjects, the calculated total amount of MOSH in the body exceeded 5 g (Barp et al., 2014). The MOSH accumulated in liver and spleen clearly differed from those in adipose tissue in terms of mass distribution and types of structures (Biedermann et al., 2015).

In a project initiated by EFSA, MOSH accumulation in terms of mass distribution and composition was investigated in Fischer 344 rats (Biedermann et al., 2015; Barp et al., 2017). The main findings were that the concentrations in human tissues were far higher than it would be extrapolated from animal experiments and estimated human exposure, presumably due to long-term accumulation and a higher absorption at lower concentrations in food. At the highest concentrations measured in human spleen, a significantly increased organ weight in the rats was noted. Maximum accumulation in rat liver and spleen was around C<sub>29</sub>, i.e. in the range of the class I oils so far considered of low concern. It was concluded that the ADI for class I oils is far too high (300–3000 times above the present exposure estimated by EFSA and already considered of “potential concern”) and that the classification should be reconsidered.

In Fischer 344 rats, granuloma formation was linked to wax components, primarily *n*-alkanes and *n*-alkyl monocyclic MOSH; a dewaxed oil of similar molecular mass distribution did not trigger granuloma formation. However, it had previously been reported that the strong accumulation of *n*-alkanes in Fischer 344 rats is not observed in other animals, particularly Sprague Dawley rats (e.g. Griffis et al., 2010). Since *n*-alkanes from natural sources (such as surface waxes of, e.g., apples) are little accumulated in human tissues, it was questioned whether Fischer 344 rats are adequate models for granuloma formation in humans. However, this would imply that the granulomas observed in human tissues would have to be formed by another mechanism, possibly not observed in Fischer 344 rats.

The genotoxicity of the MOAH fraction depends on its composition. Crude mineral oils contain genotoxic constituents (IARC, 1984); in fact, an oil used for batching jute fibers has been shown to be a tumor promoter on mouse skin (Mehrotra et al., 1987). For technical products, however, oils are refined to render them negative in the mouse skin test or the extraction test IP 346 (Mackereer et al., 2003; Gray et al., 2013). Raffination involves extraction and partial hydrogenation, which preferentially remove the less alkylated polyaromatic MOAH. At a given point, in some oils at MOAH concentrations above 15%, merely monoaromatic MOAH are left, including polycyclic hydrocarbons of which only one ring (or two isolated rings) are aromatic. There are neither indications that mono- or diaromatic MOAH include genotoxic species, nor is there generally recognized evidence of absence or data to estimate the potency of genotoxicity, which hinders the specification of a health-based guidance value.

## Conclusion

Up to a few years ago, “food grade” mineral oils were considered quasi edible. For instance, near Zürich, every week a truckload of mineral oil was delivered to an industrial bakery and used in the production of the bakery wares. This rather “loose” attitude was not supported by solid risk assessment – the concentrations in human tissues were assumed far lower than they really were. Fortunately this has changed and the large entries into food have ceased.

However, there is urgent need for better clarification of toxicity in order to implement improvement where necessary, but also to stop exaggerated alerts. Questions concern the genotoxic potency of MOAH in mineral oil products of given grades. For the MOSH, better safety assessment is needed for the concentrations occurring in human tissues. As high values are close to or even above those in animal experiments at the highest dose, no adequate safety margin can be derived. More toxicological end points should be taken into consideration, including increased organ weights.

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## N-Nitroso Compounds in Foods

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### Glossary

BMDL benchmark dose lower confidence limit

EFSA European Food Safety Authority

NOC N-nitroso compounds

SCCS Scientific Committee on Consumer Safety of the European Union

### Introduction

Knowledge about N-nitroso compounds (NOC) has been accumulated in studies dating back to the early fifties of the 20th century up to the present time. The strong carcinogenic potential of N-Nitroso-dimethylamine (NDMA) has already been described in 1956 by Barnes and Magee (Barnes and Magee, 1954). In the following decennia, more than 300 compounds were investigated chemically and biologically. Carcinogenicity and other biological effects were found to depend on structural features, and the overwhelming part was found to be mutagenic and carcinogenic in-vitro and in-vivo (Preussmann, 1990; Reed, 1996).

In 1964 (Ender et al., 1964), the occurrence of a hepatotoxic factor was reported in herring meal produced from sodium nitrite-preserved herring used as feed for sheep. Although appropriate analytical methods were not available yet at that time, NDMA was identified as the most probable causative agent, apparently being present at rather high concentrations in that specific feed. NDMA was suspected to have arisen from the treatment of the fishmeal with nitrite. These early findings triggered a large series of analytical studies to uncover the presence of N-nitroso compounds in nitrite/nitrate treated food. The then challenging and difficult analytical detection of N-nitroso compounds greatly benefitted from the advent of highly specific and sensitive detection methods, such as the thermal energy analyzer (TEA) (Fine and Rounbehler, 1975) and later coupled gas chromatography (GC) or liquid chromatography (LC)/triple mass spectrometry (LC-MS/MS/MS) methodology that allowed specific measurements at low parts per billion (ppb) levels in foods and other biological materials. N-nitroso compounds were subsequently detected in many areas of the human environment, including foods, feeds, cosmetics, consumer goods, pesticides, drugs and at working places involved in production and handling of such items, mostly (but not always) in rather low concentrations.

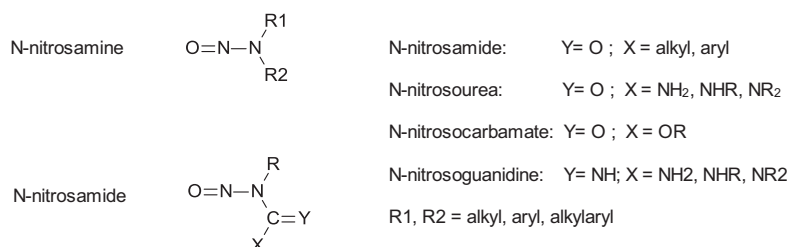
### Chemistry and Formation

There are two main categories of NOC: N-nitrosamines and N-nitrosamides (Fig. 1).

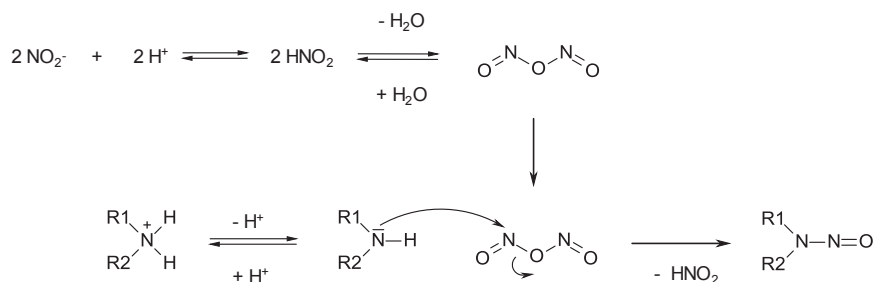
Whereas N-nitrosamines are chemically rather stable, N-nitrosamides (encompassing N-nitrosourea, -carbamate and -guanidine derivatives) are less stable, directly generating electrophilic alkylating or arylating species, depending on their structure and the respective (physico-) chemical conditions.

In practically all situations where nitrosating agents encounter N-nitrosatable amino compounds, NOC may be formed. In aqueous acidic environments, amines (classically secondary ones) react with nitrous acid. Under proton catalysis, nitrite and nitrous acid generate the actual nitrosating species, either dinitrogen trioxide ( $N_2O_3$ ) or tetroxide ( $N_2O_4$ ) or nitrous acidium (Nitrosonium) ion  $NO^+ \cdot H_2O$ . The mechanism of nitrosation is exemplified for a secondary amine in Fig. 2.

Since only the unprotonated amine nitrogen undergoes nitrosation, the  $pK_a$  value of a given amine governs the nitrosation rate. Strongly basic dialkylamines ( $pK_a > 9.5$ ) have their maximal nitrosation rate at around pH 3.5, close to the  $pK_a$  value of  $HNO_2$  (Mirvish, 1975). In general, weakly basic amines, such as morpholine ( $pK_a = 8.7$ ) are much more rapidly nitrosated than strongly



**Figure 1** Structures of NOCs.



**Figure 2** Exemplified nitrosation reaction mechanism.

basic ones. In the presence of aldehydes, especially formaldehyde, nitrosation is strongly catalyzed and proceeds even at neutral or basic pH values. This reaction has been found relevant for certain environmental conditions (including specific working places) where there is co-occurrence of amines, aldehydes and nitrosating agents (Keefer and Roller, 1973).

Early on, acid-catalyzed nitrosation has been suspected to occur in the stomach (Druckrey et al., 1961) as a possible way of in-vivo formation of N-nitroso compounds. This in-vivo formation has been found relevant especially for weakly basic amines.

Tertiary amines may also generate nitrosamines by dealkylating nitrosation, however in general at much lower reaction rates as compared to nitrosamine formation from secondary amines (Smith and Loeppky, 1967). Nitrosation of primary amines in aqueous solution results in unstable diazonium intermediates that react with water to alcohols.

The nitrosation reaction can be inhibited by NOx scavengers, such as ascorbic acid,  $\alpha$ -tocopherol, primary amines, tannins or other phenolic compounds (Loeppky et al., 1994). Total human exposure to NOC results from exogenous exposure to preformed NOC but also from endogenous exposure. The latter can result from in-vivo nitrosation by intake or in-vivo formation of precursor compounds. The relative proportion of exogenous versus endogenous NOC exposure and their respective human health relevance is still not fully elucidated.

The total “exogenous” exposure to NOC may encompass exposure related to “life style factors” (such as tobacco, food and cosmetics, drugs, household products, air) but also to specific working place conditions (e.g. in the rubber, leather, metal, chemical or related industry). The endogenous exposure, based on in-vivo formation, depends on the uptake of precursor agents (nitrate, nitrite, NOx and N-nitrosatable amino compounds) and on NOC formation kinetics predominantly in the gastro-intestinal tract.

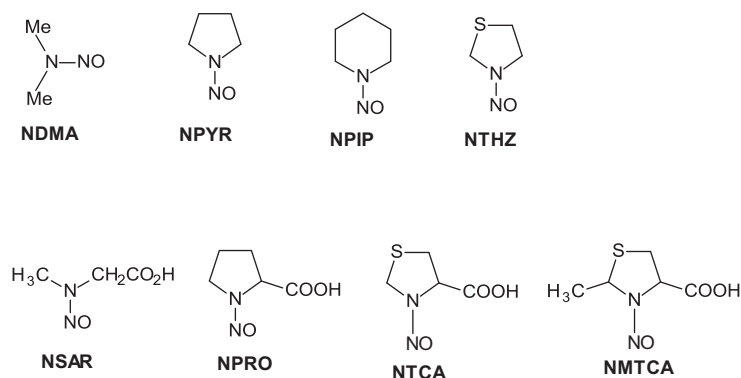
## Occurrence in Food

### Formation

Contamination of food with nitrosamines is primarily connected to various processes during manufacture, storage and/or cooking or to migration from packaging materials. The NOC formation requires the presence of N-nitrosatable amines and of nitrosating agents (Sen, 1988; Bartsch and Spiegelhalter, 1996; Pfundstein et al., 1991). Nitrogen oxides (NOx) primarily may result from addition of nitrate and/or nitrite which are interconvertible by microbial activity, for instance during the curing process of meat products. Additionally, a variety of technical processes, handling and storage conditions to which food or food constituents are exposed, such as direct drying or kilning and smoking have been found to favour NOC formation from food constituents by contact with NOx. Nitrosamines most frequently found in foods are NDMA, N-nitrosopyrrolidine (NPYR), N-nitrosopiperidine (NPIP) and N-nitrosothiazolidine (NTHZ). Certain N-nitrosated amino acids are also found, including the N-nitroso products of sarcosine (NSAR), 3-hydroxyproline and proline (NPRO), thiazolidine-4-carboxylic acid (NTCA), oxazolidine-4-carboxylic acid (NOCA) and N-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) as well as its oxazolidine analog N-nitroso-2-methyl-oxazolidine-4-carboxylic acid (NMOCA) (Fig. 3) (Tricker and Kubacki, 1992). NPRO and NTCA are most frequently found in foods. Of note, with the exception of NSAR, which is a relatively weak carcinogen, N-nitrosated amino acids are not considered mutagenic or carcinogenic.

### Meat and Meat Products

The formation of N-nitrosamines in meat and meat products can occur as a result of various processing techniques such as smoking, salting and/or curing. Curing involves the development of a curing microflora that is responsible for the generation of NO from nitrite and nitrate. Curing is an ancient technique of food preservation bringing about extended shelf life, but also heat stable colour and flavour development of cured products. The characteristic red colour of cured meats results from the formation of a NO-myoglobin complex. Curing meat with nitrite/nitrate is still a common practice. In 2017 the European Food Safety Authority (EFSA) has re-evaluated the safety of nitrate and nitrite added to food. An acceptable daily intake for nitrate of 3.7 mg/kg bw/day was derived, while for nitrite an ADI of 0.07 mg/kg bw/day was calculated, based on increased methaemoglobin as benchmark response (BMR) (EFSA et al., 2017a, 2017b). However, EFSA concluded that it was not possible to discern the amount of NOC formed by addition of nitrite at the authorized levels from those produced already in the food matrix where nitrite has not been added.



**Figure 3** Structure of NOCs found in food.

In the early eighties volatile N-nitrosamines were found in about one-third of almost 400 meat and sausage samples from the German and also from the US market (Spiegelhalder et al., 1980a, 1980b; Sen et al., 1976). Reduction of nitrite content in curing salt, decreased addition of nitrate and regular use of nitrosation inhibitors, such as ascorbic acid and/or tocopherols, brought about a significant reduction in contents of volatile N-nitrosamines in meat products as shown in studies from the late eighties where levels up to 5 µg/kg (Italy), 8 µg/kg (Sweden) or 14 µg/kg (China) were found while in Germany levels were only up to 2.5 µg/kg. Mainly NDMA, NPYR, NPIP, NDBA and NDEA were found.

Studies in model systems showed that the content of NPYR in cured meat products can increase as a consequence of thermal treatment or processing (Janzowski et al., 1978). Thermal decarboxylation of nitrosated amino acids generates the respective volatile N-nitrosamines (NPRO → NPYR; NSAR → NDMA). NPYR can be formed by heat-induced decarboxylation of nitrosoproline as well as by direct nitrosation of pyrrolidine which is reported to be present in meat products at levels up to 20 mg/kg (Pfundstein et al., 1991). Thermal decarboxylation of NPRO may also occur from N-terminal NPRO containing peptides (Janzowski et al., 1978; Tricker et al., 1984, 1985). Relative high amounts of peptide bound NPRO can be released by enzymatic degradation or chemical hydrolysis (Dunn and Stich, 1984). Non volatile NOC have been found in nitrite treated or smoked meat products in substantial concentrations, for example in cured meat up to 360 µg/kg NPRO (Janzowski et al., 1978; Tricker et al., 1984, 1985b). N-nitrosothiazolidine (NTHZ), has been found in smoke-treated products in concentrations up to 10 µg/kg (Helgason et al., 1984; Lijinsky et al., 1988; Ellen et al., 1986) and up to 1010 µg/kg in fried bacon (Massey et al., 1991). N-Nitroso-oxazolidine (NOCA) was identified in smoked mutton (40–70 µg/kg) together with its 5-methyl derivative (MeNOCA) in concentrations of 30–120 µg/kg (Tricker and Kubacki, 1992).

Formation of NOCA and NTCA in food may occur by reaction of formaldehyde generated during the smoking process with serine, threonine or cysteine present in the processed food to yield oxazolidine, 5-methyl-oxazolidine- or thiazolidine-4-carboxylic acid (OCA, MeOCA or TCA), which can subsequently be nitrosated to the corresponding NOC (Tricker and Kubacki, 1992; Sen et al., 1986). NTCA was found in different smoke treated meat products in amounts up to 1 mg/kg (Helgason et al., 1984; Sen et al., 1986), in uncooked bacon up to 1.4 mg/kg (Pensabene and Fiddler, 1985), and up to 13.7 mg/kg in fried bacon (Sen et al., 1986). Recently analysis by the Bavarian Food Surveillance Office from 2014 showed that in 40 samples of smoked bacon only 15 samples contained no NOC above the limit of detection while in 25 samples NOCs were detected. From these 25 samples, 18 contained NDBA (0.37–3.9 µg/kg), 5 samples NDMA (0.44–0.5 µg/kg), 2 samples NDPA (0.43–3.0 µg/kg) and 18 samples NPIP (0.31–7.7 µg/kg) (Bavarian Office, 2015).

### Fish and Fish Products

In fish and fish products, NDMA is the most commonly reported volatile N-nitrosamine, detected in a variety of fish products (cooked or smoked fish samples and conserved fish) in concentrations from 0.5–8.0 µg/kg (Tricker et al., 1991). Contents appear to depend on the way of preparation. Fish heated over an open gas flame (high NO<sub>x</sub>-content) showed a 30-fold increase of NDMA, whereas cooking with an electric hot plate showed no such effect (Maki et al., 1991). The apparently higher NDMA concentration reported for Asian fish products compared to western diets may be a consequence of the different preparation or conservation methods (curing, smoking) (Bartsch and Spiegelhalder, 1996). In a study from Finland on smoked fish, NDMA (up to 28.5 µg/kg) was found (Penttilä et al., 1990).

To summarise, in EU countries means of 0.2–3.0 µg/kg NDMA were observed for fish products. Data from Japan and China reported sporadically higher values (mean up to 5.9 µg/kg in certain Japanese) with even up to >130 µg/kg in certain Chinese fish products (Habermeyer et al., 2009).

### Beer and Other Alcoholic Beverages

Contamination of beer with N-nitrosamines was found to primarily result from thermal treatment of the malt (kilning). In 70% of 158 different beer samples tested, NDMA was identified at a mean concentration of 2.7 µg/kg with the highest concentration found

of 68 µg/kg. Other N-nitrosamines like NDEA and NPYR were only found very rarely (Spiegelhalder et al., 1979, 1980a). Precursors of NDMA in malt are hordenin, gramine and dimethylamine, the latter in concentrations of 5.5–12 mg/kg (Pfundstein et al., 1991). Hordenin and gramine are naturally occurring alkaloids with a tertiary amine structure and are produced in barley during the malt germination process. Gramine is nitrosated more rapidly than hordenin, however hordenin concentrations are higher (Mangino and Scanlan, 1985). It has been found that the heating technique is of major importance. Heating the air in the kilning process by direct firing resulted in substantially higher amounts of NDMA in the malt (up to 1080 µg/kg) compared to indirect heating using heat exchanger technology (Spiegelhalder and Preussmann, 1983). In particular gas burner flames operating at high temperatures (>1400 °C) generate substantial amounts of NOx that can react with amines in the malt resulting in increased NDMA formation in the kiln. Reduced NOx generation and resulting lower NDMA formation was achieved by modifications in the firing technique using indirect heating and lower operating temperatures (Tricker and Kubacki, 1992; Tricker et al., 1991). This is reflected by the observed NDMA levels in beer samples from the German market which were at a mean level of 2.7 µg/kg (<0.5–68 µg/kg) in 1977/78 (Spiegelhalder et al., 1979). The levels subsequently decreased to 0.28 µg/kg (<0.5–9.2 µg/kg) and 0.44 µg/kg (<0.5–7 µg/kg) in 1980 and 1981 (Spiegelhalder and Preussmann, 1983; Frommberger, 1985) and further down to about 0.1–0.15 µg/kg (<0.5–1.7 µg/kg) in the late eighties (Ministerium, 1983; Tricker et al., 1991). In the years 2000–2, about 15% of the beer and malt samples showed NDMA levels above the technical guidance value for Germany of 0.5 µg/kg for beer and 2.5 µg/kg for malt. Studies from this decade on beer from the German market showed the majority of the samples having NDMA levels either below the limit of detection (<0.1 µg/kg) or <0.5 µg/kg (see Table 1) (Bayrisches).

Only up to 2.2% of the samples showed NDMA above the technical guidance value of 0.5 µg/kg for beer. Similar results were observed for NDMA in the brewing malt. The reduction of NDMA concentrations in beer was observed also in other countries showing similarly reduced NDMA levels down to 0.074 µg/kg in USA/Canada 1991 (Scanlan et al., 1991), to 0.1 µg/kg in Sweden 1994 (Österdahl et al., 1994) or to 0.11 µg/kg in Spain 1996 (Izquierdo-Pulido et al., 1996).

Accordingly, for the US and Canada, a reduction of NDMA contaminations down to 1%–5% of previous levels has been reported by Scanlan and Barbour (Scanlan et al., 1991). Since NDMA levels in beer have continued to be reduced, it appears worthwhile to reconsider today's relative contribution of beer consumption to the total dietary exposure to N-nitrosamines. Approximately 30% of dietary NDMA exposure have been estimated earlier to result from beer consumption in Germany, with an estimated contribution of about 0.1 µg NDMA per person and day (Österdahl, 1988; Habermeyer et al., 2009). The daily uptake of NDMA resulting from the consumption of beer in France and Spain was estimated to be 0.02–0.03 µg per person (Mavelle et al., 1991; Izquierdo-Pulido et al., 1996). Other alcoholic beverages were also studied for contamination with volatile N-nitrosamines. Goff and Fine (Goff and Fine, 1979) examined wine, sherry, liqueur, gin, brandy, vodka, rum, and whiskey. Only the latter sporadically showed some NDMA contamination with concentrations between 0.3–3 µg/kg.

### Milk Products and Cheese

In general, only rather low concentrations of NDMA were found in cheese and milk powder. In two of eight cheese samples, NDMA was detected at concentrations of 0.8–1.1 µg/kg (Tricker et al., 1991). This confirmed results from an earlier study with 200 cheese samples where a rather low contamination was reported (Spiegelhalder et al., 1980a). Studies by Sen and Seaman (Sen and Seaman, 1981) reported a mean NDMA concentration of 0.4 µg/kg in Canadian samples, whereas in two studies in Europe, even smaller trace amounts of NDMA were found in milk powder (mean: 0.07 µg/kg NDMA) (Österdahl, 1988; Tricker et al., 1991). In the US, mean NDMA concentrations of 0.7–1.05 µg/kg were reported for milk powder products (Scanlan et al., 1994).

### Other Foods

Low amounts of N-nitrosamines (<1 µg/kg, primarily NDMA) have been reported for instant coffee, infant formula, cocoa, powdered egg, and instant soup (Österdahl, 1988; Tricker et al., 1991; Sen and Seaman, 1981). Spices showed contaminations with NDMA (mean: 0.3 µg/kg, max: 1.4 µg/kg), NPYR (mean: 1.75 µg/kg, max: 29 µg/kg) and NPIP (mean: 1.8 µg/kg, max:

**Table 1** NDMA in beer from the German market

Year/Sample	Number of samples analysed	NDMA < LOD	NDMA below technical guidance value (<0.5 µg/kg)	NDMA > 0.5 µg/kg
2011	132	124 (93.9%)	6 (4.6%)	2 (1.5%)
2012	182	180 (98.9%)	2 (1.1%)	–
2013	197	193 (98.0%)	4 (2.0%)	–
2014	314	202 (64.3%)	105 (33.4%)	7 (2.2%) <sup>a</sup>
2016 - craft beer	88	22 (25.0%)	66 (75.0%)	–
2016 - normal beer	156	31 (19.9%)	123 (78.8%)	2 (1.3%)

LOD, Limit of detection: 0.1 µg/kg.

<sup>a</sup>3 samples using a smoked malt.

Table compiled by authors from data given in Ref. Bayrisches.



23 µg/kg) (Tricker et al., 1991). Peak contaminations with NPIP and NPYR were found in ground pepper, also containing high amounts of respective precursor amines like piperidine (535–642 mg/kg) and pyrrolidine (41–91 mg/kg) (Pfundstein et al., 1991). NDMA (max: 16 µg/kg) and NPYR (max: 6.1 µg/kg) were also found in dried chillies and in dried chilli powder (Tricker et al., 1988).

In fermented vegetables (Chinese cabbage and radish) NDMA (up to 13 µg/kg), NPIP (up to 14 µg/kg), and NPYR (up to 96 µg/kg) have been reported to sporadically occur (Poirier et al., 1987a). In *Brassica oleracea* from India, NDMA (15–16 µg/kg), NPYR (19–25 µg/kg), NPRO (2.1–3.5 µg/kg), and NTCA (0.7 µg/kg) were found (Kumar et al., 1990).

### Human Dietary Exposure to N-Nitrosamines

Table 2 summarizes daily exposure to volatile N-nitrosamines from food in different countries (Habermeyer et al., 2009). NDMA is the most common N-nitrosamine found in food and thus contributes primarily to the total human exposure. As a result of the mitigation measures applied since the eighties, e. g. reduction of nitrite content in curing salt or addition of nitrosation inhibitors such as ascorbic acid or tocopherols as well as the improved kilning processes for barley malt production (Tricker and Kubacki, 1992), the mean exposure to NOC could be reduced from 1.1 µg/person/day (Germany 1980) to about 0.2–0.3 µg/kg in 1990.

Importantly, humans are not only exposed to NOC via dietary uptake, but may also be exposed at specific working places (Federal Institute for Occupational Safety and Health, 552; Federal Institute for Occupational Safety and Health, 611). This is not discussed here but may need consideration in individual risk assessments.

For most industrialized countries, the dietary intake of volatile N-nitrosamines can be estimated to reach approximately < 0.1–0.6 µg/person, respectively < 1.7–27 ng/kg body weight (Bartsch and Spiegelhalter, 1996; Osterdahl, 1988; van Maanen et al., 1996). Beer, processed meat products and fish are considered the main sources of exposure.

By contrast, based on N–NO-group specific analysis to detect the “apparent total N-nitroso compounds” (ATNC), the daily exposure to non volatile N-nitroso compounds was estimated to reach much higher levels, approximately 10–100 µg/person (Tricker and Kubacki, 1992; van Maanen et al., 1996; Challis, 1996). A substantial contribution to this exposure is attributed to beer and bacon consumption (Challis, 1996; MAFF, 1992). Of note, although many non volatile N-nitroso compounds identified in food, such as most N-nitroso amino acids are not considered to be biologically active, the whole spectrum of non volatile NOC to be expected in that group still awaits chemical identification and biological characterisation. It is assumed that the fraction of characterized non volatile N-nitroso compounds is approximately just 10% of the total ATNC content (Tricker and Kubacki, 1992).

Another important exposure pathway is the endogenous formation of NOC. Most nitrosated amino acids are not carcinogenic or mutagenic and practically quantitatively excreted in the urine. They can therefore be utilized as biomarkers for endogenous nitrosation. There have been many attempts to assess endogenous formation of NOC, predominantly by NPRO measurement in urine, stool and other biological media. Although it has been clearly demonstrated that carcinogenic NOC might easily be formed when the appropriate precursors are being taken up, endogenous formation of carcinogenic NOC has not been evaluated adequately yet with respect to their human health relevance and especially concerning human cancer risk. It is well known, that there is endogenous formation of nitrosating agents and that certain disorders, such as inflammatory diseases, bacterial, viral or parasite infections and the like can substantially increase endogenous formation of nitrosating agents and thus enhance the risk of forming carcinogenic NOC in vivo. It is still not clearly established yet, whether NPRO is a valid biomarker for endogenous formation of NOC other than those arising from nitrosation of amino acids or peptides (Habermeyer et al., 2015). It thus remains a mandatory research need to develop in the future biomarkers with better predictivity to reliably assess the overall risk of endogenous NOC formation in humans, including potentially carcinogenic NOC.

### Toxicology, Toxicokinetics

After oral intake, N-nitrosamines are well absorbed from the gastrointestinal tract. Further uptake routes encompass inhalative or dermal exposure, leading to uptake rates similar to the oral route. N-nitrosamines are distributed with the blood stream and are

**Table 2** NOC exposure estimates over time (late sixties to nineties of the last century)

Country/Region	NOC <sup>a</sup> (µg/person/day)
Various EU countries (GER, GB, FR, NL)	
Before 1981	0.1–1.8
After 1981	<0.1–0.6
Japan	
Before 1981	1.8
After 1981	0.5

Uncertainty analysis: earlier data, especially before 1981, not validated methods.

<sup>a</sup>primarily NDMA; NPYR, NPIP mostly below or close to the LOD.

Table compiled by authors from data given in Ref. Habermeyer et al., 2009.

rapidly metabolised, predominantly in the liver particularly at low concentrations (Mirvish, 1995). However other tissues are competent as well to metabolize nitrosamines. Most N-nitrosamines are precarcinogens, requiring metabolic activation. With few exceptions the elimination of intact parent compounds is minor to negligible, due to the generally fast and effective biotransformation of N-nitrosamines.

The first step in the metabolic activation consists of hydroxylation at the  $\alpha$ -C position, mediated by cytochrome P450 dependent monooxygenases (CYP450). This results in the formation of an  $\alpha$ -hydroxy-N-nitrosamine, a proximal carcinogen. The  $\alpha$ -hydroxy-N-nitrosamine is unstable, releases an aldehyde to form an even more unstable monoalkylnitrosamine. The latter rearranges into the corresponding diazonium intermediate that reacts as an electrophile with biomolecules such as DNA, RNA, proteins or glutathione, forming covalent adducts with appropriate nucleophilic centers (Fig. 4) (Shu and Hollenberg, 1997).

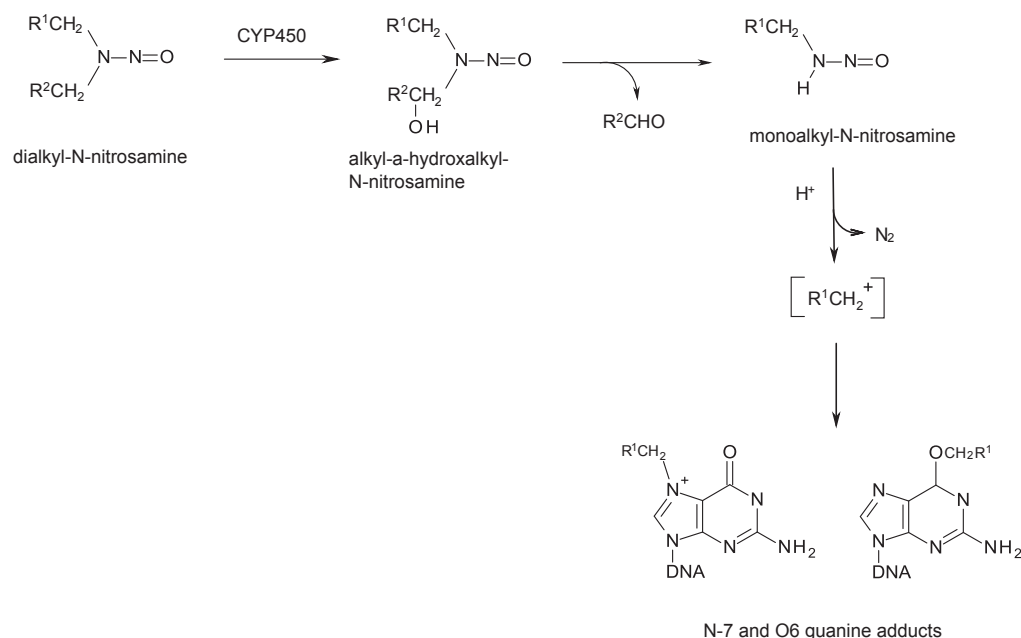
The metabolic activation of NOC by CYP450 depends on their structure, and also varies for different CYP450 isoenzymes. Of major importance for metabolic activation of N-nitrosamines in humans are CYP450 2E1, 2A3, 2A6, and 3A4 (Shu and Hollenberg, 1996; Yang et al., 1994). Due to the varying expression of CYP450 isoenzymes in different organs, N-nitroso compounds can also undergo biotransformation in extrahepatic tissues, e.g. lung, gastrointestinal tract or other organs.

N-nitrosamines with longer side chains may also be hydroxylated at positions other than the  $\alpha$ -C atom. The resulting hydroxylated metabolites might be directly excreted or further oxidized to the respective acids or carbonyl compounds or may undergo conjugation reactions by phase II enzymes, the resulting conjugates undergoing excretion via bile or urine.

Metabolically generated reactive electrophilic compounds lead to alkylation of DNA bases, mainly at N-7, O6, N-3 of guanine (see Fig. 4), N-1, N-3, N-7 of adenine the O2- and O4-position of thymine and of phosphate diester groups of the nucleic acid backbone. Although N-7 alkylguanine in general is a major DNA base adduct resulting from N-nitrosamines, this lesion is considered of rather low promutagenic potential, in contrast to alkyltransfer to the oxygen atoms of nucleobases. Especially alkylation of O6-alkylguanine, O4- and O2-alkylthymidine entail mutations that result in DNA mismatches and miscoding and represent mutagenic lesions that may result in malignant transformation.

### Further Aspects of Exposure and Risk Assessment

As a consequence of the previously described mitigation measures for foods and also for cosmetic products (not discussed here), a consistent reduction of contamination with N-nitrosamines, and thus of human exposure, down to about  $<0.1$ – $0.6$   $\mu\text{g}/\text{person}/\text{day}$  for NDMA (see Table 2), respectively  $<1.7$ – $10.0$  ng NDMA/kg body weight/day (based on a body weight of 60 kg) has been achieved. Based on these data, a margin of exposure (MOE) may be calculated. The MOE describes the ratio between human exposure and a benchmark dose (BMD) associated with a certain benchmark response (BMR) from long term animal experiments. As BMD, the T25 may be chosen, reflecting the dose rate in mg/kg/d which in a long term carcinogenicity experiment will give 25% extra tumor risk over background tumor incidence. EFSA prefers another point of departure for risk assessment: the BMDL, which is the lower one-sided confidence bound of the BMD, derived by modelling dose response data to arrive at the 95% confidence interval of a BMD, set by default at 10% extra risk over background tumor incidence (BMDL10) (EFSA, 2005; O'Brien et al.,



**Figure 4** Reaction mechanism of dialkyl nitrosamines and DNA adduct formation.

2006; EFSA Scientific Committee et al., 2017). The T25 and BMDL10 values for various carcinogenic NOC have been published in two opinions concerning risk assessment of nitrosamine contamination in cosmetics and consumer products by SCCS in 2012 (Table 3), based on available rodent carcinogenicity studies (SCCS, 2011).

According to EFSA, a MOE of 10.000 or higher would be considered of low concern with respect to human health and may confer a low priority for risk management actions (Yurchenko and Mölder, 2007). An MOE estimate for N-nitrosamines occurring in food is given in Table 4.

On the basis of the above “historical” occurrence data, the MOE can be estimated to range between 2700 and 15 900. Results of more recent research published in the following years contributes to better understand parameters governing the process-related formation of nitrosamines, for instance in meat products and thus to support mitigation.

Thanks to tremendous progress in analytical methodology this research has also provided more recent data on nitrosamine occurrence in European foods, to allow for updated estimates of human exposure (Yurchenko and Mölder, 2007; Campillo et al., 2011; Drabik-Markiewicz et al., 2009, 2010, 2011; Herrmann et al., 2015). NDMA was most often found, together with varying spectra of other nitrosamines, such as NDEA, NPYR, NPIP and, in rare cases, even NMEA and NDBA. In more than 100 samples of dry fermented sausages of the European market quantifiable levels of nitrosamines were reportedly found only in rare cases. With one exception (about 12 µg/kg NPIP in a pepper salami) total nitrosamine levels remained below 5.5 µg/kg. Also, no connection was found between contents of biogenic amines and of nitrosamines (De Mey et al., 2014). Sporadically, NMOR was found in traces below the LOQ and was hypothesized to originate possibly by migration of morpholin from packaging or anticorrosion material.

Data on nitrosamine exposure from processed meat products in Denmark informed about mean total contents (all samples) of < 1 µg/kg, corresponding to dietary exposure levels of 1.1 ng/kg bw/d in children (4–6 years) and 0.34 ng/kg bw/d in adults, based on consumption data from the Danish National Survey of Diet and Physical Activity (Herrmann et al., 2015).

With reference to the BMDL10 value of 27 µg/kg bw/d for NDMA derived by SCCS, MOE values of 24 500 and 79 000 are to be derived for volatile nitrosamine exposure (Herrmann et al., 2015).

In a recent study encompassing more than 380 foods from the Korean market (7 largest cities) sampled over 3 years, about similar contents of volatile nitrosamines were found in meat products (Park et al., 2015). NDMA was again detected most frequently, others appeared at lower concentrations. Negative to very low contents were reported for milk products and cheese.

In contrast, remarkably high individual findings (up to 15 µg/kg) were noted for fresh vegetables and mushrooms, seasoning and vegetable oil. Salted fish was reported with some exceptionally high levels (up to more than 300 µg/kg). Unfortunately no discussion was addressed to the potential causes and consequences of these findings, nor was a systematic dietary exposure assessment included, based on consumption figures, to allow for an MOE calculation. Especially the findings in salted fish may deserve further scrutiny, since salted (and dried) fish has been associated earlier with relatively high NDMA contamination. Regular consumption of salted fish was found associated with enhanced occurrence of nasopharyngeal tumors in Southeast Asia (Poirier et al., 1987b). Contamination with NDMA of salted fish has recently also been studied in China. Fifty-four samples of Chinese salted fish obtained from five provinces were analyzed. The results indicated concentrations of NDMA in 57.4% of the samples

**Table 3** Dose descriptors T25, BMDL10 and TD50 for selected carcinogenic nitrosamines

NOC	T25-method <sup>a</sup> (mg/kg bw/d)	BMDL10 <sup>b</sup> (mg/kg bw/d)
NDMA (N-Nitrosodimethylamine)	0.058	0.027
NDEA (N-nitrosodiethylamine)	0.085	0.018
NPYR (N-nitrosopyrrolidine)	0.57	0.16

<sup>a</sup>dose in animal experiment with 25% tumour incidence.

<sup>b</sup>Benchmark Dosis Lower confidence Limit for 10% enhanced tumour incidence.

Table compiled by authors from data given in Ref. SCCS (2011).

**Table 4** Margin of exposure calculation for NDMA

Carcinogen	BMDL10 [mg/kg bw/d]	Estimated human exposure [ng/kg bw/d]	MOE
NDMA	0.027	< 1.7 – 10.0	<2700–15 900

exceeding the limit (acceptable China national standard value of 4 µg/kg), with a median of 5.8 and a range of 0.2–89.4 µg/kg (Qiu et al., 2017).

## Conclusions

The majority of the estimated exposure relates to food intake. As mentioned, NOC are present in the human environment not only in foods, but may also be present in cosmetics and other non food products, giving rise to an aggregate systemic human exposure. Based on average consumption figures, sustained development and application of mitigation measures have brought about substantial reduction of food contamination levels. However if dietary exposure situations are identified that are characterized by a low margin of exposure this may create health concerns and requires targeted mitigation measures.

Of note, an important source of NOC exposure that in general outweighs dietary exposure discussed here relates to tobacco smoking and/or consumption of non smoked tobacco products (chewing tobacco, snuff etc). Tobacco related exposure results in intake especially of tobacco-specific nitrosamines, highly potent carcinogens. Tobacco smoke-related exposure occurs not only by active smoking but also by passive exposure to indoor tobacco smoke (Hecht, 1998).

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## Further Reading

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# Migration From Food Contact Materials

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## Glossary

**FCM** Food contact material; any material or article intended to come into contact with food

**Migration** The transfer of substances from an FCM to food

**NIAS** Non-intentionally added substances; substances that are not starting materials in the manufacture of FCMs i.e. oligomers, impurities and reaction products

**IAS** Intentionally added substances; starting materials in the manufacture of FCMs (e.g. monomers or additives)

**GC** Gas chromatography

**MS** Mass spectrometry

**FID** Flame ionisation detection

**GCxGC** Comprehensive two-dimensional gas chromatography

## Introduction

A food contact material (FCM) may be any article or material which is in contact with food. This contact can be during food processing (e.g. conveyor belts), food preparation and consumption (e.g. pans, plates, forks) or food storage (e.g. packaging). When FCM are in contact with food they may transfer some of their components to the food, a process which, in food science, is commonly referred to as migration. As a source of food contamination packaging in particular is generally high risk, as food is often stored for significant amounts of time in its packaging and/or hot during the filling process. The type of materials which are used as FCM cover a very wide range of chemistry and include plastics, elastomers, resin coatings, metals, adhesives, printing inks and paper/board.

Despite the modest attention FCMs receive in food safety, migration from packaging is by far the biggest source of organic contamination in food (Grob et al., 2006). There have, however, been several examples, where problems from migration from FCM have been identified and in some cases addressed. At times, this led to significant damages to food business operators and large investments from the public sector.

It is beyond the scope of a single article on migration from FCMs to be comprehensive. Therefore, examples shall be presented and possible strategies to find substances migrating from FCMs that may be of concern to consumers. Alternative sources for information on FCM can be found at the European Reference Laboratory on food contact materials (EURL-FCM) or the charity Food Packaging Forum.

## Examples

Typically, in migration a distinction between intentionally added substances (IAS) and non-intentionally added substances (NIAS) is made. To the authors' knowledge, the biggest database on IAS contains more than 10,000 entries (Van Hoeckel et al., 2017). They are substances used in the manufacture of a wide range of FCMs and were compiled from European and national legislation and the Council of Europe's Resolutions. The database is accessible through <https://fcm.wiv-isp.be/About.aspx>. A few examples shall illustrate where there have been problems with IAS in the past.

In 1991 contamination of nuts, rice and cocoa products with up to 500 mg/kg **mineral oil** were reported from jute bags used for transport (Grob et al., 1991). The bags had been treated with high-boiling mineral oil components, so called batching oil, to render the fibers flexible. This practice has been banned for food destined for the European market.

An age old and well known contamination of food by **lead and cadmium** from ceramics is even believed to have been a problem in the Roman Empire. The US food and drug agency took action against lead contamination from ceramics in 1971 after a family of six were poisoned by such articles in 1970 (Gilmore et al., 2013). This also led to a European Regulation for lead and cadmium in 1984 (84/500/EEC). Though the issue seems less severe nowadays, the release of heavy metals from ceramics remains an active area of food safety considerations to this day (Beldi et al., 2016).

A substance which caused a relatively large scale scandal in Europe was **2-Isopropylthioxanthone (ITX)**. It is a so called photo initiator which is used in UV-curing printing inks (Sagrati et al., 2006). The issue arose due to an alert of the Italian authorities in 2005 to the **European Rapid Alert for Food and Feed** system, and has led to many since. In a survey ITX was found in amounts up to

357 µg/kg food from the German market in 2007 (Rothenbacher et al., 2007). Since the issue was identified, it has been largely solved by the advent of low migration inks for food packaging (Lago et al., 2015).

When **Bisphenol-A-diglycidyl ether** (BADGE), a starting material in the manufacture of epoxy based can coatings, was first discovered to migrate into food, there were no available data on genotoxicity, despite the reactive epoxide groups (Fig. 1). (Biedermann et al., 1996) This was in stark contrast to the detected levels in food of up to 57 mg/kg. Since then it was established that BADGE is not genotoxic and there is now a European Regulation (EC No 1895/2005) setting levels of safe migration of BADGE and some other related substances.

More recently, **formaldehyde** migration from melamine plastic was rediscovered in 2005 (Bradley et al., 2005). A total of 10% of the tested articles were found not to comply with regulation on the migration of formaldehyde, which seems surprising considering the topic had been reported much earlier (Ishiwata et al., 1986; Martin et al., 1992). This may to some extent reflect the lack of attention FCM have received in the area of food safety. These findings resulted in EU-wide legislation to tackle the issue in 2011 (Commission Regulation (EU) No 284/2011).

**Plasticisers** in polyvinyl chloride plastic (PVC) regularly attract media attention. Initially, this was focussed on the group of the phthalic acid esters of which the most prominent substance is di-2-ethylhexyl phthalate (DEHP). Plasticisers are lipophilic and therefore are prone to migrate into fatty food. PVC is mainly used for FCM in cling film and sealing gaskets of glass jars. For the latter type of food contact material a pan European campaign with over 400 samples in 2011 showed that a quarter of all roughly 300 relevant samples (oily content) were non-compliant with respect to plasticiser migration (McCombie et al., 2012). The most incidences of exceedances was due to sum of plasticisers, the highest migration for the sum of plasticisers was 1314 mg/kg food. Unfortunately, the situation had not improved in a follow up campaign in 2013 (McCombie et al., 2015). To this date, there are still many sealing gaskets made from plasticised PVC on the market. The migration issue seems to be moving forward, only by the development of alternative plastics for the gaskets for food containing oil (e.g. [www.pano.de/](http://www.pano.de/)).

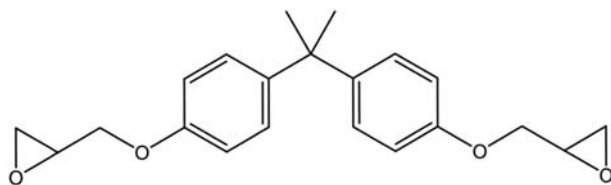
Finally, Bisphenol A (BPA) deserves a mention as an IAS, because it receives a very large amount of attention from the scientific community and the press. One of the main sources of BPA in food was from polycarbonate (most prominently baby bottles, which are banned now), for which it is a starting material. The safety assessment of this substance has been reviewed and refined many times. One of the main issues is a debate on non-linear low dose effects which are not well covered by current regulatory toxicology (EFSA, 2015).

The number of potentially migrating substances is estimated to be around ten times higher than the list of IAS mentioned earlier (Grob et al., 2006). These substances are reaction products such as oligomers and degradation products as well as impurities. These substances are usually referred to as NIAS. Many of these substances are unknown, let alone risk assessed and arguably pose the biggest risk for food safety. There have been several examples such substances being detected in food of which a selection shall be mentioned here.

At around the same time as the aforementioned formaldehyde, high levels of migrating **primary aromatic amines** (PAA) were discovered to migrate into food. These are impurities or degradation products from dyes used in colored plastic FCM, such as black nylon kitchen utensils (Mortensen et al., 2005). This problem was tackled in legislation together with formaldehyde (Commission Regulation (EU) No 284/2011).

**Mineral oil** became of major interest as a group of migrating substances a second time, many years after the batching oil problem had been solved for Europe. One of the main sources for the contamination was recycled paper and board, which is produced from newspapers, where mineral oil based printing inks are commonly used (Biedermann and Grob, 2010). Recycled paper and board, however, has been analysed in more detail, and 330 substances were detected that could be of concern (Chemisches et al., 2012). The estimation of potential concern was based on basic assumptions on the ratio of packaging to food weight and used standard cutoffs for substances used, based on EFSA guidelines. Interestingly, only two thirds of the substances could be identified by the authors. In fact for recycled paper and board it has been stated that a comprehensive safety assessment of all potentially migrating substances is not feasible (Biedermann and Grob, 2013). The most promising solution to this problem to date, seems to be the protection of food by applying a functional barrier. For packaging systems with an internal bag inside a box of recycled paper a method for testing and specifying the desired barrier properties has been proposed (Fiselier and Grob, 2012; Biedermann-Brem and Grob, 2014; Richter et al., 2014).

**Oligomers** of various plastics have also been seen to migrate into food. The short chain oligomers and low molecular weight cyclic oligomers may have toxicological properties that differ significantly from the monomers and are, therefore, not necessarily covered by restrictions on the monomers. For styrene this led to an **opinion** of the German Federal Institute for Risk Assessment (Bundesinstitut für Risikoforschung, BfR) stating health risks are unlikely. Oligomers of polyethylene terephthalate (PET) on the



**Figure 1** Bisphenol-A-diglycidyl ether (BADGE).

other hand are still poorly investigated with migration properties only recently becoming available for a wider range of oligomers (Hoppe et al., 2017). Polyolefin oligomers (polyolefin hydrocarbons, POH) constitute the highest amount of material migrating from such plastics (Biedermann-Brem et al., 2012). They are structurally similar to mineral oils and they may contain also cyclic and unsaturated substances (McCombie et al., 2016). A thorough risk assessment on these structures is not available at the time of writing, despite very high use of polyolefins as FCM. Finally, a cyclic oligomer from epoxy-based resins – the cyclic product of BPA and BADGE (cyclo-di-BADGE) – has been reported to be found to migrate up to a level of 1980 µg/kg. Though genotoxicity had been ruled out the level of migration requires more toxicological data (Biedermann et al., 2013).

The last example of an identified NIAS which may migrate into food is di-ethylhexyl maleate (DEHM). This substance is an impurity of di-ethylhexyl sulfosuccinate – an emulsifier in the varnish applied to cardboard boxes. The maximum level detected in food was 1.5 mg/kg and there was no data available on toxicology for these levels of resulting exposure (Fiselier et al., 2010).

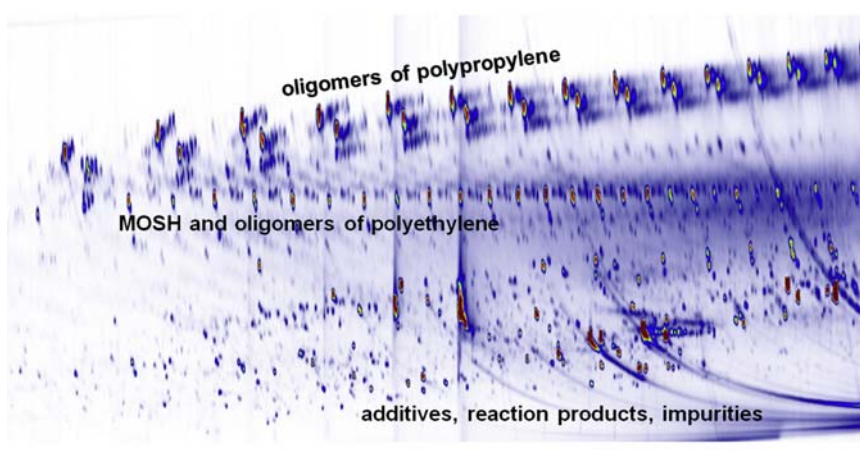
## Strategies for Finding Migrating Substances Including NIAS

Despite the aforementioned issues that were found for specifically regulated IAS, much of the focus in food science with respect to FCM deals with NIAS. These are regulated as a whole, by the requirement that they may not migrate into food at levels which could endanger human health (EU 1935/2004). The identification of NIAS that could lead to a contamination of food above a critical level presents a considerable challenge as was stated by a working group from the International Life Science Institute (ILSI) along with some ideas as to how to approach the issue (ILSI Packaging Materials Task Force, 2015). A review on strategies from 2013 may also give further insights to readers (Nerin et al., 2013; Humston-Fulmer and Binkley, 2017). Even with a focus on a subset of NIAS, such as oligomers, the challenge remains significant (Hoppe et al., 2016).

As the relevance of NIAS depends strongly on the quantity that may migrate into food, quantification is a central aspect to NIAS investigations. Commonly used detection techniques, such as electrospray mass spectrometry, are inherently difficult, as the detector response is substance dependent, ultimately making quantification of unknowns impossible (Pieke et al., 2017).

We believe the best solution to date is the use of the unsurpassed resolving power of comprehensive two dimensional gas chromatograph (GCxGC), which has been described before in the context of FCM (McCombie et al., 2016; Biedermann et al., 2014). The technology can be used with mass spectrometric detection (for identification, clues to substance structure) or with flame ionisation detection (FID). The later is unsurpassed for quantification of unknowns as the response is as close to substance independent, as is achievable. Fig. 2 shows the GCxGC chromatogram of an extract from a recycled polyolefin granulate, where each spot is a peak representing a substance. The resolving power and the richness of the data is immediately apparent. As a side effect, the exceptionally sharp peaks in GCxGC results in a sensitivity gain, compared to one dimensional GC. For the analysis of FCM we believe a column set with a polar column (50% phenyl and 50% methyl polysiloxane) in the first dimension and an apolar column (100% methyl polysiloxane) on the second dimension is particularly useful, as it permits group type separation of hydrocarbons, as marked in Fig. 2. Furthermore, the polar substances in the bottom section of the chromatogram elute with excellent peak shapes. As GCxGC is an untargeted approach IAS are also included in the analysis.

The assessment of risk associated with a given NIAS depends on the exposure and the toxicity of the substance. There are neither available data on the toxicity nor on the substance identity, for a given peak, for most NIAS. There are, however, scientifically recognised maximal exposure levels for substances with limited data, which are describe in a concept of Threshold of Toxicological Concern (EFSA Scientific Committee, 2012). The tolerable exposure threshold for genotoxic substances is at 0.0025 µg/kg b.w./day, which is 600 times lower than that of the so called Cramer III substances at 0.3 µg/kg b.w./day. In the absence of a structure it must be assumed that a NIAS is genotoxic, as its genotoxic potential cannot be assessed.

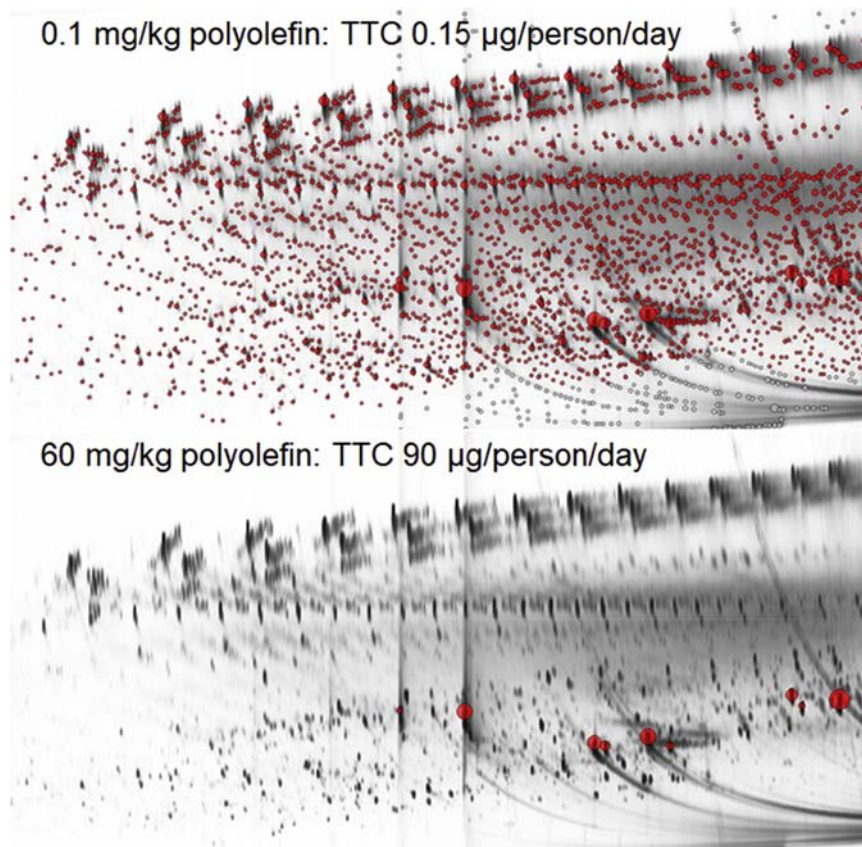


**Figure 2** GCxGC chromatogram of an extract from a recycled polyolefin.

Making some basic assumptions on the quantity of consumed food packed in the FCM of interest (150 g/day) and the amount of food in contact with the FCM (1 kg food in contact with 10 g plastic), a body weight of 60 kg and a transfer of 100% allows the picking of peaks of interest in an extract, such as the one presented in Fig. 2. The threshold can be set by adding appropriate amounts of internal standard. The results of the picking of the peaks of interest is given in Fig. 3; in the top panel the threshold has been set for genotoxic (or potentially genotoxic) substances, while it was set for Cramer III substances in the bottom panel. Note that such an estimate of peaks of interest is only valid, if the detector response shows minimal differences between analytes, as is the case for FID. In order for an in-depth risk assessment to be able to focus on the abundant substances (peaks highlighted in red in the bottom panel), genotoxicity must be ruled out for all of the peaks marked in red in the top panel. To achieve this, the minimal work would be to identify the low intensity peaks (highlighted in red in the top panel) and to perform an in-silico analysis to determine genotoxic potential. In some cases it might also be appropriate to sum up the signals from a group of substances, which are expected to act similarly on an organism such as the oligomers. Please note that the assessment of relevant peaks was performed on an extract. This has the disadvantage of one having to make certain assumptions such as 100% transfer or a the weight ratio packaging/food. However, it also has advantages such as increased sensitivity (more reliable threshold) and circumvention of polar food simulants, which are unsuitable for GC analysis. Although the assessment aspect of migrating substances is not covered by this review it should be mentioned that the strategy outlined above does not cover the full assessment currently discussed by EFSA for the approval of new IAS (EFSA, 2016).

A drawback of GCxGC is the limited range of molecular mass and polarity, which GC analysis can cover. Though the latter may be addressed by derivatisation such as acetylation or silylation. Nevertheless, complementary analytical techniques such as LC-MS are still needed for a full coverage of NIAS. The extent of the coverage of an extract can be estimated by size exclusion chromatography and evaporative light-scattering detection. The coverage of components < 1000 da (considered toxicologically relevant) in GCxGC may vary depending on the material under investigation.

In the future, it may also be possible to determine the genotoxicity of an entire mixture using a bioassay (Koster et al., 2012). There are however to date some issues with using in-vitro genotoxicity tests for mixtures and how they relate to accepted OECD tests for single substances. In particular, there are open questions on the sensitivity of such tests.



**Figure 3** The chromatogram from Fig. 2 with peaks highlighted in red which are relevant for potential migration. In one case genotoxicity is assumed for all substances (top) in the other for none (bottom).



## Conclusions

As it is not feasible to comprehensively cover the issue of migration from food packaging, this chapter is intended to highlight the underestimated issue and give the reader an idea of the scope and challenges of the field. There are thousands of unidentified or toxicologically uncharacterised, chemicals which may migrate into food from food contact materials. The levels of migration often exceed those which would required investigation according to modern scientific cutoff criteria. The examples in this review demonstrate that there have been various issues, which may very well only be the tip of the iceberg.

There is a need for more research in the area. A combination of analytical tools is required to investigate migration. To date one of the most powerful techniques is GCxGC with a resolving power to separate thousands of substances in a single run and can be combined with a variety of detectors including FID, which has a nearly substance independent detector response.

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## Process Contaminants: A Review

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### Glossary

**BMDL10 (BenchMark Dose Level)** Study endpoint that is associated with a 10% extra risk of an adverse effect in exposed test animals, as compared to the background levels of risk.

**EPA PAHs** The United States Environmental Protection Agency has classified 16 of the PAHs as priority-pollutants based on toxicity, potential for human exposure, frequency of occurrence at hazardous waste sites, and the extent of information available. Of these PAHs US EPA considers seven (benzo(a)anthracene, chrysene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, dibenz(a,h)anthracene, and indeno(1,2,3-cd)pyrene) as probable human carcinogens.

**JECFA** The Joint FAO/WHO Expert Committee on Food Additives (JECFA) is an international scientific expert committee that is administered jointly by the Food and Agriculture Organization of the United Nations and the World Health Organization. It has been meeting since 1956, initially to evaluate the safety of food additives. Its work now also includes the evaluation of contaminants, naturally occurring toxicants and residues of veterinary drugs in food.

**Maillard reaction** Heat induced reaction of reducing sugars with amino acids. Complex reaction from which aroma active substances and melanoidins originate.

**MOE (Margin Of Exposure)** The MOE is a ratio of two factors which assesses for a given population: the dose at which a small but measurable adverse effect is first observed and the level of exposure to the substance considered.

**Solid Phase Extraction (SPE)** Sample preparation method by which compounds that are dissolved in a liquid are separated from other compounds according to their physical and chemical properties. This is used to concentrate and purify samples for analysis. It can be used to remove the matrix from the samples (e.g. blood, urine, foods, soil) to eliminate interferences during chromatographic analysis.

**TAMDI (Theoretical Maximum Daily Intake)** Predicted maximum daily intake of a residue (mg per person per day), assuming that it is present at the maximum residue level and that average daily consumption of foods per person is represented by assessed regional diets.

### Nomenclature

List of nomenclature used including all definitions and units.

**AαC** 2-Amino-9H-dipyrido[2,3-b]indole

**Glu-P-1** 2-Amino-6-methyl-dipyrido[1,2-a:3',2'-d]imidazole

**Glu-P-2** 2-Amino-dipyrido[1,2-a:3',2'-d]imidazole

**IQ** 2-Amino-3-methylimidazo[4,5-f]quinoline

**MeAαC** 2-Amino-3-methyl-9H-dipyrido[2,3-b]indole

**MeIQ** 2-Amino-3,4-dimethylimidazo[4,5-f]quinoline

**MeIQx** 2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline

**PhIP** 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

**Trp-P-1** 3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole

**Trp-P-2** 3-Amino-1-methyl-5H-pyrido[4,3-b]indole

**TDI** Tolerable Daily Intake (mass kg<sup>-1</sup> bw day<sup>-1</sup>)

### Introduction

Heat treatment of foods is a key operation in the industry resulting in the development of a large range of flavors and tastes through the Maillard Reaction (MR). Besides reducing or even eliminating biological contamination the heat treatment results in modifications for improving the texture and aroma. The major concern arising from heating processes comes from the formation of compounds that are not naturally present in foods but may develop during heating or preservation processes and may reveal harmful effects such as mutagenic, carcinogenic and cytotoxic effects (Neo Formed Contaminants, NFC). In parallel to the aroma formation harmful substances can be formed. Some selected groups are discussed in this chapter; namely, the heterocyclic

aromatic amines, furan derivatives, acrylamide, polycyclic aromatic hydrocarbons (PAHs), monochloropropanediols (MCPD). The concentrations in the foods are normally low and even conditions favoring the formation of these compounds do not lead to acute toxicity but prolonged exposure could increase the risk of cancer of different organs. (Fig. 1)

In the following paragraphs we will give a brief discussion about those compounds considering important issues such as their occurrence, mechanisms of formation and techniques of mitigation, toxicology and exposure.

## Heterocyclic Amines

This group of compounds was first identified by the group of Sugimura at the National Cancer Center Research Institute in Japan in the 1970's. They are formed during intensive heating of protein rich foods as pyrolysates from amino acids or as part of the Maillard reaction from carbohydrates, amino acids, and creatine. Of the currently 30 compounds described up to date only 10 are known to be carcinogenic in rodents.

## Quantification in Foods

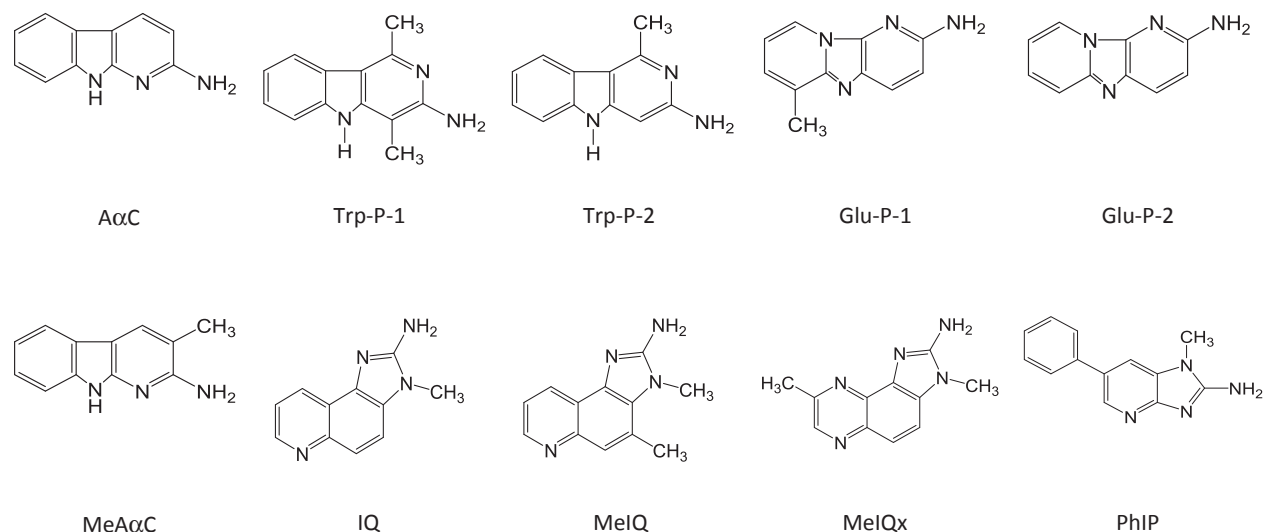
The current analytical method of choice is liquid chromatography with mass selective detection. Although it is possible to use UV detection the sensitivity is low and the selectivity very limited to obtain accurate results. The sensitivity and selectivity of triple quadrupole MS is sufficient for good accuracy. Some of the less polar substances (PhIP, A $\alpha$ C, MeA $\alpha$ C, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2) can be determined by its fluorescence which is sufficiently sensitive. In any case the clean-up using a liquid extraction with ethyl acetate and subsequent solid phase extraction (SPE) with cation exchange materials is necessary. The complex matrix of intensively heated meat and the extremely low concentrations are a challenge for the analytical chemist. A very specific SPE material – copper phthalocyanin – was used for selective enrichment of the HCAs in a series of matrices (e.g. meat, air, river water) – but was not commercially available.

## Occurrence in Foods

The concentrations found in heated meat or fish are in the low ppb range (0–10 ng g<sup>-1</sup>). Intensive heat treatment results in significantly higher concentrations. Normally, PhIP is the compound occurring at higher concentrations with values of up to 480 ng g<sup>-1</sup> in e.g. barbecued chicken breast.

## Chloropropanediols, Glycidol and Esters

3-MCPD was first identified by the group of Velisek in Prague/CZ who showed that it is formed during manufacturing of acid-hydrolyzed vegetable protein. This hydrolysate can be used for production of soy sauce. 3-MCPD has been reported to be nephrotoxic as well as toxic to reproduction. In addition, there are some reports on its potential carcinogenicity. Inconsistent toxicological data are published with a clear genotoxic potential *in vitro* shown in experiments using *Salmonella typhimurium* TA100 (with and without T9 mix, indicating base-pair substitution) and no genotoxicity in TA98 (indicating frameshift mutations) using similar conditions; no genotoxicity was determined *in vivo*.



**Figure 1** Structures and commonly used abbreviations of the carcinogenic heterocyclic amines.

The IARC classified 3-MCPD as potentially carcinogenic to humans (group 2B). The TDI of this non-genotoxic carcinogen was set to  $2 \mu\text{g kg}^{-1} \text{ bw}$ . The fatty acid esters of 3-MCPD and glycidol have the same toxicity as the non-conjugated glycerol derivatives since they are hydrolyzed completely during digestion.

### Analytical Considerations

3-MCPD is commonly analyzed after derivatization with phenylboronic acid forming dioxoboranes by GC-MS. Other derivatization agents (e.g. acetone, heptafluorobutyl imidazole) are also possible resulting in a better column life but with lower sensitivity. For the determination of esters direct or indirect (i.e. after release from the esters) methods have been applied. For the direct analysis LC-MS methods were developed which show the heterogeneity of the reaction products.

### Formation and Occurrence in Foods

The formation of chloropropanols from lipids involves a nucleophilic attack by a chloride ion on the least sterically hindered carbon of the glycerol moiety. As the free alcohol in e.g. diacylglycerols is not a good leaving group for this nucleophilic attack the most probable mechanism might be via an intermediary formed epoxide or a cyclic acyloxonium ion with which the chloride is reacting. A direct nucleophilic substitution of the ester is also possible.

Glycidyl esters are formed during refining of vegetable oils containing high amounts of diacylglycerols or monoacylglycerols. The formation of the glycidylesters is independent from the formation of the MCPD esters.

The mean daily exposure for the younger age groups was for 3-MCPD  $0.5\text{--}1.5 \mu\text{g kg}^{-1} \text{ bw}$ , for 2-MCPD  $0.2\text{--}0.7 \mu\text{g kg}^{-1} \text{ bw}$  and for glycidol  $0.3\text{--}0.9 \mu\text{g kg}^{-1} \text{ bw}$ . The daily exposure for the adult and older population ranged from  $0.2\text{--}0.7 \mu\text{g kg}^{-1} \text{ bw}$  (3-MCPD),  $0.1\text{--}0.3 \mu\text{g kg}^{-1} \text{ bw}$  (2-MCPD), and  $0.1\text{--}0.5 \mu\text{g kg}^{-1} \text{ bw}$  (glycidol).

A survey of the data shows that the highest concentrations including the esters were found in palm oil with a mean of  $2900 \mu\text{g kg}^{-1}$  for 3-MCPD,  $1600 \mu\text{g kg}^{-1}$  for 2-MCPD and  $4000 \mu\text{g kg}^{-1}$  for glycidol. Other oils than palm oil contains significantly lower amounts. Margarine contains  $670 \mu\text{g kg}^{-1}$  3-MCPD,  $240 \mu\text{g kg}^{-1}$  2-MCPD and  $580 \mu\text{g kg}^{-1}$  glycidol. Other food groups that are highly contaminated are potato crisps, hot surface cooked pastries, cookies and short crusts.

3-MCPD is formed from lipids and chloride ions during heat processing of foods. The highest concentrations are found in systems containing lecithin. The main occurrence of 3-MCPD in foods is in soy sauce produced by acid hydrolysis. For this, defatted soy beans and other proteins from cereals are treated with 4–9 M hydrochloric acid at  $103\text{--}110^\circ\text{C}$  for 20–35 h.

### Furan

Furan ( $\text{C}_4\text{H}_4\text{O}$ ) is a small organic compound with high volatility (boiling point:  $31^\circ\text{C}$ ) and lipophilicity. The presence of furan in a broad range of heat processed foods ( $0\text{--}6000 \text{ mg kg}^{-1}$ ) has received considerable since it is considered as a “possible carcinogenic compound to humans”. Multiple pathways are involved in furan formation in foods. The thermal degradation and rearrangement of sugars and amino acids, as well as the thermo-oxidation of polyunsaturated fatty acids and ascorbic acid, have been proposed as the mechanisms responsible for its generation in foods. Despite its high volatility, furan has also been found in low-moisture foods processed in open containers, such as potato chips, crackers, crisp breads, and toasted breads. A better understanding about the effect of processing conditions over furan generation in heat processed foods is necessary to develop effective mitigation technologies. Since high levels of furan were detected in tomato sauces, fruit juices, and baby foods, most research has been focused to improve the understanding about its formation in these types of food matrixes. Furan levels in food are reported to range from a few  $\mu\text{g kg}^{-1}$  to  $7000 \mu\text{g kg}^{-1}$ . The highest furan concentrations were found in roasted and instant coffee, followed by baby foods and soups which showed maximum concentrations slightly higher than  $200 \mu\text{g kg}^{-1}$ . Foods less subjected to heat processing conditions, such as milk or fruit juice, are the less contaminated items. In this sense it was reported that furan was present in a number of foods, with the highest levels being found in coffee. Just recently, the US Food and Drug Administration (US FDA) published a report on the occurrence of furan in a number of foods that undergo thermal treatment, especially canned and jarred foods. Furan levels of over  $100 \text{ mg kg}^{-1}$  were found principally in three categories of major foods; coffee, baby food, and sauces and soups; all of them sold in hermetically sealed packages that do not allow the evaporation of furan. Baby food was of particular interest as a high proportion of samples sold in jars and cans contained furan and such foods may form the sole diet of many babies. The estimated intake based on consumption of baby food from glass jars was  $< 0.2$  to  $26 \text{ mg furan per day}$  or  $< 0.03$  to  $3.5 \text{ mg kg}^{-1} \text{ bw per day}$  for a 6 month old baby weighing 7.5 kg. The daily intake for adults from canned or jarred vegetables was estimated to be 1.1 to 23 mg, and from beer was 1.3 to 50 mg. The daily intake from coffee based was 2.4 to 120 mg, making coffee the major dietary source for adults.

Furan has been considered as a possible carcinogen to humans (2B) by the International Agency for Research on Cancer (IARC). The liver is the main target organ of furan-induced toxicity in rats and mice with a clear dose-dependency and probably acting by a genotoxic mechanism. Studies performed by the JECFA have revealed the comparatively small margin of exposure (MOEs of 960 and 480 for average ( $0.001 \text{ mg kg}^{-1} \text{ bw per day}$ ) and high ( $0.002 \text{ mg kg}^{-1} \text{ bw per day}$ ) dietary furan exposures) between human exposure and the furan doses which induce liver tumor in experimental animals. Since a genotoxic mode of action could be associated with furan-induced tumor formation, current human exposure levels to this contaminant may indicate a risk to human

health and the necessity for its mitigation. Previous information clearly shows that furan exposure could represent a critical public health risk, specifically in infants since furan is a carcinogenic compound that might act via a DNA-reactive genotoxic metabolite. Moreover, the mechanism of furan induced carcinogenicity in rodents as well as the levels and effect of furan on humans has not yet been clarified. Furan was found to induce hepatocellular adenomas and carcinomas in rats and mice, and high incidence of cholangiocarcinomas in rats at doses higher than of  $2 \text{ mg kg}^{-1} \text{ bw}$ . Due to limited relevant experience with regard to toxic effects associated with neither human exposure nor epidemiology a final conclusion concerning genotoxic or mutagenic activity of this molecule has not been drawn yet.

### Furfuryl Alcohol

The identification of a new mechanism of activation of vinyllogous alcohols by sulphotransferases has raised concerns about the presence of compounds like furfuryl alcohol (FA) in foods. FA occurs in high amounts in a limited number of foods and even if the mutagenic/carcinogenic activity is low, there might be a risk due to the high exposure. FA is formed by heating of carbohydrate rich foods; the concentrations of FA can reach several hundred  $\text{mg g}^{-1}$ . Although FA can polymerize in acid media the concentration of the monomer is still high in heated foods; especially in roasted coffee. FA gives a burnt, cooked sugar and rubber like odor; when FA interacts with dihydroxy benzene or trihydroxy benzene as it is occurring during roasting of coffee it will produce a bitter taste. However, FA is used as a flavoring agent with an acceptable daily intake of up to  $0.5 \text{ mg kg}^{-1} \text{ bw}$ .

FA can be formatted from degradation of reducing sugars. In this sense, glucose or fructose can undergo isomerization reactions at high temperatures. The key intermediate in this isomerization reaction, 1,2-enediol, is also considered as the starting intermediate in the degradation reactions by  $\beta$ -elimination producing an unstable compound 3-deoxyaldoketose which then undergoes a cleavage reaction producing formic acid and a  $\text{C}_5$ -compound. The  $\text{C}_5$ -compound (2-deoxypentose) will react further by cyclization and aromatization forming FA. FA as a furan derivative is occurs mainly in roasted coffee. Though the polymerization and evaporation proceeds during the roasting of coffee the concentration of the monomeric FA is still high in the finished products. The concentration of FA is  $267 \text{ mg g}^{-1}$  in instant coffee and in  $564 \text{ mg g}^{-1}$  coffee roasted at  $210^\circ\text{C}$  for 3 min. Additionally, FA is also found in rice cakes ( $2\text{--}2.3 \text{ mg g}^{-1}$ ), bread  $190 \text{ mg g}^{-1}$ , honey  $1.6 \text{ mg g}^{-1}$ , toasted almonds ( $6.0 \text{ mg g}^{-1}$ ), non-fat dried milk stored for 3 months at room temperature  $15 \text{ mg g}^{-1}$ , popcorn ( $0.038\text{--}0.082 \text{ mg g}^{-1}$ ), corn tortilla chips ( $0.54 \text{ mg g}^{-1}$ ), roasted cocoa powder ( $0.021 \text{ mg g}^{-1}$ ), among others.

The estimated daily FA intake is  $130 \text{ mg kg}^{-1} \text{ bw}$  with a margin of safety of  $462 \text{ mg kg}^{-1}$ . FA is mutagenic to *S. typhimurium* strain TA 100 engineered for the expression of human SULT1A1. The mutagenicity of FA is dose dependent and increases its mutagenicity when the amount of FA increases. In mice which received FA with the drinking water the DNA samples of liver, kidney and lung contain DNA adducts. In rodents which were exposed to FA tumors that contained the 2-methylfurfuryl adduct were formed but at lower concentration exposure ( $0.06 \text{ mg kg}^{-1} \text{ bw}$  and day) no tumors were formed. Although the acute toxicity of FA is not relevant in the foods they could be activated with sulphotransferases to highly reactive compounds which are then mutagenic/carcinogenic whose detailed risk is not yet known and additional work is pending.

### Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a group of diverse organic compounds chemically recognized with two or more aromatic rings in their structure. PAHs generally occur in complex mixtures, which may consist of hundreds of compounds. For most of the PAHs their carcinogenic and genotoxic potential constitute the critical factor for the hazard and risk characterizations.

#### Human Exposure

The major exposure for non-smokers is from foods; for smokers the contribution from smoking can be significant. Food can be contaminated from environmental sources (air, soil, water), industrial food processing, and some domestic cooking practices.

#### The PAHs List of Interest

In 1970, a set of 16 PAHs which are frequently found in environmental samples has been proposed by the U.S. Environmental Protection Agency (EPA) to be monitored. There were acenaphthene, acenaphthylene, anthracene, fluoranthene, fluorene, naphthalene, phenanthrene, pyrene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, benzo[a]pyrene, chrysene, dibenz[a,h]anthracene, and indeno[1,2,3-cd]pyrene. In the EU, the Scientific Committee on Food (SCF) assessed the toxicity of PAHs in 2002. The SCF prioritized compounds based on the health risk rather than on occurrence in food and recommended the monitoring of 15 PAHs, including 8 high molecular weight PAHs that are also part of the U.S. EPA list. As the most suitable indicators of occurrence of PAHs in food, eight compounds (PAHs8), namely benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, benzo[a]pyrene, chrysene, dibenz[a,h]anthracene, and indeno[1,2,3-cd]pyrene were selected. Benzo[a]pyrene was suggested as a marker of occurrence of carcinogenic PAHs in food.

The IARC has identified 12 PAHs of the EU list as carcinogenic to humans (group 1), and probable (group 2A) or possible (group 2B) human carcinogens. Other PAHs not defined as carcinogens may act as synergists.

### Legislation on PAHs in Food

The most comprehensive regulations are given in the European Union for different food categories in which higher levels of PAHs could be expected. Maximum limits are set for a group of four PAHs including benzo[a]pyrene by the Regulation (EU) No 835/2011.

### Determination of PAHs

In the literature a number of in-house validated methods are reported, although there is still a lack of standardized methods for the analysis of PAHs. Because the concentrations of PAHs in food are very low, generally in the range of ppb units, the methods usually consist of several extraction and sample purification steps to reach the low detection limits. The two main instrumental analysis techniques for determination of PAHs in food are an HPLC with selective and sensitive fluorescence detection and a gas chromatography with single quadrupole (MS), tandem mass spectrometry (MS/MS), and high resolution mass spectrometry, respectively.

### Occurrence of PAHs in Food

Data from surveys indicate that the most frequently present PAHs in foodstuffs are the low molecular weight PAHs from the EPA list (particularly those of two or three benzene rings). These PAHs are less relevant from the toxicological point of view and do not contribute to the genotoxic and carcinogenic potential of PAHs. Food can be contaminated by PAHs that are present in air, soil, or water, or during food processing and cooking. The levels of PAHs found in unprocessed foods in rural areas reflect the background contamination that could originate from long distance airborne transportation of contaminated particles.

Processed foods are contaminated by PAHs during drying (seeds and kernels producing oils), smoking (meat, fish and dairy products) and cooking of food at high temperatures (grilling, frying, roasting, baking), and certain preservation techniques (milk).

With respect to food groups, the highest levels of total PAHs were detected in meat and meat products ( $26 \mu\text{g kg}^{-1}$ ), oils and fats ( $23 \mu\text{g kg}^{-1}$ ) and cereals ( $20 \mu\text{g kg}^{-1}$ ). The highest individual PAHs levels corresponded to phenanthrene ( $27 \mu\text{g kg}^{-1}$ ), naphthalene ( $26 \mu\text{g kg}^{-1}$ ), and fluoranthene ( $14 \mu\text{g kg}^{-1}$ ), while the lowest levels were reported for benzo[a]pyrene ( $1.3 \mu\text{g kg}^{-1}$ ) and benzo[k]fluoranthene ( $1.3 \mu\text{g kg}^{-1}$ ). Based on available data, food consumption was shown to be the main source of PAHs intake and thus highlighted the importance of research on PAHs in food and the development of mitigation strategies to reduce their contents in food.

### Acrylamide

Acrylamide (AA) is a hazardous contaminant classified as 'probably carcinogenic' for humans and recognized as a genotoxic carcinogen in laboratory animals. Evidence from animal studies shows that acrylamide and its metabolite glycidamide are genotoxic and carcinogenic. In 2015, the European Food Safety Authority (EFSA) confirmed that acrylamide in food potentially increases the risk of developing cancer for consumers in all age groups.

### Analytical Considerations

In complex matrices it can be determined after extraction procedures to water or other polar organic solvents by analytical methods using GC/MS after bromination. If derivatization is not used, the extract clean-up is more demanding. LC/MS analysis has the advantage that the LC technique in reversed-phase mode is directly compatible with an aqueous solvent that is best suited for extraction of acrylamide from foods. For this reason, most survey data for acrylamide have been obtained using LC-MS/MS analysis.

### Occurrence of Acrylamide in Food

The presence of acrylamide in foods is supposed not to be from external sources (environment, packaging materials), but as a consequence of natural processes via the Maillard reaction during heat treatment. It is predominantly formed from asparagine in the presence of reducing sugars in certain foods when exposed to temperatures higher than  $120^\circ\text{C}$  at low moisture conditions (baking, frying, grilling, roasting, toasting). Raw materials which contain the abovementioned precursors in significant levels are cereals, potatoes, green coffee and various kinds of vegetables and fruits. Foodstuffs prepared from these raw materials by thermal processing are suspicious of high acrylamide content, independent on commercial or home preparation.

According to the data collected and published by the EFSA in 2015, acrylamide was detected in following food categories in average content: coffee ( $520 \mu\text{g kg}^{-1}$ ) and coffee substitutes ( $1500 \mu\text{g kg}^{-1}$ ), potato crisps and snacks ( $390 \mu\text{g kg}^{-1}$ ) and potato fried products ( $310 \mu\text{g kg}^{-1}$ ), biscuits, crackers and crisp bread ( $270 \mu\text{g kg}^{-1}$ ), breakfast cereals ( $160 \mu\text{g kg}^{-1}$ ), other products based on potatoes, cereals and cocoa ( $97 \mu\text{g kg}^{-1}$ ), cereal baby foods ( $73 \mu\text{g kg}^{-1}$ ), soft bread ( $42 \mu\text{g kg}^{-1}$ ), baby foods other than cereal

(24  $\mu\text{g kg}^{-1}$ ). Among other products the extraordinary high are vegetable crisps (1900  $\mu\text{g kg}^{-1}$ ). The distribution of acrylamide levels in particular food categories is summarized in the scientific opinion of the EFSA.

### Mitigation Efforts and Tools

The effort to minimize acrylamide content in frequently consumed foods has been transformed into the recently approved Commission Regulation 2017/2158 establishing mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food which shall apply from 11 April 2018. When the benchmark levels set to the particular food category are exceeded, a food business operator (FBO) is obliged to review the mitigation measures applied and adjust processes and controls with the aim to achieve levels of acrylamide as low as reasonably achievable below the benchmark levels. Industry guidance has been developed by various food sectors at national and EU levels, e.g. The Acrylamide Toolbox by Food Drink Europe and the Guidance for Industry by the Food and Drug Administration (US FDA). Among proposed most relevant intervention steps are storage and selection of raw material with low level of precursors, adjustment of heat transfer, application of additives preventing acrylamide formation, enzymatic treatment with asparaginase in order to eliminate asparagine.

### Exposure Level and Risk

The estimates of daily acrylamide intake range from 0.4 to 1.9  $\mu\text{g kg}^{-1}$  bw for the mean exposure, and from 0.6 to 3.4  $\mu\text{g kg}^{-1}$  bw for 95th percentile exposure across surveys and age groups. Children are the most exposed age group on a body weight basis. Acrylamide is metabolized in the body to glycidamide. Both are conjugated with urinary mercapturic acid, forming adducts with hemoglobin as well as DNA. They can be used as biomarkers for measuring the exposure to acrylamide. In epidemiological studies AA intake was not associated with an increased risk of most common cancers, but the evidence is limited and inconsistent.

### Conclusions

Food processing results in chemical changes that are not always favorable. A change of the texture and the formation of aroma, the elimination of harmful or spoiling bacteria are accompanied by the formation of possibly harmful compounds. The concentrations found in foods are normally low and with limited relevance. However, when specific foods are consumed to excess or the heat treatment is too intensive the cancer risk can increase. Some of the compounds discussed in this chapter are known to be extremely carcinogenic. Careful cooking and limited uptake of potentially contaminated foods reduce the risk significantly.

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# Food Allergens: A Regulatory/Labelling Overview Including the VITAL Approach

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## Introduction

### What Is an Allergy

Our immune system has the essential function to protect us from “invaders” like bacteria or viruses. This is done by ingesting the invader by a mast cell and presenting parts of it on its surface, which triggers the formation of antibodies that specifically recognize the invader. If, at a later time, the same bacterium or virus invades again, antibodies are already there to participate in the elimination of the foreign agent. In some cases, the immune system, by a mechanism not well understood, mistakes certain proteins, including those from food, as “invaders” and triggers an immune response. These events are known as food allergic reactions. An allergic episode can involve one or several body systems, i.e., respiratory, cutaneous, circulatory and digestive. Sensitivity to food proteins and the characteristics of the reactions vary among allergic individuals, and also between episodes. The exposure to food allergens can trigger symptoms ranging from mild to severe, and even fatal.

### Who Is Affected

While the total number of food-allergic individuals globally are estimated to range between 1%–10% (Chafen et al., 2010), the number of indirectly affected persons is significantly higher. This can be easily demonstrated with the following example:

- Thomas is a 10-year-old boy and suffers from an allergy to milk. Who is affected?
- Thomas parents need to ensure that he does not consume milk-containing products.
- Thomas grandparents, uncles and aunts also need to ensure that when Thomas is around, no food containing milk proteins is served to him.
- Thomas goes to school with lunch-time catering. Therefore, the caterers need to be aware not to serve milk-containing products to him. This includes products where milk is not an obvious ingredient. Example is a potato mash, which can be made with milk or cream for smoothness.
- Thomas also plays soccer and the team goes to lunch and celebrations following their matches. The trainer needs to ensure that Thomas does not consume milk proteins in those places.
- When Thomas is invited to birthday parties, the friends’ parents also need to ensure that Thomas does not consume milk-containing products during the party.

This example demonstrates that while the number of directly affected people with food allergies may seem small, the number of indirectly affected people is significantly larger.

### What Is the Difference Between an Allergy and Gluten Intolerance

Food allergies and intolerance to gluten (celiac disease) are often mixed and used interchangeably, even sometimes by experts. This is not very surprising since gluten-containing cereals can trigger both, allergies and intolerances. Celiac disease is estimated to be around 1% in developed countries, although this figure is thought to be significantly higher due to the fact that only a fraction of affected individuals is diagnosed (Catassi and Fasano, 2008). Although there are many unknowns about the mechanisms of food allergies and celiac disease, it is known that food allergy is a hypersensitivity mediated by IgE (humoral component) while celiac disease has a more cellular basis, and it is not only limited to the digestive system.

### Allergenic Foods: Food Ingredients and Food Contaminants

Foods, that cause allergic reactions in a small percentage of the population, are healthy for the majority. The main risk for allergic individuals happens when they are inadvertently exposed to the allergenic source. For example, if allergens are not listed as ingredient on a product label (due to omission) or because the food allergen is present in the final product due to cross-contamination. If allergenic foods can be food contaminants, what is the difference between them and contaminant-only compounds like pesticides and mycotoxins? The primary difference is the acute effect of food allergens after consumption by sensitive individuals. While a dose of pesticide residues or mycotoxins which slightly exceeds the legal limit is unlikely to cause substantial, short term health effects for those consuming it, this is significantly different for food allergens. In many cases, small amounts of food allergens can trigger severe and sometimes life-threatening effects in question of seconds. The onset of the reaction usually occurs within 30 minutes after exposure to the offending food. The most sensitive allergic individuals may suffer anaphylactic shock, that may lead to death, if not treated immediately (for instance, with an epi-pen as first aid). Many examples are not described only in the scientific literature, but they also make headlines in the press. A recent example is the case of an allergic person dying from consumption of a dish in an Indian restaurant containing peanuts (Withnall, 2017). Another issue specific to food allergens is the adventitious contamination and carry-over during production. This happens for example when different foods are produced on the same production

line, and the first one contains food allergens as part of the recipe, while the second, produced on the same line and after cleaning, does not. Remains in pipes, mixers or conveyer belts from the first production can inadvertently contaminate the second product where no food allergen is part of the recipe and therefore is not labelled. Other examples are the carry-over of particles in a factory through air-flow from one production lines containing allergens as part of the recipe to another which does not. The possibility of carry-over and adventitious contamination has led to a proliferation of the so-called “precautionary allergen labeling” (PAL, also known as “May contain” labelling). This is discussed in more detail at a later point.

## Regulations

This section describes the different existing regulation of selected countries and regions. It is not to be considered as exhaustive list since many countries follow [Codex Stan 1-1985](#) (REV 2010) and it can reasonably be assumed that more countries will follow. As it can be seen by Europe and Canada, some countries add additional allergens to the list of those required to be labelled on pre-packaged foods. All the rules require the labeling of certain food allergens when used as food ingredients.

### Europe

Europe has one the most extensive lists of food allergens requiring labelling, if one excludes the voluntary items on the Japanese food allergen labelling list below. How did Europe get to this list and what were the regulatory developments from when food allergens initially entered the regulatory arena? Europe is the region which has most likely seen the largest number of changes to allergen labelling between its first introduction and the current regulations, forcing food industry and consumers to constantly catch up with the changes ([Popping and Diaz-Amigo, 2018](#)).

Let’s take a step back to when the first attempts of harmonizing food labelling were made back in 1979: The [79/112/EEC](#) was a general directive on how food sold to the final consumer had to be labelled. Neither allergens nor gluten were mentioned in this directive. This directive experienced a major overhaul in 2000 by the enactment of the [Directive 2000/13/EC](#), and still, there was no mention of food allergens or gluten. Two years later, the so-called Food Safety Regulation was introduced. The [178/2002/EC](#) was a regulatory milestone, and article 14 stated clearly:

#### Article 14

Food safety requirements

1. Food shall not be placed on the market if it is unsafe.
2. Food shall be deemed to be unsafe if it is considered to be:
  - (a) injurious to health;
  - (b) unfit for human consumption.

While one may think that this automatically triggers labelling of food allergens, this was not the case. Regulators had the average, healthy, European consumer in mind and tried to protect from food fraud or tainted foods.

A year later, European manufacturers and consumers woke up to the first allergen labelling requirement: the European Commission published an amendment to the general food labeling [Directive \(2000/13/EC\)](#): the [Directive 2003/89/EC](#) became the “Allergen Labeling Directive”. The European Commission explained in the “whereas” to this Directive that the objective was aimed to “achieve a high level of health protection for consumers and to guarantee their right to information”. In annex IIIa, allergens and substances of intolerance that now required labeling were listed. It is important to notice here that this Directive applies to food ingredients. This 12-strong group contained:

- Cereals containing gluten (i.e., wheat, rye, barley, oats, spelt, kamut, or their hybridized strains) and products thereof;
- Crustaceans and products thereof;
- Eggs and products thereof;
- Fish and products thereof;
- Peanuts and products thereof;
- Soybeans and products thereof;
- Milk and products thereof (including lactose);
- Nuts, i.e., almond (*Amygdalus communis* L.), hazelnut (*Corylus avellana*), walnut (*Juglans regia*), cashew (*Anacardium occidentale*), pecan nut [*Carya illinoensis* (Wangenh.) K. Koch], Brazil nut (*Bertholletia excelsa*), pistachio nut (*Pistacia vera*), and macadamia nut and Queensland nut (*Macadamia ternifolia*), and products thereof;
- Celery and products thereof;
- Mustard and products thereof;
- Sesame seeds and products thereof; and
- Sulfur dioxide and sulfites at concentrations of more than 10 mg/kg or 10 mg/L expressed as SO<sub>2</sub>.

While there are twelve groups listed for labelling, from the analytical perspective, these were significantly more as each of the cereals, each of the fish species, the different mustards (yellow, brown, black) and each of the nuts need to be detected.

Another remarkable issue was that this Directive did not list any exemptions, but rather included all “products thereof”. This means that under this Directive, even highly refined oils and even pure vitamins derived from these plants require labeling, although the allergen triggering proteins are no longer present.

The suppliers of raw materials and food ingredients launched an initiative to remedy this situation. They argued that the labeling of highly refined products would be unnecessary and detrimental to the quality of life of allergy sufferers as it further limits their already small list of safe products to select from.

The European Commission followed their argumentation and issued a temporary exemption list through yet another amendment of the [Directive 2000/13/EC](#) (and indirectly also to the [Directive, 2003/89/EC](#)). The exemptions in the [Commission Directive 2005/26/EC](#) were the following:

### **Cereals Containing Gluten**

- Wheat-based glucose syrups, including dextrose;
- Wheat-based maltodextrins;
- Glucose syrups based on barley; and
- Cereals used in distillates for spirits

### **Eggs**

- Lysozyme (produced from egg) used in wine; and
- Albumin (produced from egg) used as fining agent in wine and cider

### **Fish**

- Fish gelatin used as a carrier for vitamins and flavors; and
- Fish gelatin or isinglass used as fining agents in beer, cider, and wine.

### **Soybean**

- Fully refined soybean oil and fat;
- Natural mixed tocopherols (E306), natural D- $\alpha$ -tocopherol, natural D- $\alpha$ -tocopherol acetate, and natural D- $\alpha$ -tocopherol succinate from soybean sources;
- Vegetable oil-derived phytosterols and phytosterol esters from soybean sources; and
- Plant stanol ester produced from vegetable oil sterols from soybean sources.

### **Milk**

- Whey used in distillates for spirits;
- Lactitol; and
- Milk (casein) products used as fining agents in cider and wine.

### **Nuts**

- Nuts used in distillates for spirits; and
- Nuts (almonds and walnuts) used (as flavor) in spirits.

### **Celery**

- Celery leaf and seed oil; and
- Celery seed oleoresin.

### **Mustard**

- Mustard oil;
- Mustard seed oil; and
- Mustard seed oleoresin.

It is important to remember that this amendment was a temporary one.

In the meantime, European Commission seemed to have identified two additional products that can trigger allergic reactions in susceptible individuals: lupins and mollusks. This triggered another amendment, the [Commission Directive 2006/142/EC](#), to the already amendment-rich [Directive 2000/13/EC](#) (before its repeal in 2011, the 2000/13 EC counted more than 40 amendments and three corrections).

In 2007, the time for the temporary exemptions had run out and the 2000/13/EC experienced a major overhaul regarding its requirement for allergen labelling. All previously mentioned amendments were consolidated in the [Commission Directive 2007/68/EC](#), which again, amended the Directive 2000/13/EC. With that, European regulators had arrived at a now 14-strong group of food allergy- and intolerance triggering substances that had a number of exemptions:

1. **Cereals containing gluten** (i.e., wheat, rye, barley, oats, spelt, kamut, or their hybridized strains) and products thereof, *except* wheat-based glucose syrups, including dextrose; wheat-based maltodextrins; glucose syrups based on barley; and cereals used for making distillates or ethyl alcohol of agricultural origin for spirit drinks and other alcoholic beverages.
2. **Crustaceans** and products thereof.
3. **Eggs** and products thereof.
4. **Fish** and products thereof, *except* fish gelatin used as carrier for vitamin or carotenoid preparations; and fish gelatin or isinglass used as fining agents in beer and wine.
5. **Peanuts** and products thereof.
6. **Soybeans** and products thereof, *except*: fully refined soybean oil and fat; natural mixed tocopherols (E306), natural D- $\alpha$  tocopherol, natural D- $\alpha$  tocopherol acetate, and natural D- $\alpha$  tocopherol succinate from soybean sources; vegetable oil-derived phytosterols and phytosterol esters from soybean sources; and plant stanol ester produced from vegetable oil sterols from soybean sources.
7. **Milk** and products thereof (including lactose), *except* whey used for making distillates or ethyl alcohol of agricultural origin for spirit drinks and other alcoholic beverages; and lactitol.
8. **Nuts**, i.e., almonds (*A. communis* L.), hazelnuts (*C. avellana*), walnuts (*J. regia*), cashews (*A. occidentale*), pecan nuts [*C. illinoensis* (Wangenh.) K. Koch], Brazil nuts (*B. excelsa*), pistachio nuts (*P. vera*), macadamia nuts and Queensland nuts (*M. ternifolia*), and products thereof, *except* nuts used for making distillates or ethyl alcohol of agricultural origin for spirit drinks and other alcoholic beverages.
9. Celery and products thereof.
10. Mustard and products thereof.
11. Sesame seeds and products thereof.
12. Sulfur dioxide and sulfites at concentrations of more than 10 mg/kg or 10 mg/L expressed as SO<sub>2</sub>.
13. Lupin and products thereof.
14. Mollusks and products thereof

It is very noticeable that lysozyme and albumin from egg, and mustard oils and oleoresins are no longer on the list of derivatives exempt from labelling.

Another major milestone, not only for food allergen labelling, but for food labelling in general, was the so-called Consumer Information [Regulation 1169/2011](#), published in 2011. A major difference is that while the 2000/13/EC was a Directive, allowing “national” flavors of implementing local legislation, the 1169/2011 is a Regulation, which must be adopted by all European member states immediately and without change.

The [Regulation 1169/2011](#) maintained the list of groups listed in the [Directive 2007/68/EC](#) requiring labeling while still expanding the scope by including not only packaged but also non-prepackaged products (e.g., in bakeries). It also requires catering outlets to provide allergen information to customers. This applies to restaurants and canteens alike. European Commission also made clear, that the [Regulation 1169/2011](#) is also prone to changes as new evidence comes to light. It reads in Article 21, Section 2: “In order to ensure better information for consumers and to take account of the most recent scientific progress and technical knowledge, the Commission shall systematically reexamine and, where necessary, update the list in Annex II by means of delegated acts, in accordance with Article 51.” But like all previously mentioned Directives, this Regulation also only addresses the labelling requirements for ingredients and does not consider adventitious contamination with allergens through carry-over, leaving a significant regulatory gap causing insecurity with food manufacturers and consumers alike. Instead, in July, the European Commission explained to food manufacturer and consumers, what “milk” and “egg” and “nuts” mean in the [EC/1169/2011](#). [Commission notice C \(2017\) 4864](#) states:

“Egg” in Annex II, point 3 refers to eggs from all farmed birds.

“Milk” in Annex II, point 7 refers to milk from the mammary gland of farmed animals.

“Nuts” as listed in Annex II, point 8 are to be understood as an exhaustive list.

Even though it is acknowledged that ostrich eggs are unlikely to be used as ingredients in most products, it means that analytically, there is still a need to have detection systems available. Equally, minks, being farmed mammals, are not known to produce significant amounts of milk that is used as ingredient. Still, there is at least theoretically a need to develop analytical tools to prove absence.

While this clarification may not contribute significantly to clarify the situation for food manufacturers and affected consumers, it certainly leaves one of the major issues, the precautionary allergen labelling (PAL), unresolved.

At this point it should be noticed that the DG JRC and DG SANCO met in 2016 to discuss the issue. One of the key statements is that any PAL needs to be risk assessment based, closing the door to ingredient manufacturers which like to be covered for any eventualities without necessarily evaluating their risk of having contaminated products appropriately ([O’connor et al., 2017](#)). The report from this meeting was published in November 2017.

Also, it is worth noting that Germany already introduced in 2014 *de facto* action levels for food allergen labelling through its enforcement bodies ALTS committee (Waiblinger and Schulze, 2018).

Gluten-free labelling had previously been regulated in the Regulation EC/41/2009 but it made perfect sense to integrate this also into the Consumer Information Regulation EC/1169/2011. To do this, several legal acts were necessary: Regulation (EC) No. 609/2013 repealed several regulations dealing with food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control. It included repealing gluten-free labeling Regulation (EC) No. 41/2009, effective July 20, 2016. Approximately 6 weeks later, on August 21, 2013, the Commission issued a Commission-delegated Regulation (EC) No. 1155/2013 amending Regulation No. 1169/2011. The third regulation (EC) No. 828/2014 then stated the “requirements for the provision of information to consumers on the absence or reduced presence of gluten in food.”

## USA

In the US the applicable regulation is the Food Allergen Labeling And Consumer Protection Act of 2004, or short FALCPA. Like Europe, the basis for allergen labelling in the United States is the Codex Standard CODEX STAN 1-1985 Framework. FALCPA adopted the Codex “big eight” groups of major food allergens with some modifications, i.e., it only includes wheat from the list of cereals containing gluten:

- milk
- eggs
- fish (e.g., bass, flounder, cod)
- crustacean shellfish (e.g., crab, lobster, shrimp)
- tree nuts (e.g., almonds, walnuts, pecans)
- peanuts
- wheat
- soybeans.

Because FALCPA only included examples for fish, crustacean shellfish and tree nut, instead of an exhaustive list of major allergic foods in these groups, in 2006, the US FDA made a list of what was consider a “tree nut”. This list includes: Almond (*Prunus dulcis* (Rosaceae)), beech nut (*Fagus* spp. (Fagaceae)), Brazil nut (*Bertholletia excelsa* (Lecythidaceae)), butternut (*Juglans cinerea* (Juglandaceae)), cashew (*Anacardium occidentale* (Anacardiaceae)), chestnut (Chinese, American, European, Seguin) (*Castanea* spp. (Fagaceae)), Chinquapin (*Castanea pumila* (Fagaceae)), coconut (*Cocos nucifera* L. (Arecaceae alt. Palmae)), filbert/hazelnut (*Corylus* spp. (Betulaceae)), ginkgo nut (*Ginkgo biloba* L. (Ginkgoaceae)), hickory nut (*Carya* spp. (Juglandaceae)), lichee nut (*Litchi chinensis* Sonn. Sapindaceae), macadamia nut/bush nut (*Macadamia* spp. (Proteaceae)), pecan (*Carya illinoensis* (Juglandaceae)), pine nut/pinon nut (*Pinus* spp. (Pinaceae)), pili nut (*Canarium ovatum* Engl. in ADC. (Burseraceae)), pistachio (*P. vera* L. (Anacardiaceae)), shea nut (*Vitellaria paradoxa* C.F. Gaertn. (Sapotaceae)), walnut (English, Persian, black, Japanese, California), heartnut, butternut (*Juglans* spp. (Juglandaceae)). Similarly, the US FDA further clarified that fish and crustacean shellfish include those species listed in [The Seafood List](#).

## Canada

At the time of preparing this article the latest amendment to the Canadian Food and Drug Regulations “1220 — Enhanced Labelling for Food Allergen and Gluten Sources and Added Sulphites” was done in December 2016. Looking at the allergens which require labelling, Canada list is similar to the European but does not require labelling of celery and lupine.

## AUS/NZ

The Food Safety Australia and New Zealand (FSANZ) also requires the labeling of an extended “big eight” list of priority food allergens: peanuts, tree nuts, milk, eggs, sesame seeds, fish, shellfish, soy, lupin and wheat, according to the [Australia New Zealand Food Standards Code](#) – Standard 1.2.3 (Information requirements – warning statements, advisory statements and declarations). Like in the European labelling regulation, FSANZ also exempt certain derived products from labelling. These include:

- glucose syrups made from wheat starch (subject to low limits)
- fully refined soy oil
- soy derivatives (tocopherols and phytosterols)
- fish isinglass
- distilled alcohol from wheat or whey.

## Japan

The currently active labeling act for food allergens is Food Labeling Act No. 70 (Shoji et al., 2018). Japan has one of the more dynamic labelling regulations. The regulatory authorities revise the list of regulated allergens based on the result of food allergy prevalence surveys, which are conducted periodically (2001–02; 2011–12). Japan has in fact two different lists:

- List of food allergens subjected to “mandatory” labeling: Egg, milk, wheat, buckwheat, peanut, shrimp, and crab.
- List of “recommended” allergens for labeling: Abalone, squid, salmon roe, orange, cashew nuts, kiwi fruit, beef, walnut, sesame, salmon, mackerel, soybean, chicken, banana, pork, matsutake, peach, yam, apple, and gelatin.

Japan, as one of the few countries setting labelling threshold for allergens, requires products with more than 10 mg/kg of the target allergen (soluble protein) in the finished product to be labelled. The determination of these quantities can only be performed by Japanese Official methods, which have been validated in the course of a national ring trials (Shoji et al., 2018).

## China

China is also following Codex. As it states in the National Food Safety Standards GB 7718-2011 on pre-packed food labels, section 4.4.3.1, regulators require “the use of easily identifiable names in the list of ingredients” on the package if any of the following are present:

- Gluten-containing cereals and their products (such as wheat, rye, barley, oats, spelt or their hybrids);
- crustaceans and their products (such as shrimp, lobster, crabs, etc.);
- fish and their products;
- eggs and their products;
- peanuts and their products;
- Soybeans and products thereof;
- milk and dairy products (including lactose);
- nuts and nuts products.

## VITAL

When it comes to labeling thresholds, Japan with its 10 mg/kg (Shoji et al., 2018) and Germany with its action level ALTS list (Waiblinger and Schulze, 2018) are exemptions. Most countries do not provide threshold guidance for those levels at which labeling for allergens should occur.

This gap was identified by the Allergen Bureau. It developed in 2007 an allergen management strategy called Voluntary Incidental Trace Allergen Labelling (VITAL), which is based on risk analysis. A group of experts analyzed existing literature, especially clinical trials for eliciting doses, and determine eliciting doses for triggering food allergens in 1% or 5% of the allergenic population. In 2014, these levels were updated to the following values described in Table 1 (Taylor et al., 2018).

For other allergens, insufficient data or data of insufficient quality had been available to make a recommendation. To determine how much ingested food would trigger an allergic reaction based on the values of Table 1, they need to be multiplied with the amounts individuals are likely to consume in a single serving. For this, the Allergen Bureau has developed a toolset that is available from their Website as subscription service. These recommendations for labeling thresholds are currently used as reference by the food manufacturing industry.

**Table 1** Amount of allergenic protein recommended by the VITAL Scientific Expert Panel (Taylor et al., 2018)

Allergen	Protein level in mg
Peanut	0,2
Milk	0,1
Egg	0,03
Hazelnut	0,1
Soy	1,0
Wheat	1,0
Cashew	2,0 (provisional)
Mustard	0,05
Lupin	4,0
Sesame	0,2
Shrimp	10



And to take this full circle, the German ALTS action levels mentioned earlier (Waiblinger and Schulze, 2018), are actually based in their majority on the VITAL-recommended values. Unfortunately, at this point, the European Food Safety Authority (EFSA) has not provided a Europe-wide recommendation as to such levels and is unlikely to do so before 2021.

Additional Information on food allergen regulations, food allergen management, survey and detection methods can be found in the open access Special Section of the Journal of AOAC International (2018, 101(1)) on Food Allergens (Diaz-Amigo and Popping, 2018).

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## Food Allergens: An Update on Analytical Methods

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### Glossary

**Coeliac disease** an autoimmune condition caused by ingestion of gluten

**Digital PCR** A PCR carried out with massive partitioning of the initial DNA in microwells or droplets such that a binary result (present or absent) is achieved from each microwell or droplet. From this if the molecule population follows the Poisson distribution the distribution of target molecule within the sample can be accurately approximated allowing for a quantification of the target

**DTT** dithiothreitol, a reducing agent used to reduce the disulfide bonds in proteins

**ELISA** enzyme-linked immunosorbent assay, a technique using antibodies to detect and quantify allergens;

**iFAAM** Integrated Approaches to Food Allergen and Allergy Risk Management – a 4 year (2013 to 2017) €9 million EU-funded project, built on an earlier €14.3 million research (EuroPrevall). IFAAM was led by the University of Manchester (UK) and involved 38 partners. <http://research.bmh.manchester.ac.uk/iFAAM>

**In silico** performed on computer or via computer simulation by analogy with *in vivo*, and *in vitro*, commonly used to refer to experiments done in living organisms, and in laboratory settings respectively.

**Intercalating dye** compound which binds with DNA and thus enables detection of amplified PCR product, generally by fluorescence, examples are SYBR Green and EvaGreen,

**LC-MS/MS** liquid chromatography mass spectrometry/mass spectrometry, a technique that uses liquid chromatography to separate analytes of interest followed by their detection and characterisation by fragmentation pattern and charge to mass ratio in tandem mass spectrometers;

**LFD** lateral flow device - low cost rapid simple point of use assays based on ELISA.

**LOD** limit of detection, the lowest quantity of a substance in a sample that can be distinguished from a sample in which the substance is absent, usually within a stated confidence interval;

**LOQ** limit of quantitation, the lowest concentration at which the analyte can not only be reliably quantified with an acceptable level of uncertainty;

**MRM** Multiple reaction monitoring

**PCR** polymerase chain reaction, a process in which thermal cycling separates double stranded DNA, and with the introduction of short single strands of nucleic acid allows the synthesis of a new set of double stranded DNA, repetition of the cycle amplifies the DNA amount to levels at which it can be reliably detected and characterised

**Post translational modification** covalent and generally enzymatic modification of proteins following protein biosynthesis, includes phosphorylation, glycosylation, nitrosylation, methylation, acetylation, lipidation and proteolysis and influences almost all aspects of normal cell biology

**RM** reference material, a material, sufficiently homogeneous and stable with reference to specified properties, which has been established to be fit for its intended use in measurement

**Shotgun proteomics** Also known as "Bottom-up" proteomics and refers to the characterization of proteins by analysis by LC-MS/MS of peptides released through proteolytic digestion of intact proteins.

### Introduction

What is food allergy? Food allergy is an increasing public health problem, particularly in developed nations. It is a subset of conditions collectively known as food hypersensitivity. Some adverse reactions to food are predictable, for example any individual might succumb to food poisoning. But there are reactions to food which affect only some people; moreover these are unpredictable, that is to say, they affect the susceptible person unexpectedly. When those reactions subsequently occur every time the person eats a given amount of the trigger food the reactions are 'reproducible' and termed 'food hypersensitivity'. Thus food hypersensitivity is: '*objectively reproducible symptoms or signs initiated by a defined stimulus at a dose tolerated by 'normal' subjects*' (Johansson et al., 2001). Food hypersensitivity can take many forms but if mediated by the immune system is food allergy, especially if an immune protein, immunoglobulin E, IgE (Schroeder and Cavacini, 2010) is involved. There are other forms of food allergy called 'non-IgE-mediated gastrointestinal food-induced allergic disorders', for further discussion of these and food allergy itself (see Walker and Gowland, 2017).

Food hypersensitivity also includes coeliac disease, an autoimmune adverse reaction to ingested gluten in which the small intestine becomes inflamed and unable to absorb nutrients. It can cause a range of symptoms including diarrhoea, abdominal pain and bloating. Gluten is a protein found in wheat, barley and rye (McIntosh et al., 2011). There are also a range of food intolerance

conditions, for example lactose intolerance (Walker and Gowland, 2017). Sensitivity to sulphites, a widely used set of food additives, can cause severe, even life threatening asthmatic reactions in sensitive subjects (Vally and Misso, 2012; EFSA, 2014).

### Sensitisation and Elicitation

Two processes are involved in IgE mediated food allergy – sensitisation and elicitation. Susceptible individuals undergo sensitisation, which can occur at any age, when an immunologically driven reaction develops to specific food proteins. Thereafter ingestion of those food proteins elicits, within a short time, release of inflammatory mediators such as histamine causing symptoms of, in increasing seriousness, mild lip tingling, pruritus (itch), urticaria (hives, a raised, itchy rash), diarrhoea, vomiting, asthma, and angioedema (swelling caused by a build-up of fluid). The most severe allergic reaction is anaphylaxis (Burks et al., 2012), an often rapid onset clinical emergency in which a multi-organ systemic allergic reaction precipitates life threatening airway, breathing or circulatory problems. Although fatal food anaphylaxis is rare there are well-documented detriments to the quality of life for allergic consumers and their families and carers (Ventner et al., 2015). There are burdens on health care, on businesses (food recalls, for example) and on regulators (Madsen et al., 2012). In less developed countries where, owing to poor labelling and awareness, significant challenges may exist. Currently, those with food allergies must adhere to lifelong avoidance of the eliciting food(s).

### Food Allergens

Jurisdictions that manage food allergy risks by labelling legislation include sulphites in such law however all other legislated allergens are large molecule proteins (Gendel, 2012).

### Protein Families

Proteins are made up of amino acids linked by amino-carboxylate condensation ('peptide bonds') into polyamides, also more frequently called polypeptides, and serve many functions in living organisms. Protein structure includes the sequence of the constituent amino acids, and the way in which amino acid side chains covalently link the polypeptide chains making up the primary structure of the protein. The most important of the side chain links is the disulfide bond between two cysteine residues. The amino acid chains are coiled and folded in an ordered way largely maximising their hydrogen bonding (secondary and tertiary structure). If more than one polypeptide chain is present these are packed in an ordered manner known as the quaternary structure. Common structures include the  $\alpha$ -helix,  $\beta$ -pleated sheet and the irregular globular proteins (Simmonds, 1992; Aalberse, 2000). Food allergens belong to a somewhat limited group of protein families of plant or animal origin. Important plant protein families that frequently include allergenic proteins include the cereal seed storage prolamins, cupins and the profilin and Betv1 families. Significant animal food allergens are present in milk, egg, and seafood. Mammalian milk allergens are found in three protein families,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and caseins. In hens' eggs the protein ovomucoid is important and in seafood there are two major groups of allergenic proteins, the tropomyosins of crustaceans and molluscs and the parvalbumins in fish and amphibians. Allergenicity is often associated with protein thermal stability and resistance to proteolysis, the ability to form oligomers, and the tendency to aggregate (Breiteneder and Mills, 2005).

### Allergen Nomenclature

Systematic allergen nomenclature was initiated by Marsh, Løwenstein and Platts-Mills in 1980 (Breiteneder and Chapman, 2014). Each allergen name is now derived from the first three letters of the genus and the first letter of the species followed by a numeral to indicate the allergen in the chronological order of purification. Thus as peanut belongs to the genus *Arachis* and the species *Arachis hypogaea*, the allergens are Ara h 1, Ara h 2 and so on. Ara h 2 is member of the prolamin protein superfamily. An allergen nomenclature subcommittee exists under the auspices of the World Health Organization (WHO), and the International Union of Immunological Societies (IUIS), with criteria for including allergens in the systematic nomenclature (the Website is <http://www.allergen.org/index.php>). Other examples include milk from the cow, *Bos domesticus*, for example Bos d 8, the caseins, and chicken, *Gallus domesticus* hence hens' egg contains Gal d 2, ovalbumin. The AllFam database is a resource for classifying allergens into protein families (<http://www.meduniwien.ac.at/allfam/>).

### Risk Assessment and Risk Management

Official risk management of food allergy depends mainly on food labelling. Food ingredients that may trigger food allergy (priority major allergens) or hypersensitivity reactions (gluten and sulfites) are specified in law (Gendel, 2012). If included in a foodstuff the designated ingredients must be disclosed in the list of ingredients of the food. In the European Union, EU, these ingredients must be highlighted, for example in **bold print** in the list of ingredients (European Parliament and Council, 2011). The global standard for food allergen labelling is that of the Codex Alimentarius (Codex Alimentarius, 2010) which lists eight allergens, although individual jurisdictions such as the EU legislate for more, Table 1. A review of other jurisdictions is provided by Gendel (2012). However,

**Table 1** Allergens legislated for in the European Union, Regulation 1169/2011, Annex II

<i>Annex II entry</i>	<i>Examples</i>
Cereals containing gluten and products thereof	Wheat Rye Barley Oats
Crustaceans and products thereof	Shrimp/prawn Crab Lobster Crayfish
Eggs and products thereof	
Fish and products thereof	
Peanuts and products thereof	
Soybeans and products thereof	
Milk and products thereof	Skimmed milk powder Cheese
Nuts, and nut products namely	Almond Hazelnut Walnut Cashew Pecan Brazil Pistachio Macadamia
Celery and products thereof	
Mustard and products thereof	
Sesame seed and products thereof	
Sulfur dioxide/sulfites	
Lupin and products thereof	
Molluscs and products thereof	Mussels Scallops Cockles Oyster Clam

There are limited exceptions cited in Annex II of ingredients that do not contain sufficient allergenic protein to elicit a reaction.

unintended allergens, which might cross contaminate the supply chain during harvest, transport, storage or processing, are treated differently. Food business operators must implement a risk assessment in order to establish whether a hazard is likely to occur, and seek to either eliminate this risk, or reduce the risk of contamination to acceptable levels below which only the most sensitive allergic subject might react. Advisory (“may contain ...”) labelling is often used but should only be applied when there is a demonstrable and significant risk of allergen contamination (European Parliament and Council, 2002; Monks et al., 2010). Walker et al. (2017a,b) have reviewed the management of food allergens in the UK Retail Supply Chain.

Risk assessment approaches have been developed by the Allergen Bureau Voluntary Incidental Trace Allergen Labelling, VITAL and the Integrated Approaches to Food Allergen and Allergy Management (iFAAM) consortium to manage food allergen risk. These apply milligram per kilogram allergen protein ‘action levels’ derived from the estimated eliciting dose extrapolated from dose-distribution relationships for the allergen and the food serving size. The eliciting dose is the predicted amount of allergenic food, that may provoke an allergic reaction in a given percent of the population. Table 2 shows estimated reference doses and the related action levels calculated for a specific food serving size (Taylor et al., 2014).

Thus analysis for food allergens may be required from percentage down to milligram per kilogram (parts per million) concentrations, with the emphasis on the latter.

## Food Allergen Analysis

### Why Is Food Allergen Analysis Needed?

Food allergen analysis is a key component in protecting people with allergy from harm and ensuring businesses are able to provide food that is safe for them. Regulatory risk management of food allergens hinges on labelling disclosure to facilitate avoidance. Thus surveillance and enforcement of allergen labelling frequently turns on analysis to support and protect consumers and responsible businesses.

**Table 2** Reference doses and typical concentrations

Food	Reference dose (EAACI food allergy & anaphylaxis guidelines)	Typical <sup>b</sup> action levels mg kg <sup>-1</sup>
Peanut ED 1%	0.2 mg peanut protein	2
Cow's milk ED 1%	0.1 mg milk protein	1
Egg ED 1%	0.03 mg egg protein	0.3
Hazelnut ED 1%	0.1 mg hazelnut protein	1
Soya ED 5%	1.0 mg soya protein	10
Wheat ED 5%	1.0 mg wheat protein	10
Cashew ED 5%	2.0 mg cashew protein	20
Mustard ED 5%	0.05 mg mustard protein	0.5
Lupin ED 5%	4.0 mg lupin protein	40
Sesame seed ED 5%	0.2 mg sesame protein	2
Shrimp ED 5%	10 mg shrimp protein	100
Fish ED 5%	0.1 mg fish protein <sup>a</sup>	1

ED  $x$  %, Eliciting Dose for  $x$  % of the allergic population.

<sup>a</sup>provisional.

<sup>b</sup>assuming a portion size of 100 g.

Irresponsible replacement of almond powder with peanut powder has caused death thus with increasing need to demonstrate traceability and integrity in the supply chain analysis is needed to check that food is what it is claimed to be, and encourage systems to reduce exposure to fraud.

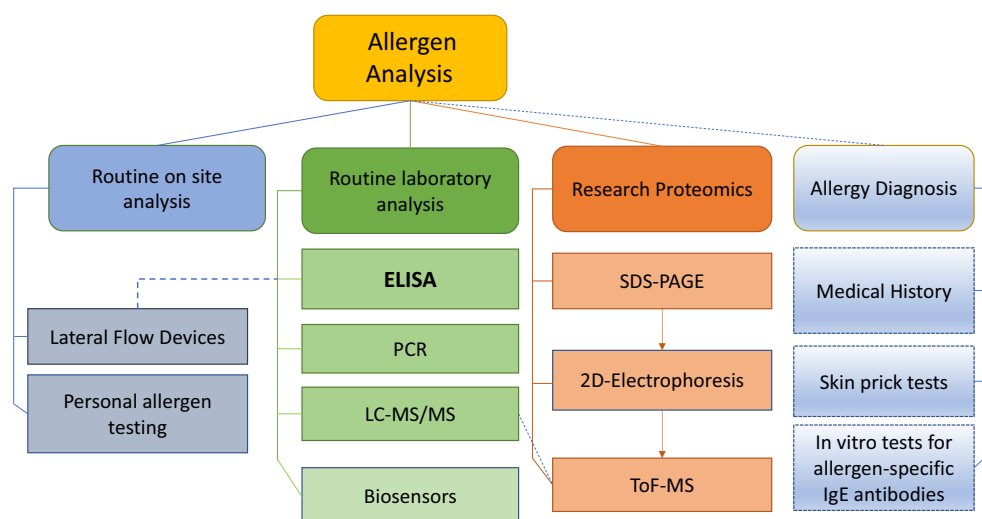
Validation and verification of factory cleaning requires allergen analysis.

If something does go wrong investigation of recalls and incidents may require allergen analysis. Allergen analysis has provided key evidence in criminal or civil action in the courts. Investigation of adverse reactions may require analysis to find out what caused the reaction, and therefore enable the individual to avoid it in the future. Investigation of fatalities may require analysis for example of food seized at the incident, stomach contents or other forensic exhibits (Gowland and Walker, 2014).

It should be remembered that food allergy can develop to any consumed food, not just those foods included in labelling legislation.

### How Is Food Allergen Analysis Carried out?

Fig. 1 shows an over view of analytical approaches applied to food allergens in different disciplines. A distinction must be made between research tools, routine industrial or enforcement laboratory analysis, on-site and personal allergen testing and testing for allergy diagnosis. Many techniques have been applied, Table 3 illustrates most of the commonly applied approaches (Walker et al., 2008).



**Figure 1** An over view of analytical approaches applied to food allergens.

**Table 3** Main and subsidiary techniques for allergen detection

<b>Routine techniques</b>	
ELISA	Routinely used, capable of standardisation, quantified results, good precision but results can be kit dependent. Targets proteins (in some, but not all, cases allergens) therefore of direct relevance to allergic consumers. Relatively inexpensive if operated batch wise.
Lateral Flow Devices, LFD	LFD are based on ELISA and offer low cost rapid simple point of use assays capable of use by non-technical staff. Exhibiting similar specificity and limits of detection to ELISAs they are semiquantitative with subjective quantitation and reproducibility can be less than with ELISA.
Real time PCR	Detects DNA rather than protein therefore not probative of the presence of an allergen but sensitive. DNA more stable than proteins under harsher extraction conditions. Quantitative results can be obtained.
LC-MS/MS	Mass spectrometry platforms are becoming the gold standard technologies for protein characterisation and quantification. Shotgun and targeted protein identification and quantification by mass spectrometry have been successfully applied to the analysis of allergens in processed food matrices. Mass spectrometry allows harsher extraction conditions than can be used with ELISA. The basis of routine liquid chromatography -mass spectrometry (LC-MS or LC-MS/MS) approaches are that the extracted protein is broken down into its constituent peptides using enzymatic digestion. The peptides can then be chromatographed and detected as with small molecule MS. Expensive to set up and run but multiplexing automated runs are the norm and metrological traceability is achievable.
<b>Less routine or research techniques</b>	
PCR	See above - not quantitative.
PCR-ELISA	PCR but with ELISA endpoint. As for PCR but can be laborious owing to the extra steps introduced by the ELISA.
Biosensors	Trade examples are Biacore, (surface plasmon resonance) Bioveris, Luminex, and can be used to detect protein or DNA. Some applications – hazelnut, egg and milk noted. Expensive set-up costs but rapid real time throughput achievable.
SDS-PAGE	Used to characterise allergens. Elaborate and time consuming, use human sera but antibodies can be raised in animals after allergen characterised.
Immunoblotting	Has been used but not widely owing to elaborate procedures necessary. In an antibody-containing gel standard or sample allergen proteins migrate according to their electrophoretic mobility forming rocket-shaped precipitates. The height of the 'rockets' is proportional to the amount of allergen protein.
Rocket Immuno-electrophoresis (RIE)	Simple and inexpensive screening of food samples, semi quantitative. Standards and sample extracts are spotted onto membrane strips. Detection is by incubation with enzyme-labelled antibodies which bind to the target antigens. The spots are visualized by addition of a substrate which is transformed by an enzymic reaction into a coloured product. The intensity of the spots is proportional to the amount of antigen
Dot immunoblotting	
RAST, EAST	Radioallergosorbent and Enzyme allergosorbent - <i>in-vitro</i> diagnostic tests which have been applied to food analysis (historic interest only).

## Research Proteomics

Gel electrophoresis, commonly used for protein separation, depends on the differential mobility of proteins in a gel under an applied electric field and as a function of their size, shape and net charge. The gel is usually a polyacrylamide, hence Polyacrylamide Gel Electrophoresis, PAGE. If the extracted proteins are dissolved in an anionic detergent, e.g. sodium dodecyl sulphate (SDS), SDS-PAGE can be performed to separate proteins largely on the basis of their molecular weight. This arises because almost all non-covalent interactions in the native proteins are disrupted by SDS, each anionic moiety of which binds to a low number of amino acids yielding a highly negatively charged protein-SDS complex of generally spherical shape. Mercaptoethanol or dithiothreitol, DTT, can also be added to reduce disulphide bonds (which otherwise stabilize folded protein conformations substantially, Creighton, 1988). Reduction of disulfide bonds folded within the protein structure can also be achieved by more harsh conditions such as high temperature, or by 6 M guanidine chloride or 8 M urea, in effect denaturing the protein (Monera et al., 1994).

Visualisation of the separated proteins is by staining, with reagents such as silver, fluorescent dyes, coomassie brilliant blue, or less frequently now, radio-labelling. SDS-PAGE yields information on the extracted proteins, such as if they are aggregated or modified. However more information can be obtained by enzymatic digestion of each protein 'spot' in the gel and liquid chromatography – tandem mass spectrometry, LC-MS/MS (see below) which has largely replaced Edman degradation. The latter uses sequential reaction of the N-terminal amino acid with phenyl isothiocyanate for cleavage and subsequent identification (Edman, 1950; Mann, 2016).

Extracted proteins can be analysed by 2D-electrophoresis which allows the resolution of complex mixtures of proteins, and qualitative and semi quantitative protein profiles. Protein extracts can also be characterized by 1D-Polyacrylamide Gel Electrophoresis, PAGE, in combination with immunoblotting using allergen specific animal antibodies where available. Mass spectrometry has a significant role to play in allergen characterisation, profiling post translational-modifications such as glycosylation or phosphorylation which occur naturally and or may be induced thermally or chemically during food processing (Monaci and Visconti, 2009).



## Routine Allergen Analysis

### Immunological Approaches

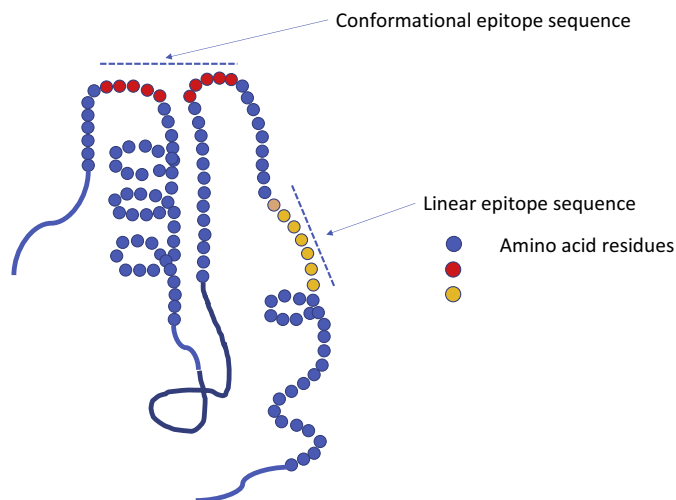
Immunological approaches make use of the ability of certain proteins, called antibodies, to bind with high selectivity to targets, called antigens. Antibodies are generally raised in animal hosts making use of the animal's immune system which, necessarily, recognises only large molecules. Small molecules (haptens) must be covalently bound to carrier proteins to elicit recognition and antibody production in the host animal. In the clinical format, radioimmunoassay, RIA, developed in 1960 by Berson and Yalow in New York, and Ekins in London, detection and quantity estimation depended on counting emissions from radioactive labelled antibodies or antigens. Resource requirements were demanding, special laboratory facilities to handle radioactivity, expensive counting instrumentation, and safety and waste disposal considerations. A less demanding label was needed. Although it had been thought that attaching a bulky enzyme would sterically hinder the immunochemical reaction between antigen and antibody, enzyme labels were proposed independently by Perlmann and Engvall in Stockholm (Engvall and Perlmann, 1971) and Schuurs and van Weemen in the Netherlands (van Weemen and Schuurs, 1971). The former developed enzyme linked immunosorbent assay, ELISA, and the latter enzyme immune assay, EIA, both building on foundational work by many research groups. Clinical applications in the form of both commercial kits and in-house set-ups burgeoned with an exponential rise in the number of clinical papers published between 1976 and 1990 (Lequin, 2005). The first commercially produced immunoassay kits for food analysis appeared in the mid 1980's (Allen, 1986), with applications to analytes such as aflatoxins, and antibiotic and other veterinary residues (Franek and Hruska, 2005). Early environmental applications were for the triazine herbicides which the author well recalls using in a court case on soil contamination in the 1990s.

Antibodies recognise sequences of amino acids in the target protein called epitopes. Such sequences may be linear or conformational (Fig. 2). Disruption of the higher order protein structure may cause conformational epitopes to become unrecognisable to the targeting antibodies (Fig. 3).

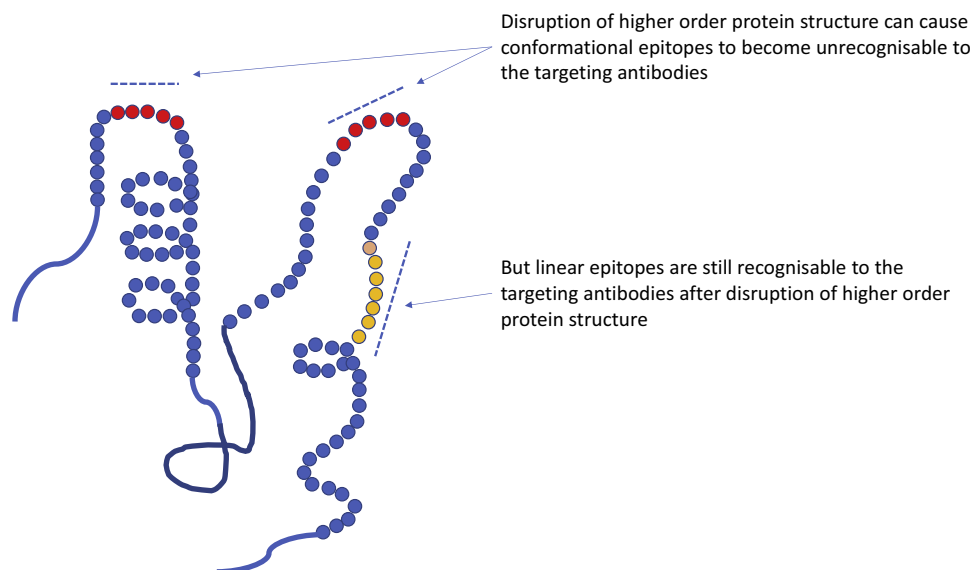
Antibodies may be either polyclonal or monoclonal. Polyclonal antibodies are purified from the serum of a suitable mammal (for example, rabbit, goat or sheep), that has been injected with the antigen. The immune response in the animal produces a mixed population of antibodies that bind to numerous different recognition sites (or epitopes) on the protein. This is an advantage in constructing assays since a knowledge of the precise orientation of an analyte is not a prerequisite for success. However the finite stock of antibodies from an animal is easily consumed, and batch-to-batch variability is an issue. Monoclonal antibodies are derived from a single hybridoma cell line tailored to recognise a single epitope with high immunogenicity. Typically the antigen is introduced into a mouse and the cells that produce antibodies (B lymphocytes) are extracted from the spleen of the mouse and added to a culture of cancer (myeloma) cells. The fusion of the B cell and myeloma cell is termed a hybridoma. The antibodies from each hybridoma line will recognise a single epitope which gives monoclonal antibodies a selectivity advantage over polyclonal antibodies albeit monoclonal antibodies are more expensive given their laborious method of production (Pang, 2010a).

### Enzyme Linked Immunosorbent Assay, ELISA

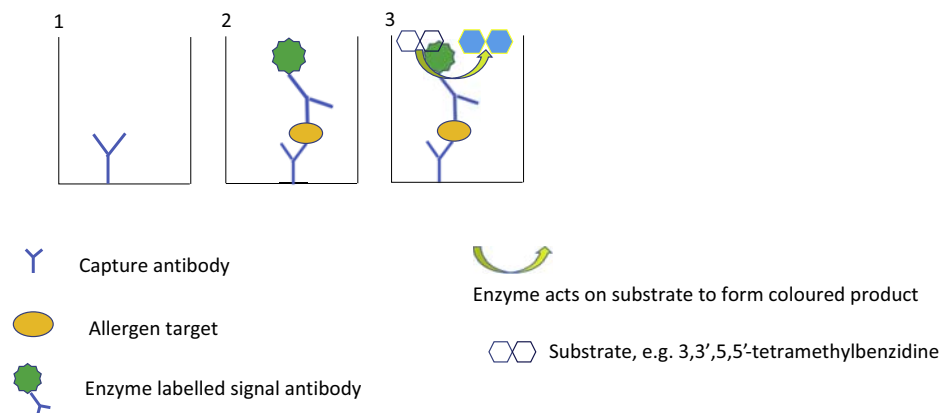
ELISA first made it routinely possible to detect food allergens. Since the technology resides in the antibodies rather than the apparatus (Allen, 1986) ELISA is relatively easy to use, largely specific to the target allergenic foods, reasonably rapid, not expensive if applied batch-wise and exhibits suitable limits of detection for many allergens. Thus, currently, routine food allergen analysis is



**Figure 2** Linear and conformational amino acid sequences.



**Figure 3** Disruption of higher order protein structure causes conformational epitopes to become unrecognisable to the targeting antibodies.



**Figure 4** Basic principle of a 'direct sandwich' ELISA.

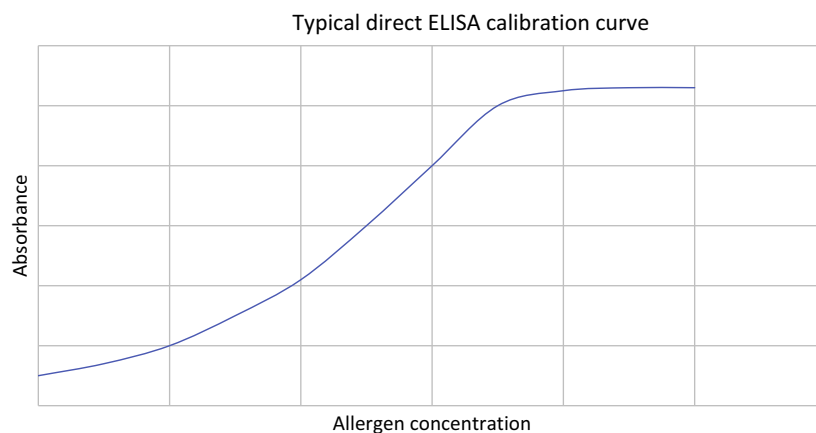
dominated by ELISA which, although there are limitations, yields quantification which, with certain caveats, is relevant and largely accepted by the food industry.

It is possible to construct an ELISA if the antibodies to the analyte of interest can be commercially sourced and many research groups do this routinely. The complexity of food as a matrix, the problems of allergen analysis and the expertise and experience of commercial ELISA kit manufacturers renders buying a kit and validating it in-house the optimum course of action for most routine purposes. The processes of construction an ELISA are well described in trade publications (for example [Bio-Rad, 2017](#)).

**Fig. 4** illustrates the basic principle of a 'direct sandwich' ELISA. Firstly, capture antibodies are bound to the well of a microtitre plate, secondly, a solution containing either calibrant allergen or an extract of the food under test is incubated in the well. Allergen, if present, becomes bound to the capture antibody. A washing step to dispose of unbound components is followed by introduction of further antibodies which bind to the captured allergen forming a 'sandwich'; a linked enzyme after further washing catalyses a colour change in an introduced chemical substrate which is detected spectrophotometrically. It is usual to include an acidic 'stop' reagent which also alters the colour and hence the detection wavelength ([Pang, 2010b](#)). A calibration curve is constructed from calibration solutions commonly supplied with the commercial kit; a typical curve is illustrated in **Fig. 5**.

There are other ELISA formats (see for example [Bio-Rad, 2017](#)) but the main alternative to the sandwich ELISA is the competitive format, essential when there is only one binding epitope on the target, for example hydrolysed products containing protein fragments or peptides. In the competitive format any assayed allergen in a sample extract or calibration solution is allowed to compete with the same allergen pre-bound to a microtiter plate well or enzyme. An inverse relationship between the analytical signal and the concentration of assayed allergen is produced.

The well-bound format works like this. A fixed amount of the allergen intended to be targeted is bound to each well, the sample extract or calibration solution is introduced followed by a standard amount of enzyme linked antibody and incubated. If there is no target allergen in the solution added to the well all the well-bound allergen will be conjugated to the introduced enzyme linked



**Figure 5** Typical direct ELISA calibration curve.

antibody. On adding the enzyme substrate the maximum amount of coloured reaction product ('signal') will be produced. An increasing amount of target allergen in the added solution competes with the well-bound allergen for the enzyme linked antibody resulting in decreasing enzyme remaining in the well after washing. Thus when the signal substrate is added decreasing amounts of coloured reaction product will be produced, hence the inverse relationship between the analytical signal and the concentration of assayed allergen.

## Practical Considerations

### Extraction

Extraction of the sample matrix to yield a solution for presentation to the ELISA is a complex issue. There are often structural changes in the target allergen molecules by modification, aggregation and interactions with other food components (e.g., lipids, starches, polyphenols) in complex processed food matrices that hinder simple extraction and inhibit subsequent antibody binding. High temperature processing and extremes of pH pose the same difficulties (Mills et al., 2009). Chocolate is recognized as a difficult matrix from which to recover allergen proteins for the above reasons. The addition of non-target protein to the test sample is used to reduce polyphenol-binding of the target allergen proteins to enhance extraction efficiency, skimmed milk powder or fish gelatin are used for this purpose. The surfactant sodium lauryl sulfate (SDS), is used for protein solubilization, and the reducing agent 2-mercaptoethanol is useful for protein dissociation by disulfide bond cleavage and both have been employed to enhance target allergen extraction (see also the section on 'Research Proteomics' above). Owing to its malodour and hazard categorization 2-mercaptoethanol has been successfully replaced by sodium sulfite in some instances (Ito et al., 2016).

The extraction of gluten is a problem that has received particular attention. The most commonly used solvent in gluten extraction is aqueous alcohol (60% ethanol or 50% propanol). However, to deal with disulphide bonds formed in processed foods reducing agents such as 2-mercaptoethanol or tris(2-carboxyethyl)-phosphine (TCEP) and disaggregating agents such as guanidine or sodium dodecyl sulphate (SDS) are used in combination with aqueous alcohols. Depending on the food matrix, defatting with n-hexane may be required for products with more than 10% fat. In general the compatibility of the extraction solvent with the subsequent analytical procedure obviously needs to be verified for each procedure (McIntosh et al., 2011; Scherf and Poms, 2016).

### Validation and Familiarisation With the Assay

Validation of the ELISA in the laboratory using it is essential. It is also important for staff using any commercial ELISA to become thoroughly familiar with the assay before attempting to produce data for reporting to clients. This can take place during validation of the ELISA in-house. Guidance on validation of allergen ELISAs is available (Abbott et al., 2010) and, in brief, should include the usual parameters (Table 4) with special focus on cross reactivity, intermediate precision and matrix interference.

### Pipetting

Pipetting accuracy in ELISA has been aided by multichannel micropipettes. However all micropipettes must be used and stored with care, and must be serviced and calibrated regularly (Blues et al., 2004), and see also micropipette manufacturers' guides (for example Gilson (undated)). Care must be taken as regards placement at the microtiter plate and avoidance of aerosol splashing.

### Plate Washing

Washing the microtiter plate is an essential step in any ELISA and must be undertaken thoroughly and with care according to the kit instructions.

**Table 4** Validation

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(a) accuracy;
(b) applicability (matrix and concentration range);
(c) limit of detection;
(d) limit of determination;
(e) precision:
(e1) repeatability;
(e2) reproducibility;
(f) recovery;
(g) selectivity;
(h) sensitivity;
(i) linearity;
(j) measurement uncertainty;

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**Follow the Procedure**

ELISA manufacturers work hard to perfect the procedure for each kit and although there are shortcomings in presentation and editing for some kits the kit procedure must be followed exactly. Since antibody–antigen reactions may not come fully to equilibrium in the timescale of an assay particular attention must be paid to timings. Similarly incubation and ambient temperatures are crucial for enzyme reactions and must be adhered to. It is usual to apply multiple replicates on the microtitre plate (duplicate or triplicate aliquots). Calibration curves must be constructed for each microtitre plate used on the day of assay and should follow the kit instructions – some kit manufacturers provide access to a spreadsheet for this purpose.

For particularly important results it is advisable to repeat the quantification of an allergen by a different ELISA platform.

**Laboratory Environment**

It is important to ensure the laboratory environment does not cross contaminate the sample. For example, blenders used for homogenization of the sample can be difficult to clean thoroughly, and atmospheric aerosols or dust can contaminate the sample with other foods, or pollen that might be homologous with an assayed allergen can enter via open windows.

For assays for which there are significant consequences, such as legal action, it may even be important to carry out the work of evidence recovery and analysis in a dedicated restricted-access containment suite. Such a facility is designed to guard against any possibility of laboratory cross-contamination. When working in the containment suite analysts must gown-up in disposable (colour-coded) forensic coveralls. Positive-pressure air-handling should be considered and environmental swabbing to ensure the area remains free of allergens must be carried out. Maximum use should be made of disposable equipment, extending to the recycling of blenders to non-sensitive areas of other work if they have been used with a positive sample or control. Separate safety cabinets should be used for different aspects of the work. The analysts must take care over their own diet to ensure that a major food allergen has not been recently consumed when working in the containment suite (Walker, 2012).

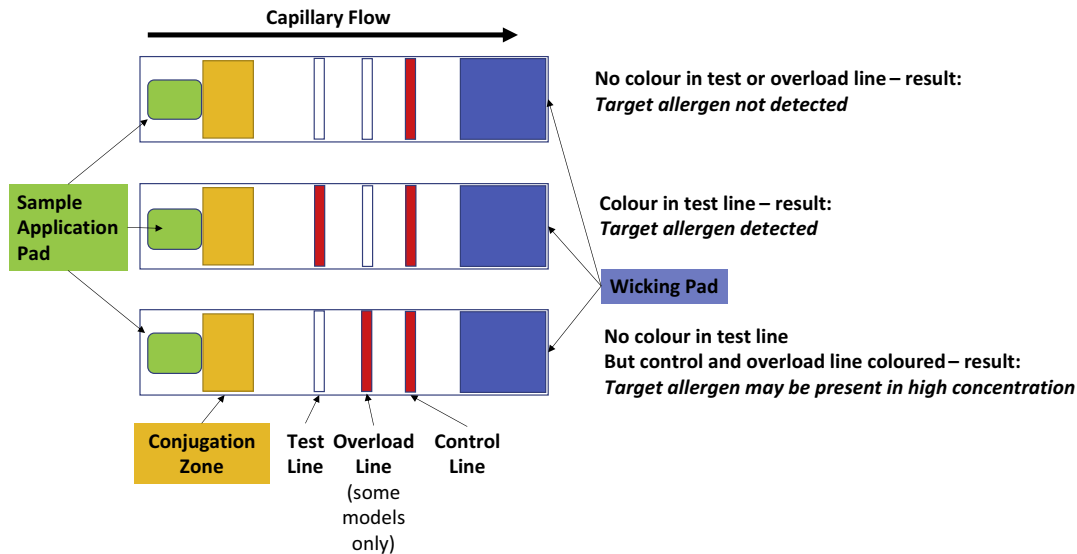
**Reporting**

A report should state the method of analysis and be in the format [X] mg/kg (or mg kg<sup>−1</sup>) as Y, where [X] is the best estimate of the concentration of allergen found in the analysis of the sample received after in-laboratory homogenisation, extraction and analysis by a validated method, and Y is either the allergen protein or the name of the food. But if the whole food is the reporting basis the conversion factor from allergen protein to whole food must be given. Conversion factors should be reported with literature references to the typical protein contents of the allergen. As a matter of routine the basis of data as allergen or (preferably) allergen protein should be specified every time a datum is given in a method or report.

**Lateral Flow Devices**

Lateral flow devices, LFD, probably most familiar as pregnancy test kits, are based on ELISA. LFD offer low cost rapid simple point of use assays capable of use by non-technical staff. Exhibiting similar specificity and limits of detection to ELISAs as a form of immunochromatography they offer some tolerance of interferences. They are, however, semiquantitative with subjective quantitation and reproducibility can be less than with ELISA.

LFDs consist of a strip of material such as nitrocellulose, nylon or other materials that support capillary flow with different compartments from a sample application zone at one end to a wicking zone at the other end which pulls along and captures the applied liquid sample extract and reagents. LFDs can be used to check incoming ingredients for target allergens, to assess



**Figure 6** Basic principle of a lateral flow device.

production line cleaning regimes by swabbing or testing rinse water or for any of the uses ELISA is normally put to. Validation is required before use and manufacturers should be consulted about sample extraction.

Fig. 6 shows typical LFD format. The sample liquid is applied to the sample application pad where it flows to a conjugation zone containing allergen-specific antibody coupled to a label to enable visualization of results. The label can be coloured latex beads, colloidal metals such as gold, increasingly in nanoform, or a fluorescent compound. Any allergen protein present in the sample binds to the labelled allergen-specific antibody which flows onto a test line. The test line contains immobilized allergen-specific antibody which captures the allergen protein with its attached labelled antibody. The accumulated label shows up as a colour or fluorescence in the test line which is proportional to the amount of allergen in the sample. Unbound labelled allergen-specific antibody continues to travel along the strip to a control line containing immobilized antibody raised against the species used to produce the allergen-specific antibody. If the test has worked (i.e. capillary flow has occurred unimpeded by sample matrix or malfunction) the species-antibody captures the labelled allergen-specific antibody and colour accumulates in the control line. A positive result therefore consists of a coloured test line and a coloured control line. A negative result consists of an uncoloured test line and a coloured control line.

However if the target allergen protein is present at a very high concentration the labelled allergen-specific antibody may be saturated with allergen protein leaving no sites at which the immobilized allergen-specific antibody in the test line can bind. Allergen protein may also travel ahead of the allergen-bound-labelled allergen-specific antibody to saturate the capture antibody in the test line. Either way, this is known as the 'hook effect' and would result in a blank test line and a coloured control line – i.e. a false negative. If this is suspected 100 to 1000 times serial dilutions of the sample extract should be tested. Some LFDs include an 'overload line' where an additional allergen-specific antibody present in the conjugate zone can be detected. Absence of colour in both the overload and test lines could signal a high allergen concentration which should be investigated by serial dilution and retest (Baumert and Tran, 2015).

The first LFD for an allergen appears to have been developed by Mills et al. (1997). The LFD employed a simple one-step extraction method and was able to detect as little as 0.01% (w/w) of peanut in marzipan and 0.1% (w/w) of peanut in chocolate. Roasted nuts were also detected, down to a concentration of 0.1% (w/w) in both foods. Polyclonal antibodies were raised to conarachin, the 7S globulin of peanut, and used as both the capture and detector elements of the assay. No significant cross-reaction with proteins from a range of nuts and legumes was seen. The final steps utilized an avidin-biotin detection system and tetramethylbenzidine as the substrate.

## Reference Materials

The importance of reference materials, RM, for allergen analysis has been emphasized by many. Commercially available ELISA kits exhibit variable and manufacturer specific sensitivities and cross-reactivity. In proficiency testing multimodal datasets for allergen ELISAs are common and different assigned values have to be generated for the different kits used. PCR and LC-MS/MS assays require appropriate calibrators for quantification and reference materials for method validation (Walker et al., 2016).

Taylor et al., 2009 were the first to describe with examples the preparation of naturally incurred standards as allergenic food residues incorporated into various representative food matrices and then processed in a manner similar to 'real-world' food processing and this approach has stood the test of time in this and other contexts.

Reference materials produced by National Measurement Institutes exhibit the highest standards. However there are few RMs produced solely with allergen analysis in mind. The Joint Research Centre (JRC), the European Commission's science and knowledge service, is a major producer of reference materials (JRC, 2017). The JRC produced a material for peanut analysis including 6 vials containing peanuts prepared at different roasting temperatures and times (IRMM – 481). Several milk powder and dried whole egg reference materials are available from JRC (BCR – 685) and the U.S. National Institute of Standards and Technology (NIST, 2017) (NIST – SRM 1549a) (NIST – SRM 8445). NIST produced a peanut butter standard reference material certified for the content of proteins (NIST – SRM 2387). LGC (LGC, 2017) has made available a mechanically defatted light roasted peanut flour in 5 g vials under Argon (LGCQC1020) which is from the same source and batch of peanut flour used for recent protein detection studies. Values are provided for nitrogen and water content and the material will prove useful for allergen protein measurement (Lee et al., 2018a, 2018b). LGC also have available a quality control set based on a chocolate dessert mix used as the vehicle for oral food allergen challenges in clinical studies (Johnson et al., 2014). Each set contains a blank unit and a unit with 10 mg kg<sup>-1</sup> gravimetrically incurred peanut protein (LGCQC101-KT). Within the iFAAM project, the material was used as a ring test sample for ELISA and Mass spectrometry analysis. A consortium of LGC, University of Manchester and Romer Laboratories commenced work in 2018 on a multi-allergen reference material on contract to the UK Food Standards Agency.

The MoniQa Association (MoniQa) have produced the first validated reference material for food allergen analysis. They manufactured milk powder biscuits (cookies) at two agreed concentrations, 3.5 mg of milk protein per kg of cookie (LOW-MQA 102016) and 35 mg of milk protein per kg of cookie (HIGH-MQA 082016). The materials are sold alongside the dry skimmed milk powder (SMP-MQA 092014) and a blank cookie (BLANK-MQA 082015). In the near future, the production of egg and soy-based reference materials is expected.

The FAPAS (Food Analysis Performance Assessment Scheme) produced a cake mix reference material containing gluten, egg, and milk (FCAL7-PRO10RM). Differently from the MoniQa material, egg and milk were added as a known amount of total ingredient to a commercial cake mix manufactured to be gluten-free, egg-free and milk-free. The material is currently not available. FAPAS produces two quality control materials: cooked biscuits containing hazelnut and peanut (T27171QC) and chocolate containing hazelnut (FCAL8-CON2QC). For the products described, the protein concentration assignment was done by consensus assessment based on ELISA analysis.

Work undertaken by the JRC (Rzychon et al., 2017) has assessed the variation in results between ELISA measurements of gluten and its likely impact on the enforcement of the EU gluten-free legislation. The study systematically examined the feasibility of harmonizing gluten ELISA assays by the introduction of (a) a common extraction procedure, (b) a common calibrator, such as a pure gluten extract and (c) an incurred matrix reference material. The comparability of measurements was limited by a weak correlation between kit results caused by differences in the selectivity of the methods. This lack of correlation produced bias that was not corrected by using a reference material alone. The use of a common calibrator reduced the between-assay variability to some extent, but variation due to differences in selectivity of the assays was unaffected. The authors advocate consensus on robust markers and their conversion to “gluten content”.

## DNA Based Methods - PCR

DNA, deoxyribonucleic acid, is the hereditary material in almost all organisms, where nearly every cell has the same DNA. DNA is located in the cell nucleus (nuclear DNA). Smaller amounts of DNA can also be found in the mitochondria (mitochondrial DNA or mtDNA). While each cell has one nucleus which aids quantification, mitochondria are more abundant per cell hence mtDNA is more attractive with regard to sensitivity. The information in DNA is stored as a code made up of four chemical bases: adenine (A), guanine (G), cytosine (C), and thymine (T). The sequence of these bases determines the information available for expressing proteins essential to the organism. DNA bases pair with each other, A with T and C with G, to form base pairs. Each base is also attached to a sugar molecule and a phosphate molecule. Together a base, sugar, and phosphate are called a nucleotide. Nucleotides are arranged in the now familiar double helix. An important property of DNA is that it can replicate, or make copies of itself. Each strand of DNA in the double helix can serve as a pattern for duplicating the sequence of bases (U.S. National Library of Medicine, 2017a).

DNA PCR analysis can be thought of as a series of procedural steps: sampling, sample preparation, DNA extraction, DNA quantitation, PCR setup, equipment operation, software analysis, manual analysis, and user interpretation. The collection of samples, their handling and continuity of evidential material, as well as the spatiotemporal separation of the laboratory into pre- and post-PCR areas are all important to successful results especially when working to a forensic standard (Valdivia and Burns, 2010; Goodwin et al., 2011).

DNA extraction kits are commercially available. A cetyltrimethylammoniumbromide (CTAB) protocol is a common starting point for DNA extraction and Valdivia and Burns (2010) have described in an approachable manner the effective sample preparation of nuts, to maximise the quantity and quality of DNA extracted. Quantitation of the extracted DNA in order to facilitate appropriate aliquoting for amplification and subsequent quantification of the assayed species is required, for example by surface tension systems enabling the analysis of microlitre sample volumes (e.g. the NanoDrop spectrophotometer). UV spectrometry or fluorometric approaches (e.g. PicoGreen) may also be used. As with ELISAs, polyphenols (e.g. in cocoa products) may inhibit extraction. Extracted DNA to which amplification is applied has been purified however inhibition of amplification by carried over lipids, polysaccharides and certain mineral salts, as well as extraction chemicals themselves, must be guarded against.



The Polymerase chain reaction (PCR), developed by Kary Mullis in the 1980s amplifies nucleic acid sequences with high specificity thus enabling the identification of plant and animal species. Amplification occurs enzymatically via repeated cycles of.

- DNA denaturation (double strand separation into single strands by heat denaturation),
- oligonucleotides or 'primers' selective for the species are added and anneal to each single strand and
- DNA polymerase promotes extension.

The DNA polymerase enzyme can add extra nucleotides to the primer by using the genomic DNA as a template. Subsequent heat denaturation and annealing of the second primer to the newly synthesized single-strand DNA allows synthesis of a complementary DNA strand (U.S. National Library of Medicine, 2017b). After sufficient cycles (up to 30–40) the amplified product can be visualised in several ways. For example an end-point PCR with capillary electrophoresis (CE) detection of soya, maize, cotton, sugar beet and lupin is described by Busby and Burns (2014) illustrating method development including the sourcing of primers or their *in silico* design using 'primer BLAST' based on sequence data deposited in GenBank. Basic Local Alignment Search Tool, BLAST, finds regions of similarity between biological sequences (NIH, 2017).

The most common approach is now real-time PCR (quantitative real-time PCR, qPCR). This is based on the measurement of a fluorescent signal that increases during the amplification of PCR products, for example by 'TaqMan' chemistry. In this approach a target-specific oligonucleotide probe has a fluorescent 'reporter' dye and a 'quencher' attached. The proximity of the quencher to the dye prevents the detection of fluorescence. When the probe binds to the amplified target DNA the quencher is separated from the reporter dye allowing measurement of the fluorescence which is proportional to the amount of amplified PCR product (Navarro et al., 2015). A sigmoid curve is obtained when the fluorescence level is plotted against the number of amplification cycles ( $C_q$ , previously  $C_t$ ). This can be used to quantify the target DNA present in the sample before the reaction by the threshold PCR cycle at which the fluorescence signal can be distinguished from the background noise. The more DNA originally present the fewer cycles required for the fluorescence to exceed the threshold.

Design of primers and probes is one of the most crucial factors affecting the success and quality of qPCR analyses and should meet specific criteria. The formation of primer-dimers and other non-specific products should be avoided or reduced. Multiple primers and probes software programs and websites are available many of them free. These tools often consider the default requirements for primers and probes, although new research advances in primer and probe design should be progressively added to different algorithm programs. After a proper design, a precise validation of the primers and probes is necessary (Rodríguez et al., 2015).

Melt curve techniques applying intercalating dyes play an important role in screening for allergen species. The melting point (dissociation of double stranded DNA) of PCR product is a function of its constituent nucleotides and can be instrumentally exhibited as an inflection in the fluorescence–temperature curve. The first derivative thereof generates 'peaks' at the melting temperature which are diagnostic for the species of interest. See also 'Complementary Techniques' below.

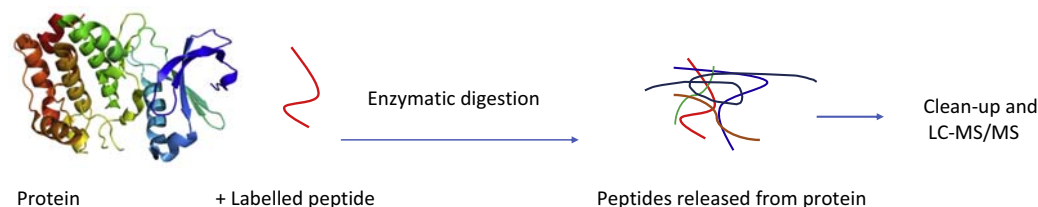
For some allergen targets DNA PCR is the only routine approach currently available. A well-known example is celery for which a specific ELISA is not possible owing to cross reactivity within the Apiaceae (or, Umbelliferae) family particularly to carrot and parsley (Daems et al., 2017). DNA-based techniques often have advantages in terms of better sensitivity and greater specificity. In addition, as an analyte DNA is generally more stable than proteins although DNA degradation does occur by extremes of, for example, temperature and pH (Gryson, 2010). Primers and probes of base pair sequence length too long to anneal to degraded DNA are best avoided, base pair sequence lengths of 150–200 are generally optimum. A PCR assay is more rapid to develop than an ELISA when novel antibodies need to be generated and PCR opens the possibility of multiplex analysis. While amplification of the base pair sequence that codes for the allergenic protein is the ideal many PCR assays amplify a gene specific only for the species and while this is strong evidence for the presence of the allergenic protein PCR has been criticised as it does not detect the actual protein(s). It should also be remembered that small primer sequences may be found in genomes other than the intended target and that sequence databases are incomplete and some entries may be misattributed. Thus method validation, including exploration of potential cross reactivities is mandatory. For two allergens, egg and milk, DNA methods are not applicable since DNA from (a) hens' eggs and chicken meat and (b) cows' milk and beef are indistinguishable. See also 'Complementary Techniques' below.

See Holzhauser and Röder (2015) for further discussion of PCR including a list of the major published methods for individual allergens.

In summary, real-time PCR methods allow the detection of allergenic ingredients in food products at levels of 10 mg kg<sup>-1</sup> or lower. Although they do not directly measure the hazard (protein), they are especially valuable for foods for which no ELISA based test is available (e.g. celery) or where ELISA results are ambiguous (Prunus species). PCR assays are probative of the source species DNA (which may not be present e.g. egg white) rather than the allergen protein and are essentially qualitative at present. Quantification based on copy number can be derived from cycle thresholds but requires reference materials to construct a calibration curve although digital PCR may circumvent this difficulty (Burns et al., 2010).

## Mass Spectrometry

The principles and applications of mass spectrometry, MS, and hyphenated techniques coupling chromatographic techniques to MS (LC-MS and LC-MS/MS) are dealt with elsewhere in this encyclopedia.



The basis of routine liquid chromatography-mass spectrometry (LC-MS or LC-MS/MS) approaches are that the extracted protein is broken down into its constituent peptides using enzymatic digestion.

**Figure 7** Basic principle liquid chromatography-mass spectrometry (LC-MS/MS).

Mass spectrometry, MS, the gold standard technology for small molecule identification and quantification, is increasingly applied to protein characterisation and quantification. Given the challenges faced by other allergen quantification approaches there is a need for methods that confirm molecular identity and are capable of validated quantification. MS methods, e.g. multiple reaction monitoring of peptides arising from enzymatic digestion of proteins, offer such advantages along with the possibility of multiplexed high throughput analysis. Shotgun and targeted protein identification and quantification by MS have been successfully applied to the analysis of allergens in processed food matrices. Shotgun proteomics, also known as “bottom-up” proteomics refers to the characterization of proteins by analysis of peptides released (or ‘cleaved’) through proteolytic digestion of intact proteins. In a typical shotgun proteomics experiment, the peptide mixture is then fractionated and subjected to LC-MS/MS analysis with the use of isotopically labelled internal standards, [Fig. 7](#). Peptide identification is achieved by comparing the tandem mass spectra derived from peptide fragmentation with theoretical tandem mass spectra generated from *in silico* studies making use of protein databases. [Zhang et al., 2013](#) describe in detail protein extraction, proteolytic digestion, and MS peptide ionization, separation, and fractionation methods and the identification of post translational modifications. Quantification is also addressed by Zang et al. Proteolysis is typically carried out by trypsin but at least 10 other enzymes with differing cleavage specificities have been applied.

Shotgun proteomics provides information on protein/peptide structure and on chemical/thermal modifications occurring during food processing. MS offers a number of advantages for the detection of protein allergens in food matrices. Firstly, it offers the stringent specificity required for confirmation of the presence of the analytes of interest by monitoring specific peptides as markers of allergenic proteins, especially when employing selected reaction monitoring (SRM) experiments on triple quadrupole instruments. Secondly, it offers a wide dynamic range and high sensitivity can be achieved if the correct sample preparation techniques are implemented. Thirdly, the sample preparation and method can be designed to target the particular class of molecule of interest. In the application of mass spectrometry for the detection of allergens, ideally, the target analyte would be the specific molecule giving rise to the allergenic response and the detection of peptides for the detection of allergens in foods has been reported ([Sealey-Voyksner et al., 2016](#)). The targeted SRM approach is capable of multiplex analysis. However, the technique requires a high level of expertise and costly equipment. More importantly, the complexity of most food matrices represents a significant challenge in the development of MS methods where matrix homogeneity, protein content, protein stability and solubility and process induced modifications of the proteins are generally less well understood. The effects of the tertiary and quaternary structure of the allergen proteins need also to be considered. Extraction efficiency is often overestimated with difficulties stemming from thermal and processing induced protein insolubility and protein reaction with matrix compounds such as tannins and polyphenols although MS allows harsher extraction conditions than can be used with ELISA. There is difficulty in determining recovery owing to lack of reference methods and reference materials. There is lack of metrological traceability to secure standardisation and harmonisation of results.

Guidelines have been published ([Johnson et al., 2011](#)) dealing with criteria for the selection of target protein analytes, peptides, MS transitions, optimization of protease digestion, quantification (e.g. through standard addition or isotopically labelled peptide standards), and effective validation of methods and harmonization of results through the use of naturally-incurred reference materials spanning several types of food matrix.

MS approaches are the only means of obtaining metrologically traceable absolute quantification of allergen proteins. This is the property of an analytical result which allows measurements made under different conditions (e.g. at different times, by different people, in different locations, using different procedures) to be compared in a meaningful way. It is achieved through a series of unbroken ‘calibration chains’ for each parameter in the measurement process which influences the final result, and its associated measurement uncertainty, within an international infrastructure, the International System of Units (the SI) ([BIPB, 2006](#); [Barwick and Wood 2010](#)). SI traceable assignment of the concentrations of allergenic proteins in food matrices has been achieved ([Cryar et al., 2012, 2013](#); [Groves et al., 2017](#)) but further work of this nature would greatly facilitate the standardisation of current analytical techniques for food allergens.

Non-exhaustively the literature contains useful papers on MS approaches to allergen analysis. The following studies illustrate the approaches being developed and the problems being tackled. [Popping and Godefroy \(2011\)](#), introduced a set of papers in the Journal of AOAC International flagging allergen detection by mass spectrometry as ‘the new way forward’. The edition included a study from [Monaci et al. \(2011\)](#) on the detection of milk allergens based on the identification of unique peptides in the tryptic digests of cookie and wine extracts using an RP-HPLC separation coupled to an Exactive nonhybrid mass spectrometer using

Orbitrap technology. The high mass accuracy and resolution provided by the Orbitrap analyzer allowed a fast preliminary identification of four previously proposed peptide markers of caseins using only accurate values of the  $m/z$  of their ions. No interference was observed, despite the complexity of the analyzed matrixes. Moreover, the availability of a high-energy, collisionally activated dissociation cell integrated in the mass spectrometer enabled acquisition of peptide MS/MS-like spectra through post-source fragmentation. Confirmation of peptide marker identity could then be achieved by a comparison between experimental and predicted product ions. See also Monaci et al. (2011b). Lutter et al. (2011) contributed studies on the development and validation of a method for the quantification of milk proteins in food products based on LC-MS.

Heick et al. (2011a) reported the first screening method for the simultaneous detection of seven allergens by LC-MS/MS. The method was based on extraction of the allergenic proteins from a food matrix, followed by enzymatic digestion with trypsin. The chosen marker peptides were implemented into one method that is capable of the simultaneous detection of milk, egg, soy, hazelnut, peanut, walnut and almond. The method was used to detect all seven allergenic commodities from incurred bread material, baked according to a standard recipe from the baking industry. Detected concentrations ranged from 10 to 1000 mg kg<sup>-1</sup>. Heick et al. (2011b), went on to compare the LC-MS/MS method with commercially available ELISA test kits. Carrera et al. (2012) described the rapid (less than 2 h) direct detection of the major fish allergen, parvalbumin, by selected MS/MS ion monitoring mass spectrometry. Trypsin digestion under an ultrasonic field provided by High-Intensity Focused Ultrasound (HIFU) was used with monitoring of nineteen parvalbumin peptide biomarkers in a linear ion trap mass spectrometer.

By contrast, Cucu et al. (2012) developed a MALDI-based identification of stable protein derived tryptic marker peptides for hazelnut which can be often present as a cross-contaminant. The authors suggest the identified peptides could be further used as analytical targets for the development of more robust quantitative MS methods for hazelnut detection in processed foods.

Di Stefano et al. (2012) published a mini-review of applications of LC-MS/MS for food analysis including for allergens. Abdel Rahman et al. (2013) described the proteomics of characterizing and quantifying allergenic proteins from shrimp as a means of controlling occupational asthma in the seafood industry. The proteins were extracted from shrimp tissue and profiled by gel electrophoresis. Allergenic proteins were identified based on their reactivity to patient sera and were structurally identified using tandem mass spectrometry. Tropomyosin, arginine kinase, and sarcoplasmic calcium-binding protein were found to be the most significant allergens. Multiple proteolytic enzymes enabled 100% coverage of the sequence of shrimp tropomyosin by tandem MS. Monaci et al. (2013) studied multi-allergen quantification of fining-related egg and milk proteins in white wines by high-resolution MS. Selected tryptic peptides were used as quantitative markers for milk (casein) and egg-white (lysozyme and ovalbumin) proteins, added to commercial white wines at sub-ppm levels. An evaluation of protein digestion yields was also performed by implementing the <sup>15</sup>N-valine-labelled analogues of the best peptide markers identified for  $\alpha_{S1}$ -casein and ovalbumin. The tryptic digests of the dialyzed wine extracts were run on liquid chromatography/high resolution mass spectrometry (LC/HRMS) with peptides providing the most intense electrospray ionization (ESI)-MS response chosen as quantitative markers of the proteins under investigation. Six-point calibrations were performed by adding caseinate and egg-white powder in the concentration range between 0.25 and 10  $\mu$ g mL<sup>-1</sup>, to an allergen-free white wine. The following three peptide markers, LIEWTSSNVMEER, GGLPINFQTAADQAR and ELINSWVESQTNGIIR, were highlighted as best markers for ovalbumin, while GTDVQAWIR and NTDGSTDYGLILQINSR for lysozyme and YLGYLEQLLR, GPFPPIV and FFVAPFPEVFGK for caseinate. Limits of detection (LODs) ranged from 0.4 to 1.1  $\mu$ g mL<sup>-1</sup>.

Uvackova et al. (2013) studied an independent quantitative mass spectrometry (MS<sup>E</sup>) approach in quantifying important allergenic proteins and detected relevant peptides carrying known epitopes in wheat grain extracts. These authors looked at 76 wheat allergenic sequences downloaded from the AllergenOnline database ([www.allergenonline.org](http://www.allergenonline.org)) as a starting point. Alcohol soluble extracts of gliadin and glutenin proteins were analyzed. This resulted in identification and quantification of 15 allergenic protein isoforms that belong to amylase/trypsin inhibitors,  $\gamma$ -gliadins, and high or low molecular weight glutenins. Additionally, several peptides carrying four previously discovered epitopes of  $\gamma$ -gliadin B precursor were detected. These data were validated against the UniProt database, which contained 11764 *Triticeae* protein sequences. The identified allergens are discussed in relation to Baker's asthma, food allergy, wheat dependent exercise induced anaphylaxis, atopic dermatitis, and celiac disease (i.e., gluten-sensitive enteropathy). Posada-Ayala et al. (2015) looked at a novel LC-MS method for the sensitive determination of the mustard allergen Sin a 1 in food. Mustard allergen Sin a 1 was purified from yellow mustard seeds and was detected with a total of five peptides showing a linear response (lowest LOD was 5 ng). Sin a 1 was detected in mustard sauces and salty biscuit ( $19 \pm 3$  mg kg<sup>-1</sup>) where mustard content was not specified.

Popping and Diaz-Amigo guest edited a further special edition of J AOAC International in 2017 on 'a global reflection on food allergen regulations, management, and analysis'. the present author's research group contributed three papers on (a) the science behind the ground breaking analysis for allergens by ELISA, molecular biology, and protein mass spectrometry during the investigation of the almond and mahaleb incidents in 2015 (see below), (b) managing food allergens in the UK retail supply chain and Groves et al. (2017) on recovery of food allergens from solid processed matrices applying SI (International System of Units) traceably quantified milk protein solutions and a novel extraction method. There were contributions to the J AOAC Int special edition from five continents on topics as diverse as food allergen labelling and regulation, quantitative ELISA, targeted and novel mass spectrometry approaches to allergen analysis and analytical devices for use by consumers. The edition is open access and available on the J AOAC International Website.

## Clinical Testing

The diagnosis of food allergy is still based primarily on a detailed medical history and comprehensive physical examination. Clinical or laboratory tests only serve as an add-on tool to confirm the diagnosis. The standard techniques include skin prick testing and *in-vitro* testing for specific IgE-antibodies, and oral food challenges. Properly done, oral food challenges continue to be the gold standard in the diagnostic workup. Recently, unconventional diagnostic methods are increasingly used. These include food specific IgG, antigen leucocyte antibody and sublingual/intradermal provocation tests, as well as cytotoxic food and applied kinesiology and electrodermal testings. These, however, lack scientific rationale, standardisation and reproducibility (Gerez et al., 2010).

## Complementary Methods

It is now well known that an incident investigated in the UK in 2015 of cumin alleged to be contaminated with almond, a risk for people with almond allergy, was caused by the *Prunus* species, *Prunus mahaleb*. It turned out that the initial findings of almond were based on almond-ELISAs that cross reacted with an almond-related species *P. mahaleb*. The author's investigation of the analytical aspects of the incident provides an illustration of the need for complementary, or 'orthogonal' approaches to the resolution of some problems of allergen analysis where one technique alone is insufficient to provide a definitive answer. The utility of ELISA solely as a screening tool for the *Prunus* family and extent of cross reactivity were confirmed. Two novel PCR assays were developed, one specific for *P. mahaleb* (Burns et al., 2016) and the other a screening method capable of identifying common *Prunus* DNA (Nixon et al., 2016). Peptides unique to almond and mahaleb were identified along with the conditions required for their extraction, tryptic digestion, chromatography and mass spectrometry and criteria for peptide identification to forensic standards (Inman et al., 2018).

The publication of an overview of the *Prunus* investigation (Walker et al., 2017a,b) enables a staged approach to be taken to any future incident thought to involve *Prunus* species and provides a template for the investigation of similar incidents. Successful resolution of the incidents described was aided by access to a multidisciplinary team of forensically aware analytical chemists, molecular biologists, protein mass spectrometrists, statisticians and food law specialists. A similar investigation of peanut and almond contamination of cumin in North America bears out the complex nature of the spice supply chain and the need for orthogonal confirmation of ELISA screening results (Garber et al., 2016). Work by Faeste et al. (2010) on celery prefigured the need for orthogonal methods for allergens although at the time no methods for the specific detection of celery protein in foods had been published. Faeste et al. (2010) developed a sandwich celery ELISA using polyclonal anticelery antibodies with an LOD of 0.5 mg kg<sup>-1</sup> in buffer; however, it was applicable only for the screening of food products because of extensive cross-reactivity with potato and carrot proteins. Using nanoLCion-trap MS/MS, a number of proteins in the three vegetable species were identified as candidates for causing cross-reactions due to amino acid sequence homologies. Among others, a novel patatin (Sola t 1)-like protein was detected in celery and a flavin adenine dinucleotide binding domain-containing protein (Api g 5)-like protein was identified in carrot. The utility of triple-quadrupole MS/MS for specific and quantitative analysis of celery, potato, and carrot allergens was evaluated using whole protein extracts. Several unique precursor ion-to-product ion transitions were determined for each species, suggesting the feasibility of developing an MS-based screening method to specifically detect celery allergens in foods.

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## Further Reading

- EAACI Food Allergy and Anaphylaxis Guidelines: <http://www.eaaci.org/resources/guidelines/faa-guidelines.html>.
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## Relevant Websites

- Allergen Nomenclature: <http://www.allergen.org/index.php> The official website for the systematic allergen nomenclature that is approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee.
- The AllFam database is a resource for classifying allergens into protein families, <http://www.meduniwien.ac.at/allfam/>.
- British Mass Spectrometry Society, BMSS, a registered charity whose purpose is to promote and disseminate knowledge of mass spectrometry and allied subjects for the benefit of the public, <http://www.bmss.org.uk/>.
- European Academy of Allergy & Clinical Immunology, EAACI, <http://www.eaaci.org/> an non-profit association including over 50 National Allergy Societies, more than 10,000 academicians, research investigators and clinicians, from 121 different countries, aimed at promoting basic and clinical research, collecting, assessing and disseminating scientific information, functioning as a scientific reference body for other scientific, health and political organisations, encouraging and providing training and continuous education and promoting good patient care in this important area of medicine.
- EAACI is a organisation. Income derived from fees, congress and meeting profits are devoted to current activities, research and travel awards, and initiatives of interest to members.
- EUR-Lex- Direct access to European Union law and other official documents <http://eur-lex.europa.eu/homepage.html>.
- iFAAM, Integrated Approaches to Food Allergen and Allergy Risk Management <http://research.bmh.manchester.ac.uk/iFAAM>.
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# Food Allergens: Seafood, Tree Nuts, Peanuts

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## Introduction

Although firm prevalence data are lacking, there is a strong impression that food allergies have increased in prevalence in the last 2–3 decades, and appear to disproportionately affect people in industrialized/westernized regions. Food allergies are more common in children compared to adults, and a rather short list of foods account for the more serious disease burden, namely peanut, tree nuts, fish, shellfish, egg, milk, wheat, soy, and seeds (Sicherer and Sampson, 2017).

To help consumers with food allergen avoidance, most developed countries mandate labelling of the most common allergenic foods such as peanuts, tree nuts, milk, eggs, fish, crustacea/shellfish, soy and wheat or cereals containing gluten, as well as ingredients derived from those foods in accordance with the 1999 Codex Alimentarius Commission (Allen et al., 2014).

Although prevalence is said to increase, some food allergies have a high rate of resolution in childhood: milk, egg, wheat and soy with continued resolution into adolescence. Nonetheless other food allergies typically have low rates of childhood resolution and persist in adulthood: peanut and tree nuts allergy. Allergy to seeds, fish and shellfish are also considered persistent, but studies are lacking to define clearly their natural course (Savage et al., 2016).

The most common food allergy among adults is shellfish, affecting an estimated 3.6% of US adults according to a self-report survey. This marks a 44 percent increase from the 2.5% prevalence rate published in Sicherer et al. (2004). Similarly, these data suggest that adult tree nut allergy prevalence has risen to 1.9% from 2008 estimate of 0.5%, an increase of 260%. Today, peanut allergy prevalence is estimated at 2.4% amongst adults (Gupta et al., 2017) while it was reported at 1.2% in 2002 (Sicherer et al., 2004).

We compiled in this review the latest scientific knowledge progress on persistent food allergies: the seafood, tree nut and peanut allergies, in particular.

## Seafood

Seafood plays an important role in human nutrition and health, as it contains excellent source of highly assimilated proteins, vitamins and polyunsaturated fatty acids. The consumption of fish and shellfish is continuously increasing worldwide, but can provoke serious IgE antibody-mediated adverse reactions in sensitive individuals. The seafood allergy and anaphylaxis epidemic is particularly serious. Seafood allergy, including shellfish (crustacean and molluscs) and fish, is typically life-long affecting up to 5% of all children and 2% of all adults (Sharp and Lopata, 2014).

Attention should be paid to the ingestion of seafood infested with *Anisakis* larvae, as it can cause a disease called anisakiasis with symptoms similar to true seafood allergy. Other adverse reactions to seafood, including histamine fish poisoning and intolerance to histamine can also trigger clinical symptoms which, although non IgE-mediated, are similar to true seafood allergy (Prester, 2016).

While shellfish and fish allergy are often discussed concurrently, the allergens causing allergic sensitization are entirely different: parvalbumins for fish and tropomyosins for crustaceans and molluscs.

## Fish Allergy

If fish consumption has been shown to increase worldwide each year, rising above 20 kg a year in 2016 for the first time (FAO, 2016), fish intake varies considerably between different regions. Traditionally, consumption is highest in coastal areas, but this pattern may become less pronounced.

Unlike other food allergies, which are typically observed in babies and young children, an allergy to fish may not become apparent until adulthood; as many as 40% of people reporting a fish allergy had no problems with fish until they were adults (Sicherer et al., 2004).

Prevalence rate of self-reported fish allergy range from 0.2% to 2.29% in the general population, but can reach up to 8% among fish processing workers. Where the gold standard challenge criteria has been used, the worldwide prevalence rate for fish allergy ranges between 0% and 0.3% (Moonesinghe et al., 2016). Fish allergy seems to vary with geographical eating habits, type of fish processing, and fish species exposure.

Fish allergy is typically known to be life-long, in contrast to clinical allergies to milk, egg, wheat and soy which are typically outgrown despite frequently persistent skin prick test (EFSA, 2014). However, fish allergy may sometimes resolve, as reported for 3.5% of fish-allergic patients in one American study (Sicherer et al., 2004). Recently tolerance induction to different types of fish through desensitization has been shown to be successful (Casimir et al., 1997; Nakajima et al., 2005; D'Amelio et al., 2017) providing hope to these sensitive patients. But unless a specific fish species was shown safe to eat by provocative challenge, patients should avoid all fish species as cross-reactivity among various species of fish may exist (Helbling et al., 1999).

Fish-allergic symptoms are the same than other IgE-mediated food allergies and can range from mild (e.g. runny nose, headache, skin rashes) to moderate (e.g. nausea, vomiting, and diarrhea) to a potential fatal reaction: anaphylaxis. Fish meat is one of the foods most commonly provoking severe anaphylaxis (Lopata and Lehrer, 2009).

The major fish allergen characterized is parvalbumin in addition to several less well-known allergens (see Table 1). Parvalbumins have been reported to be the major and only fish allergen for 95% of patients suffering from IgE-mediated fish allergy (Swoboda et al., 2002). Parvalbumins are heat resistant and were shown to keep their IgE-binding capacity after heating at 100 °C for 10 min, and after digestion with proteolytic enzymes or denaturation with chemicals (EFSA, 2014).

Latest data estimated the dose eliciting reactions in 10% of the allergic population (ED<sub>10</sub>) at 27.3 mg of fish proteins (Ballmer-Weber et al., 2015).

### Shellfish Allergy

Similarly to the consumption of fish, which has increased steadily in the past four decades, shellfish (crustacean and mollusk) consumption has increased more than threefold. This is mainly due to the increased production from aquaculture and the constant decrease in their price (FAO, 2016).

The prevalence of seafood allergy varies considerably between regions and between children and adults. Generally, shellfish allergy is more frequent worldwide than fish allergy. In decreasing frequency, shrimp, crab, lobster, clam, oyster, and mussel are the most common shellfish allergens reported (Prester, 2016). Based on self-reporting or questionnaire based methods, prevalence may reach 10% of the general population, but based on challenge studies, the reported prevalence decreases to a maximum of 0.9% in the general population (Moonesinghe et al., 2016).

Crustacea causes most shellfish reactions, and these tend to be severe. Shellfish allergy will generally develop within minutes to an hour of eating shellfish. The symptoms may be mild but shellfish allergy may also trigger anaphylaxis. It is reported to be amongst the four major causes of food anaphylaxis, with peanuts, tree nuts, and fish (Sampson, 2000).

According to Ballmer-Weber et al. (2015), the ED<sub>10</sub> for shrimp, which is the best studied crustacean allergen and the one showing highest allergy frequency for crustaceans, is 2500 mg shrimp proteins. This eliciting dose does surprisingly not follow the dose distribution of other known major allergens and is not in full agreement with Taylor et al. (2014), who set the ED<sub>05</sub> for shrimp at 10 mg shrimp proteins.

So far, tropomyosins have been considered major allergens in shellfish allergy, but some crustacean allergic individuals only show IgE-binding to non-tropomyosin allergens (Abramovitch et al., 2013). Several allergenic shellfish proteins have been identified in shrimp, crab, lobster, abalone, mussel, or oyster, these are listed in Tables 2 and 3. In a crustacean-allergic individual, the probability of reacting to another crustacean species has been estimated to be 75%, but cross-reactivity between tropomyosin from crustacean and tropomyosin from mollusks seems to be more limited as the sequence homology is markedly lower than within crustaceans (EFSA, 2014). As clinical data is lacking, no eliciting dose for mollusks has been established so far. Nonetheless, there

**Table 1** Fish allergens

Biochemical name	Allergen	Common name	Scientific name	Source	Molecular weight <sup>a</sup>
β-Parvalbumin	Clu h 1	Atlantic herring	<i>Clupea harengus</i>	Fish meat	12 <sup>b</sup>
	Cyp c 1	Carp	<i>Cyprinus carpio</i>		
	Gad c 1	Codfish	<i>Gadus callarias</i>		
	Gad m 1	Atlantic cod	<i>Gadus morhua</i>		
	Lat c 1	Barramundi	<i>Lates calcarifer</i>		
	Lep w 1	Whiff	<i>Lepidorhombus whiffiagonis</i>		
	Onc m 1	Rainbow trout	<i>Oncorhynchus mykiss</i>		
	Sal s 1	Atlantic salmon	<i>Salmo salar</i>		
	Sar s 1	Pacific pilchard	<i>Sardinops sagax</i>		
	Seb m 1	Ocean perch	<i>Sebastes marinus</i>		
	Thu a 1	Yellowfin tuna	<i>Thunnus albacares</i>		
	Xip g 1	Swordfish	<i>Xiphias gladius</i>		
Tropomyosin	Ore m 4	Mozambique tilapia	<i>Oreochromis mossambicus</i>	Fish meat	33 <sup>c</sup>
β-Enolase	Gad m 2	Atlantic cod	<i>Gadus morhua</i>	Fish meat	47.3 <sup>c</sup>
	Sal s 2	Atlantic salmon	<i>Salmo salar</i>		47.3 <sup>c</sup>
	Thu a 2	Yellowfin tuna	<i>Thunnus albacares</i>		50
Aldolase A	Gad m 3	Atlantic cod	<i>Gadus morhua</i>	Fish meat	40
	Sal s 3	Atlantic salmon	<i>Salmo salar</i>		40
	Thu a 3	Yellowfin tuna	<i>Thunnus albacares</i>		40
Vitellogenin (β' component)	Onc k 5	Chum salmon	<i>Oncorhynchus keta</i>	Fish roe	18 <sup>b</sup>

<sup>a</sup>Molecular weight (SDS-PAGE).

<sup>b</sup>Approximate – slight variation exists between species.

<sup>c</sup>kDa.

Extracted from EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies) (2014).

**Table 2** Crustacean (*Animalia: Arthropoda*) allergens

Biochemical name	Allergen	Common name	Scientific name	Molecular weight <sup>a</sup>
Tropomyosin	Cha f 1	Crab	<i>Charybdis feriatius</i>	4
	Cra c 1	Common shrimp	<i>Crangon crangon</i>	38 <sup>b</sup>
	Hom a 1	American lobster	<i>Homarus americanus</i>	–
	Lit v 1	European white shrimp	<i>Litopenaeus vannamei</i>	36
	Mac r 1	Giant freshwater prawn	<i>Macrobrachium Rosenbergii</i>	37 <sup>b</sup>
	Met e 1	Greasyback shrimp	<i>Metapenaeus ensis</i>	–
	Pan b 1	Northern red shrimp	<i>Pandalus borealis</i>	37 <sup>b</sup>
	Pan s 1	Spiny lobster	<i>Panulirus stimpsoni</i>	34
	Pen a 1	Brown shrimp	<i>Panulirus stimpsoni</i>	36
	Pen i 1	Indian shrimp	<i>Penaeus indicus</i>	34
	Pen m 1	Black tiger prawn	<i>Penaeus monodon</i>	38
	Por p 1	Blue swimmer crab	<i>Portunus pelagicus</i>	39 <sup>b</sup>
	Cra c 2	Common shrimp	<i>Crangon crangon</i>	45 <sup>b</sup>
Arginine kinase	Lit v 2	European white shrimp	<i>Litopenaeus vannamei</i>	
	Pen m 2	Black tiger prawn	<i>Penaeus monodon</i>	
Myosin light chain 2	Hom a 3	American lobster	<i>Homarus americanus</i>	23
	Lit v 3	European white shrimp	<i>Litopenaeus vannamei</i>	20
Sarcoplasmic calcium-binding protein, SCBP	Pen m 3	Black tiger prawn	<i>Penaeus monodon</i>	–
	Cra c 4	Common shrimp	<i>Crangon crangon</i>	25
	Lit v 4	European white shrimp	<i>Litopenaeus vannamei</i>	20
	Pen m 4	Black tiger prawn	<i>Penaeus monodon</i>	–
Myosin light chain 1	Pon l 4	Narrow clawed crayfish	<i>Pontastacus leptodactylus</i>	24 <sup>b</sup>
	Art fr 5	Brine shrimp	<i>Artemia franciscana</i>	17.5
	Cra c 5	Common shrimp	<i>Crangon crangon</i>	17.5
Troponin C	Cra c 6	Common shrimp	<i>Crangon crangon</i>	21
	Hom a 6	American lobster	<i>Homarus americanus</i>	20
	Pen m 6	Black tiger prawn	<i>Penaeus monodon</i>	–
Troponin I	Pon l 7	Narrow clawed crayfish	<i>Pontastacus leptodactylus</i>	30 <sup>b</sup>
Triosephosphate isomerase	Arc s 8	Shrimp	<i>Archaeopotamobius siberiensis</i>	28 <sup>b</sup>
	Cra c 8	Common shrimp	<i>Crangon crangon</i>	28

<sup>a</sup>Molecular weight (SDS-PAGE). Approximate – slight variation exists between species.<sup>b</sup>kDa.

Extracted from EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies) (2014).

**Table 3** Mollusk (*Animalia: Mollusca*) allergens<sup>a</sup>

Biochemical name	Allergen	Common name	Scientific name	Molecular weight <sup>b</sup>
Tropomyosin	Buc u 1	Common whelk	<i>Buccinum undatum</i>	
	Chl n 1	Japanese scallop	<i>Chlamys nipponensis</i>	
	Cra g 1	Oyster	<i>Crassostrea gigas</i>	
	Ens m 1	Razor clam	<i>Ensis macha</i>	
	Hal d 1	Abalone	<i>Haliotis discus</i>	
	Hal m 2	Abalone	<i>Haliotis midae</i>	
	Hel as 1 <sup>c</sup>	Garden snail	<i>Helix aspersa</i>	
	Hal r 1	Abalone	<i>Haliotis rufescens</i>	
	Mim n 1	Scallop	<i>Mimachlamys (Chlamys) nobilis</i>	
	Oct v 1	Octopus	<i>Octopus vulgaris</i>	
	Per v 1	Mussel	<i>Perna viridis</i>	
	Pin a 1	Fan shell	<i>Pinna atropurpurea</i>	
	Tod p 1 <sup>c</sup>	Japanese flying squid	<i>Todarodes pacificus</i>	
	Tur c 1	Turban shell	<i>Turbo cornutus</i>	
NA	Hal m 1 <sup>c</sup>	Abalone	<i>Haliotis midae</i>	49

<sup>a</sup>The list is not comprehensive.<sup>b</sup>Molecular weight (SDS-PAGE). Slight variation exists between species.<sup>c</sup>Allergens listed in the IUIS database.

NA: not assigned.

Extracted from EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies) (2014).

is one report describing improvement of shrimp allergy following immunotherapy for dust mite (Cortellini et al., 2011), showing that treatment of this allergy is possible.

## Tree Nuts

Tree nuts is the collective term used to describe nuts that grow on trees. Tree nuts are defined in Europe as almonds (*Amygdalus communis* L.), hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*), cashews (*Anacardium occidentale*), pecan nuts (*Carya illinoensis* (Wangenh.) K. Koch), Brazil nuts (*Bertholletia excelsa*), pistachio nuts (*Pistacia vera*), macadamia or Queensland nuts (*Macadamia ternifolia*). In the USA, tree nuts requiring mandatory ingredient labeling include additionally beech nut, butternut, chestnut, chinquapin, coconut, ginko nut, hickory nut, lichee nut, pine nut or pinon nut, pili nut, and sheanut.

Tree nuts are one of the most common foods causing acute allergic reactions and nearly all tree nuts have been associated with fatal allergic reactions. Prevalence of individual tree nut allergies varied significantly by region with hazelnut known as the most common tree nut allergy in Europe, walnut and cashew for the USA and Brazil nut, almond and walnut most commonly reported in the UK (McWilliam et al., 2015). Similar to peanut allergy, tree nut allergy tends to persist into adulthood. Resolution rates for tree nut allergy might be lower than those for peanut allergy, with 9% resolution rate only (Couch et al., 2017).

Allergic reactions to tree nuts can result from primary IgE-mediated mechanisms or, alternatively, via secondary cross-reactivity mechanisms to birch pollen, in a form of food allergy known as oral allergy syndrome or pollen food syndrome. In individuals with birch pollen sensitization, birch pollen-specific IgE can cross-react with similar proteins found in a range of fresh fruits, vegetables and nuts (apple, apricot, carrot, celery, hazelnut, peach, peanut, pear, potato and plum) resulting in oral pharyngeal symptoms (McWilliam et al., 2015).

Allergic reactions occur typically within minutes after ingestion of small amount of the offending nut with symptoms including hives, angioedema, or vomiting. Reactions may become life-threatening and involve anaphylaxis. Tree nuts and peanut together account for 70%–90% of reported food-induced anaphylaxis fatalities, with tree nuts alone accounting for around 18%–40% (McWilliam et al., 2015).

With few exceptions (notably limited peanut cross-reactivity with pistachio and walnut), peanut antigens do not serologically cross-react with tree nuts. Walnut, pecan, and hazelnut form a group of strongly cross-reactive tree nuts. Hazelnut, cashew, Brazil nut, pistachio, and almond form a group of moderately cross-reactive tree nuts. Cross-reactivities between these groups are less pronounced (notably limited cross-reactivity of walnut and pecan with Brazil nut). The strongest cross-reactivities among tree nuts follow botanical family associations: (1) walnut and pecan in the family Juglandaceae and (2) cashew and pistachio in the family Anacardiaceae (Goetz et al., 2005). Considering the potential severity of the allergy and issues with accurate identification of particular nuts in prepared foods, caution would seem prudent, and total elimination of the nut family (perhaps with the exception of previously tolerated nuts eaten in isolation) is suggested (Sicherer, 2001).

The majority of nut allergens are seed storage proteins, such as vicilins, legumins and 2S albumins. Other nut allergens are PR proteins (chitinases, Bet v 1 homologues and LTPs) and structural proteins (profilins and oleosins). Profilins are panallergens (present in pollens, nuts, seeds, fresh fruit and other vegetables). Additional proteins have recently emerged as allergens in tree nuts, including manganese superoxide dismutase (MnSOD), 60S acidic ribosomal protein P2, and cytosolic small heat shock protein. Full details about allergenic proteins from different tree nuts are available in Table 4.

If tree nuts have often been implicated as responsible for allergic reactions, two studies confirm significant increase in tolerance to the offending tree nuts following immunotherapy: Enrique et al. (2005) demonstrates successful tolerance to hazelnut after sublingual immunotherapy, as assessed by double-blind placebo controlled food challenge. The mean hazelnut quantity provoking objective symptoms increased from 2.29 to 11.56 g. In Scurlock et al. (2016), walnut oral immunotherapy induced clinical desensitization to both walnut and a test tree nut (pecan, cashews, hazelnut, pistachio) after 38 weeks on therapy.

With 202 data points, sufficient low-dose clinical challenge data was made available for hazelnut so that it is today the only tree nut allergen for which an eliciting dose was derived. The ED<sub>01</sub> (Taylor et al., 2014) is set at 0.1 mg hazelnut proteins (Taylor et al., 2014) while the ED<sub>10</sub> is according to Ballmer-Weber et al. (2015) set at 8.5 mg hazelnut proteins.

## Peanut

Peanut is commonly believed to belong to the nut family, it is in fact a groundnut and classified as a legume. Legumes represent 27% of the primary crop production worldwide. Legumes are excellent sources of proteins, however they can also elicit dangerous allergic reactions. Peanut is here a typical example.

The prevalence of peanut allergy has risen significantly across the developed world over the past few decades, with a recent estimate of around 2% among UK children. Severe reactions to peanut are rare, but peanut allergy remains a leading cause of fatal food-related anaphylaxis. While there have been important advances in peanut desensitization, primary prevention remains the most attractive strategy for addressing this rise (Foong et al., 2017).

Peanut is reported as the most common food causing severe IgE mediated reactions in children and adolescents in the USA and Europe. It is estimated that 0.8% children in the USA and 1.5% children in the UK suffer from peanut allergy (Venter et al., 2010). Peanut allergy prevalence is reported to decline with increasing age to an estimate of 0.26% (Ben-Shoshan et al., 2010) to 0.48%

**Table 4** Tree nut allergens

Common name (Scientific name)	Allergen	Biochemical name	Superfamily/family	Molecular weight <sup>a</sup>
Almond ( <i>Prunus dulcis</i> )	Pru du 3	ns-LTP 1	Prolamin	9
	Pru du 4	Profilin	Profilin	14
	Pru du 5	60s Acidic ribosomal prot. P2		10
	Pru du 6	Amandin, 11S globulin (legumin-like protein)	Cupin	360
Brazil nut	Ber e 1	2S Albumin (sulfur-rich)	Prolamin	9
	Ber e 2	11S Globulin	Cupin	29
Cashew nut ( <i>Anacardium occidentale</i> )	Ana o 1	Vicilin-like protein	Cupin	50
	Ana o 2	Legumin-like protein	Cupin	55
	Ana o 3	2S Albumin	Prolamin	14
Chestnut ( <i>Castanea sativa</i> )	Cas s 1	PR-10	Bet v 1	22
	Cas s 5	Chitinase		
	Cas s 8	ns-LTP 1	Prolamin	12–13
	Cas s 9	Cytosolic class I small heat-shock protein	Hsp17.5	17
Hazelnut ( <i>Corylus avellana</i> )	Cor a 1	PR-10	Bet v 1	17
	Cor a 2	Profilin	Profilin	14
	Cor a 6	Isoflavone reductase homologue	Bet v 5	35
	Cor a 8	ns-LTP 1	Prolamin	9
	Cor a 9	11S Globulin (legumin-like)	Cupin	40
	Cor a 10	Luminal binding protein	Hsp70	70
	Cor a 11	7S Globulin (vicilin-like)	Cupin	48
	Cor a 12	Oleolin	Oleolin	17 <sup>b</sup>
	Cor a 13	Oleolin	Oleolin	14–16 <sup>b</sup>
	Cor a 14	2S Albumin	Prolamin	15–16 <sup>b</sup>
Pecan ( <i>Carya illinoensis</i> )	Car i 1	2S Albumin	Prolamin	16
	Car i 2	Vicilin-like protein	Cupin	55
	Car i 4	11S Globulin (legumin-like)	Cupin	55.4 <sup>c</sup>
Pistachio ( <i>Pistacia vera</i> )	Pis v 1	2S Albumin	Prolamin	7
	Pis v 2	11S Globulin subunit	Cupin	32
	Pis v 3	7S Globulin (vicilin-like)	Cupin	55
	Pis v 4	Manganese superoxide dismutase	Superoxide dismutase	25.7
	Pis v 5	11S Globulin subunit	Cupin	36
Walnut - Black walnut ( <i>Juglans nigra</i> )	Jug n 1	2S Albumin	Prolamin	15
	Jug n 2	Vicilin	Cupin	56
	Jug n 4	11S Globulin (legumin-like)	Cupin	33, 22
Walnut – English walnut ( <i>Juglans regia</i> )	Jug r 1	2S Albumin	Prolamin	15–16
	Jug r 2	Vicilin	Cupin	44
	Jug r 3	ns-LTP 1	Prolamin	9
	Jug r 4	11S Globulin	Cupin	58
	Jug r 5	PR-10	Bet v 1	20
	Jug r 6	Vicilin-like protein	Cupin	47

<sup>a</sup>Molecular weight (SDS-PAGE).<sup>b</sup>KDa.<sup>c</sup>Subunit of hexameric protein.

Extracted from EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies) (2014) and WHO/IUIS Allergen Nomenclature (1984).

(Emmet et al., 1999) proven by challenge in adults. Peanut allergy does in some cases resolve with age, with a 20% rate of resolution among young patients at age 4 (Sicherer and Sampson, 2017).

The clinical expression of peanut allergy is fairly predictable, and it has the tendency to be severe, although the severity may vary with different episodes of ingestion (Al-Muhsen et al., 2003). Reported symptoms include typical Ig-E mediated allergy symptoms such as facial swelling, rash, itch, wheeze, vomiting, breathing difficulty, abdominal pain and cramps, cyanosis and collapse but may also lead to fatality. Severe symptoms were reported to be more common in adults (Hourihane et al., 1997).

According to Allen et al. (2014), the ED<sub>01</sub>, which is the dose eliciting reactions in 1% of the peanut allergic population, is 0.2 mg peanut proteins. This figure is based on data from 750 subjects. The reference dose for peanut is 25 times lower than the lowest dose that Wainstein et al. (2010) reported as leading to anaphylaxis. Recently, Ballmer-Weber et al. (2015) confirmed the ED<sub>01</sub> published by Allen et al. (2014) providing a peanut ED<sub>10</sub> value at 2.8 mg peanut proteins.

Peanut is the best characterized legume in regard to its allergenic content, with 16 allergens registered by the World Health Organisation/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee (Table 5).



**Table 5** Peanut (*Arachis hypogea*) allergens

Allergen	Biochemical name	Superfamily/family	Molecular weight <sup>a</sup>
Ara h 1	7S Globulin (vicilin-type)	Cupin	64
Ara h 2	Conglutin (2S albumin)	Prolamin	17
Ara h 3	11S Globulin (legumin)	Cupin	60.34 (fragment)
Ara h 5	Profilin	Profilin	15
Ara h 6	Conglutin (2S albumin)	Prolamin	15
Ara h 7	Conglutin (2S albumin)	Prolamin	15
Ara h 8	PR-10	Bet v 1	17
Ara h 9	ns-LTP 1	Prolamin	9.8
Ara h 10	Oleosin	Oleosin	16 <sup>b</sup>
Ara h 11	Oleosin	Oleosin	14 <sup>b</sup>
Ara h 12	Defensin	–	8 kDa (reducing), 12 kDa (non-reducing), 5.184 kDa (mass)
Ara h 13	Defensin	–	8 kDa (reducing), 11 kDa (non-reducing), 5.472 kDa (mass)

<sup>a</sup>Molecular weight (SDS-PAGE).<sup>b</sup>kDa.

Extracted from EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies) (2014).

Clinically relevant cross-reactivity between peanut and at least one other legume was found to be low to moderate, with 10%–15% of children allergic to peanuts also allergic to soybean and 28% of peanut allergic patients in a French study cross-reacting with lupine (Cabanillas et al., 2017).

Peanuts are consumed boiled in certain countries, such as China, and in some southern US states such as Florida, Georgia, and Alabama, while this kind of treatment is quite uncommon for peanut processing in other areas of the world. However, boiling has been extensively studied in peanut due to the increased evidence that this treatment can alter peanut allergenicity. On the contrary, it has been largely demonstrated that roasting has the capacity to increase the IgE-binding properties of peanut allergens up to a 90 fold (Cabanillas et al., 2017).

There is today no cure for peanut allergy, but oral desensitization therapy as well as proactive early introduction of peanuts in the baby's food are promising routes for decreasing its prevalence. The Learning Early About Peanut (LEAP) study, a randomized, open-label, controlled trial of which 640 high-risk (defined as having severe eczema and/or egg allergy) infants from 4 to 11 months were randomized to either consume peanut regularly or avoid it until 5 years of age, provided robust evidence that early introduction of peanuts significantly decreased the frequency of the development of peanut allergy among children at high risk for this allergy. This study has overall changed worldwide recommendations related to early foods (including allergenic foods) introduction (Du Toit et al., 2015). As an example, the latest US pediatrician guidelines recommends the introduction of peanut into the diet of children at around 6 months of age as long as they do not have egg allergy or severe eczema (Foong et al., 2017).

## Concluding Remarks

Although prevalence of seafood, tree nut and peanut allergies is said to have been increasing in the past decades, there is today hope that immunotherapy treatment will help induce desensitization.

In the meanwhile, the unique way of preventing allergic reactions in sensitized patients remains avoidance of the offending food and accurate food labelling plays an essential role in the safeguard of allergic patients' health. In 2007, the Voluntary Incidental Trace Allergen Labelling (VITAL) risk assessment system (Allergen Bureau, 2007) was launched. This system ensures uniform precautionary labelling at a defined level of risk based upon scientific principles. In this view, the need of determining the compliance of food labels has prompted the development and improvement of analytical methods for the detection and quantification of food allergens. Up to know, the main limiting factors of method validation were the lack of reference methods and reference materials, but early 2017, the MoniQA Association (2011) has successfully started to sell validated reference material for the analysis of milk allergen. According to MoniQA Association (2011), further reference materials are in preparation; still seafood, peanut and tree nuts reference materials need to be developed. Once available, the harmonization of suitable methodologies for the detection of these allergens could be started.

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## Relevant Websites

- <http://allergenbureau.net/> – Allergen Bureau.
- <http://acaai.org/> – American College of Allergy, Asthma and Immunology.
- <http://www.eaaci.org/> – European Academy of Allergy and Clinical Immunology.
- <https://www.foodallergy.org/> – Food Allergy Research and Education (FARE).
- <https://farrp.unl.edu/> – Food Allergy Research and Resource Program (FARRP).

## Food Counterfeiting: A Growing Concern

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### Counterfeiting and Intellectual Property Rights

When dealing with laws and regulations, the term “counterfeiting” has a very specific application to intellectual property rights (IPR). *Intellectual property rights* (IPRs) are “rights given to persons over the creations of their minds (They usually give the creator an exclusive right over the use of his/her creation for a certain period)” (WTO, 1994). IPRs are comprised of four categories: trademark, patent, copyright, trademark, and trade secret. Summarizing the definitions:

- **Trademark:** “a symbol, word, or words legally registered or established by use as representing a company or product” (Merriam-Webster, 2004).
  - **Geographical Indication:** This is a type of trademark that identifies the location of origins such as a country, region, or territory.
- **Patent:** “a right or title for a set period, especially the sole right to exclude others from making, using, or selling an invention” (Merriam-Webster, 2004).
  - **Trade Dress:** a combination of trademark and patent that protections of the use of where a shape, image, or decoration that represents the company or product.
- **Copyright:** “the exclusive legal right, given to an originator or an assignee to print, publish, perform, film, or record literary, artistic, or musical material, and to authorize others to do the same” (Merriam-Webster, 2004).
- **Trade Secret (Protection of Undisclosed Information):** “a secret device or technique used by a company in manufacturing its products” (Merriam-Webster, 2004).

As defined by the International Standards Organization (ISO), a *counterfeit* is a “material good imitating or copying an *authentic material good*” where *material good* is “manufactured, grown product or one secured from nature” (ISO, 2018). Also, *authentic material good* is “material good produced under the control of the legitimate manufacturer, the originator of the goods or rights holder” (ISO, 2018). Then *rights holder* is the “legal entity either holding or authorised to use one or more intellectual property rights” (ISO, 2018). The counterfeiting concept is usually associated with trademarks or the logos of material goods. A related term is *piracy* that is usually applied to a copyright or patent violation for a published work such as a book, song, or movie.

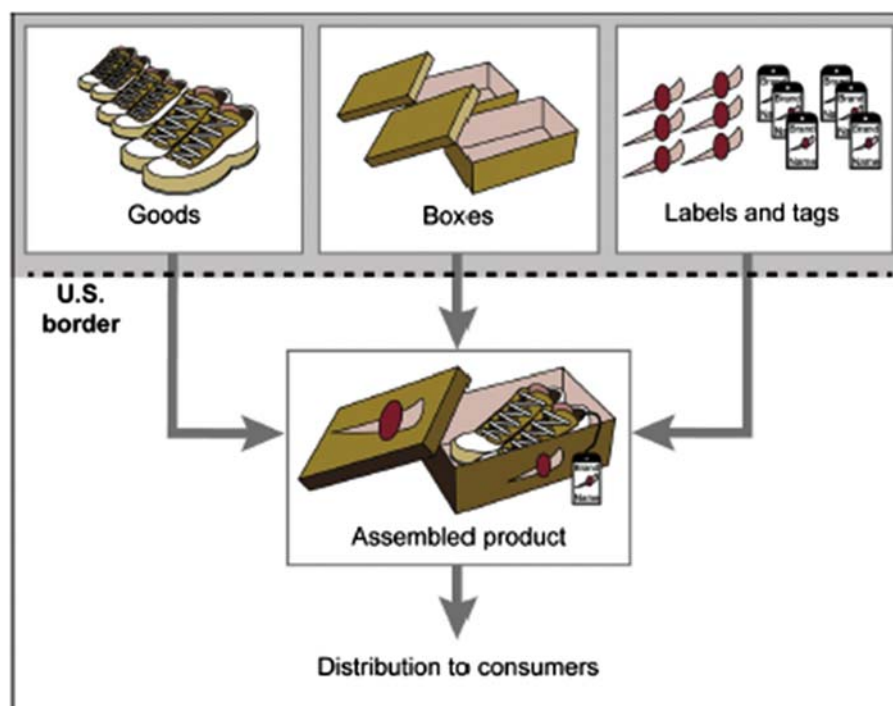
To consider the full challenges for securing legal IPR protection it is important to further review concepts such as the IPR being “validly registered,” if there is a law against manufacturing IPR violating product or it may be just illegal to export, and then also considering the laws and law enforcement priority for the importing country. Definitions are included with emphasis added since each point is critical in the legal protection (WTO, 1994):

- **Counterfeit trademark goods:** “shall mean any goods, including packaging, bearing without authorization a *trademark* which is identical to the trademark *validly registered* in respect of such goods, or which cannot be distinguished in its essential aspects from such a trademark, and which thereby infringes the rights of the owner of the trademark in question under the law of the *country of importation*.”
- **Pirated copyright goods:** “shall mean any goods which are *copies* made without the consent of the right holder or person duly authorized by the right holder *in the country of production* and which are made directly or indirectly from an article where the making of that copy would have constituted an infringement of a copyright or a related right *under the law of the country of importation*.”

In some cases, it may NOT be illegal to produce the product in one country, and the production is only illegal upon importation by another country. If the product is never exported or imported, then there may be no legal IPR violation. In other instances, what is illegal in one country may not be illegal in another. To avoid a customs agency seizure of the most expensive part of the cost of goods, the counterfeiters may import “genuine, generic” materials or finished goods and then send a small package of the counterfeit trademark components such as labels and tags. The components can be assembled inside a country to avoid customs investigations and reduce the amount of product that would be seized in a raid (Fig. 1).

Considering another challenge, the trade secret protection is important, but much more difficult to control. In some cases, a rights-holder may try to protect the IPR through confidentiality and non-disclosure agreements rather than register a patent or copyright to avoid the required public disclosure which would place the sensitive information in the public domain – a counterfeiter would be able to find detailed instructions to counterfeit the product. Also, the term “fake” and “knockoff” are sometimes used but should be avoided because they do not have a formal definition and so can cause confusion.

At this point in the chapter, it is important to establish that criminal or public health incidents related to food are handled under two separate types of laws: *food* or *material goods*. A “food law” typically focuses on the safe and secure production, manufacturing, and distribution with a priority based on minimizing the public health threat. A violation can be a strict liability so guilt even without knowledge or intent. On the other hand, the “material goods” laws focus on other aspects of the product, such as trademark counterfeit violation, where the intent is often necessary for guilt or prosecution.



**Figure 1** Domestic Assembly of Foreign Manufactured Counterfeit Goods. From GAO (2017).

The regulatory or criminal law enforcement response is activated from the food/public health perspective and most likely results in an immediate recall. This recall is important for reducing the public health threat but now leads the criminal investigation to be very challenging since the criminal is altered and the evidence (the fraudulent product) is recalled or destroyed by the criminal. The government and law enforcement priority – high to lower – is public safety (drugs, guns, violence), public health, large-scale economic crimes that disrupt markets or governments, then declining economic crimes, and then violations that are only technical. A counterfeit food that has a public health threat would be in the “public health” category and thus take precedence before the economic crime.

Regarding applying the IPR laws, the prosecution of cases is usually challenging and complex because often “two states of mind” need to be proven which include “intentional trafficking in counterfeits” AND “knowing use of a counterfeited mark” (Hilliard et al., 2007). If the plaintiff can establish that they did not know the product was counterfeit – such as parallel trade or diversion – “this eliminates the application of the counterfeit laws” (Hilliard et al., 2007).

An example of a typical food fraud investigation is the INTERPOL-Europol Operations OPSON. The seventh annual operation includes over 50 countries and over 20 companies. The scope balances the maximum economic impact while the priority is on the most severe public health threats. The focus on public health is evidenced in that some countries such as Austria included an inter-agency team enforcement agencies (police - law enforcement, customs -import controls, and Food Administration Vienna -food agency) while other countries such as Denmark and the US only include food agencies (Veterinary and Food Administration - food agency and Food and Drug Administration - food agency, respectively). The priority and assigned participants was reflected in the efforts and results of seizures: Deceiving consumer (26%, e.g., falsely labeled extra virgin olive oil), Food safety (22%, e.g., infringement related to storage conditions of the goods), Fiscal infringement (19%, e.g., Alcohol duties), and then finally Intellectual Property Rights - Counterfeiting (1%, e.g., all IPR, including appellations of origins).

Counterfeiting (IPR) is one type of food fraud in the hierarchy of adulterant-substances (dilution, substitution, concealment, unapproved enhancements), mislabeling/misbranding, grey market production/theft/diversion, and counterfeiting (IPR) (Spink and Moyer, 2011). The overall food fraud vulnerability assessment can consider counterfeiting and coordinate countermeasures and control systems in the food fraud prevention strategy. While the challenges of defining and prosecuting counterfeiting have been presented, the review emphasizes the efficiency of applying crime prevention concepts not to try to “catch bad product” but to try to “prevent food fraud from occurring in the first place.”

## Food IPR Counterfeiting

The magnitude of the economic impact of counterfeiting and piracy is very challenging to estimate to the point that it is probably impractical even to try to create a statistically significant estimate. While there are no very accurate or precise estimates, it is generally believed that for all products counterfeiting to be in the hundreds of billions of dollars. Overall there is an estimate that a broad



definition of counterfeits equates to “5%–7%” of world trade, with some pharmaceutical estimates above 10% worldwide, and other individual markets well above 50%.

A study of the economic impact of counterfeiting and piracy found that quantitative estimates were based on three core reference documents including the International Chamber of Commerce, the OECD report, and a press release from the U.S. Federal Bureau of Investigation (Spink and Fejes, 2012). Each of these documents was based on qualitative estimates such as “5%–7% of world trade” and repeated so often that the estimates reached a “mythological stature even among scholars” (Spink and Fejes, 2012). There was an assumption that since the estimate was repeated so many times that it must have been based on a sound statistical methodology and data collection. While this may be the belief, the core references were clear in their statements. For example, in the OECD report, it stated: “The overall degree to which products are being counterfeited and pirated is unknown, and there do not appear to be any methodologies which could be employed to develop an acceptable overall estimate” (OECD, 2008).

The U.S. Government Accountability Office (GAO) published “Observations on Efforts to Quantify the Economic Effects of Counterfeit and Pirated Goods” which was originally specified to establish an economic impact estimate (GAO, 2010). The GAO report reviewed global estimates and concluded that the data for the previous estimates had not been systematically evaluated or collected and did “rely excessively on fragmentary and anecdotal information; where data are lacking, unsubstantiated opinions are often treated as facts” (GAO, 2010). GAO stated that quantifying counterfeiting as a whole “is difficult, if not impossible” (GAO, 2010). They also stated, “One expert characterized the attempt to quantify the overall economic impact of counterfeiting as ‘fruitless,’ while another stated that any estimate is highly suspect since this is covert trade and the numbers are all ‘guesstimates’” (GAO, 2010).

Thus, for food counterfeiting – and for the overall food fraud – establishing methodologically sound, accurate and precise economic impact estimates is probably impossible. But, considering the general focus on vulnerabilities, the lack of a hard estimate is not a critical problem and actually is not even a hindrance. A focus on the vulnerability and “fraud opportunity” does not require a lot of data but does require deeper insight on the type of fraud, type of fraudsters, and actual incidents. These first need a review of the types of market and products that are being counterfeited.

The industry-led food fraud prevention initiatives focus on vulnerability – in both assessing the problem and also for selecting risk treatments – so this lack of an economic impact is not an insurmountable challenge. When addressing this question for food products, the OECD report stated “Unfortunately, the survey has shed very little light on the actual magnitude and scope of counterfeiting in the food and drink sector. Part of the reason for this is that there seems to be genuine and widespread misunderstanding in this sector of what is actually covered by counterfeiting [such as IPR versus all fraud]” (OECD, 2008).

The confusion on classification includes when there is a mention of “counterfeit food.” For example a report of “seizures of counterfeit (trademark infringing) fresh strawberries, cinnamon and coconut oil” defined the violation as the trademark of the brand and not the product, itself (OECD, 2016). The counterfeit strawberries, cinnamon, and coconut oil were actually strawberries, cinnamon, and coconut oil but the brand name was used without authorization.

Counterfeit material goods can be split into deceptive and non-deceptive categories.

- **Deceptive counterfeits** “are intended to deceive the consumer into believing they are purchasing a genuine product” (Spink et al., 2013).
- **Non-deceptive counterfeits** “are not intended to deceive consumers – in many instances products are promoted as ‘fakes,’ ‘knock-offs,’ or ‘counterfeits’” (Spink et al., 2013).

Next, the counterfeit product threat is present in two basic market extremes with many variations in between primary market and secondary markets. On one end, the **primary market** is through authorized resellers which are for “Consumers who demand goods of genuine, non-infringing origin establish a market that is referred to in this report as the primary market.” Then, “The only way that counterfeiters and pirates can penetrate this market is to deceive consumers into believing that their products are authentic (deceptive counterfeits).” At the other end is the **secondary market** or black market that were “Under certain conditions, consumers are often willing to purchase products they know are not legitimate.” Then, in this case, the customers are seeking counterfeit product (non-deceptive counterfeits). There are other markets in between such as products not sold through authorized resellers, legal diverted product, or other types of “gray markets.”

A **black market** is “An illegal market for goods that are controlled or prohibited by the government, such as the underground market for prescription drugs” (Black’s Law Dictionary, 2015). A **gray market** is “A market in which the seller uses legal but sometimes unethical methods to avoid a manufacturer’s distribution chain and thereby sell goods (especially imported goods) at prices [usually] lower than those envisioned by the manufacturer” (Black’s Law Dictionary, 2015). To note, the parallel **trade** or **diversion** is “Goods bearing valid trademarks that are manufactured abroad and imported into another country to compete with domestically manufactured goods bearing the same valid trademarks” (Black’s Law Dictionary, 2015).

For food products, consumers may be seeking a good deal, but they are – regardless of the market type – almost universally seeking genuine products.

To address all types of counterfeiting and for all products, to a “The Economic Impact of Counterfeiting and Piracy” was published by the Organization for Economic Co-Operation and Development (OECD) (OECD, 2008). Their survey of industry included several insights that pertain to the food industry:

- “The incidence of infringement is considerably lower when physical counterfeiting is necessary to misrepresent the product (as opposed to simply copying the packaging, labeling, trademark and the general appearance of items being substituted)”



- The physical counterfeiting of a deceptive product that has high consumer detection is more challenging because “as this requires a considerably more sophisticated enterprise to successfully carry out the substitution and convince retailers and the buying public that they are original items.”
- “For example, the counterfeiting of frozen or canned food is much more difficult [than counterfeiting other product such as luxury goods or apparel] as this would require specialized equipment to produce the substitute items, as well as to store and transport them, and this severely reduces the potential incentive for counterfeiting.”
- “In the experience of the industry, products most subject to counterfeit action are those that are the simplest to replace with passable substitutes, and whose substitutes would not be readily detected by the consumer (and sometimes the wholesalers and retailers). Tea, rice and vodka were examples of such items provided by respondents to the OECD survey.”
- “Several respondents noted that counterfeiting and infringement of trademarks in food are relatively low compared to other products, due to generally low-profit margins and the significant logistical challenges associated with the production, handling, transport, and distribution of food products.”
- “[The challenges of the food manufacturing characteristics would be further magnified for perishable products, which require even more sophisticated handling and distribution chains capable of handling these products efficiently.”

So a “*professional*” type of counterfeiter (who only focuses on counterfeiting as a their profession) would find the IPR counterfeiting of food to be more challenging than for other products because of the lower margins, perishable product, high quality needed to deceive consumers that the counterfeit is a genuine product, the challenge of inserting the counterfeit product in the legitimate supply chain, the challenge of procuring so much of the raw materials, and the specialized manufacturing equipment. That said, an “*occupational*” type of counterfeiter (who is in the food industry as a profession and supplements their income conducting fraud) would not face most of those challenges and could leverage the criminology concept of Routine Activities Theory to be aware of new, emerging, or nuanced fraud opportunities. This insight is valuable when considering the “fraud opportunity,” food fraud vulnerability assessment, and the food fraud prevention strategy. This insight would help identify the vulnerable nodes in the supply chain as well as where and how the fraudulent product would try to enter the supply chain.

## Conclusion

The intellectual property rights counterfeited food products are a significant threat with a very challenging prevention strategy. Fortunately, the industry-led food fraud vulnerability assessment and food fraud prevention strategy efforts include IPR counterfeiting as a “type of fraud” and integrate countermeasures and control systems across a company. When the entire food risk continuum is considered there can benefit from other efforts – such as random quality control checks in a marketplace – that help reduce the fraud opportunity for counterfeit foods. This food fraud prevention strategy focus already includes criminology theory such as situational crime prevention that already considers the counterfeit product threat. The anti-counterfeiting efforts are most efficiently implemented when considered in the hierarchy of all fraud and all products.

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# Food Defense

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## Introduction

Ongoing terrorist activity has reinforced the need to enhance the security of the United States across all critical infrastructures including the food and agriculture system. After September 11, 2001, the United States' Congress responded by passing the Public Health Security and Bioterrorism Preparedness and Response Act in 2002 which was signed into law by President Bush later that year. Per the USDA, Food defense is the protection of food products from contamination or adulteration intended to cause public health harm or economic disruption (USDA, <https://www.fsis.usda.gov/wps/portal/fsis/topics/food-defense-defense-and-emergency-response>). However, the definition provided by the USDA and more broadly the US government does not adequately address all the concerns of food producers and consumers. This chapter's definition of food defense is the protection of food, plants and animals from intentional contamination with the intent to cause harm.

The Bioterrorism Preparedness and Response Act required that the Food and Drug Administration (FDA) implement several regulations applicable to the food defense: (1) Administrative Detention of Imported Food; (2) Establishment and Maintenance of Records; (3) Food Facility Registration; and, (4) Prior Notice of Imported Food. Food Defense requirements were further expanded when the Food Safety Modernization Act (FSMA) was signed into law by President Obama in 2011. FSMA aims to further enhance the safety of the United States food supply by shifting FDA's focus from responding to contamination incidents after they occur, to ensuring that private industry food companies mitigate food defense risks and prevent food defense events from occurring.

FSMA also provided FDA with new enforcement authorities designed to achieve higher rates of compliance with food defense risk identification and mitigation as defined by the FDA at qualifying food facilities. FSMA also gave the FDA to regulate imported foods similarly to domestic foods. FSMA Section 103 requires facilities registered with the FDA to:

- analyses to identify and evaluate known or reasonably foreseeable hazards that may be associated with the facility, including hazards that may be introduced intentionally; and,
- develop and implement comprehensive, science-based preventive controls to provide assurances that the identified hazards will be significantly minimized or prevented and addressed, and that food and feed manufactured, processed, packed or stored by such facilities will not be adulterated or misbranded.

Importantly, FSMA includes the requirement that FDA develop regulations concerning how food facilities are to assess whether they are required to conduct vulnerability assessments, develop and implement mitigation strategies to address intentional contamination events. Additionally, food-defense provisions of FSMA Section 106 state that such regulations shall apply only to food and feed for which there is a high risk of intentional contamination, as determined by the FDA, in consultation with the Secretary of Homeland Security, that could cause serious adverse health consequences or death to humans or animals. Foods subject to the requirements include those foods for which the Agency has identified clear vulnerabilities (i.e., a food product with a short shelf-life, vulnerability to intentional contamination at critical control points, and foods produced in bulk or batch form). Under FSMA, FDA is directed to issue regulations to protect against the intentional adulteration of food and animal feed, including the establishment of science-based mitigation strategies to prepare and protect the food supply chain at specific vulnerable points. The goals of food defense are to prevent foodborne contamination, force increased corporate responsibility, identify food system vulnerabilities, analyze the risks in food production systems and processes, and implement effective mitigation strategies. Lastly, food defense aims to improve immediate response systems and improve crisis communication to the public.

## Attacker Motivation

Food Defense is protection against intentional contamination of food products. While identifying who may want to attack a food system can aid specific food defense mitigation strategies, knowing the specific motivations of attacker's is not necessary to implement effective food defense mitigation strategies. To properly develop effective preventative controls to mitigate food defense risks it is not necessary to know who wants to attack the system or why since the food defense vulnerabilities and risks in the food system do not vary based on the attacker's motivations. Vulnerabilities and risks in food production system remain constant unless the food production system is altered in some way or unless there is some change that alters human behavior in the food production environment. However, understanding the attacker's motivation can help those involved with assessing and mitigating risks identify vulnerabilities in the food production system.

Attacker motivations can vary greatly. In terrorism, intentional contamination is motivated by drawing attention to the terrorist act itself. In the case of economically motivated adulteration (e.g., counterfeiting, ingredient substitution) the criminal typically does not want the act to be noticed, since the longer it is undetected the longer the adulterator avoids punishment and receives economic benefits. There are almost an infinite number of motivations to intentionally contaminate food; however, the most frequently identified motivations are: (1) Terrorism aim to kill or harm as many people as possible publicly; (2) Economically Motivated Adulteration (EMA) aims to increase the profitability of a product; (3) Eco-Terrorism an interest group economically damages

an industry or company without killing people; (4) Extortion aims to obtain financial gain. To develop an effective food defense system, understanding the security issues associated with humans is necessary to better understand how to identify vulnerabilities in the food production system, mitigate food defense risks, and investigate and respond to intentional contamination events when they occur.

## Food Defense Plans

The food defense plan (FDP) is a document that is used to operationalize food defense strategy. Typically, the FDP is operationalized via trained personnel carrying out the standard operating procedures (SOPs) described in the company's plan. The FDP can be assembled collaboratively by a team within the company that takes the results of the facility's vulnerability assessment, ranks the issues per their importance to food defense, and develops mitigating preventive controls that will likely guard against intentional contamination events. The FDA has software to assist in the vulnerability assessment, programs to aid in the development of the FDP, and has laboratory and database resources that can assist with analysis of suspects or known harmful agents.

A properly executed FDP helps a food company identify effective food defense mitigation strategies. A FDP may increase food defense preparedness and it can be particularly helpful in responding to intentional contamination events. The FDP should be reviewed and updated bi-annually to remain relevant. This includes properly supervising and mentoring managers responsible for overseeing food defense and verifying that the plan is effectively mitigating food defense risks by measuring and evaluating the outcomes of Red Team Assessments. A Red Team Assessment is an independent group within the organization that challenges an organization to improve its effectiveness by assuming an adversarial role or point of view. Often, the Red Team Assessment occurs during normal food production conditions and the employees within the organization are unaware that the Red Team Assessment is occurring. Benefits of the assessment include challenges to preconceived notions and clarifying the problem state that planners are attempting to mitigate. More accurate understanding can be developed of exploitable vulnerabilities and identify instances of bias among employees responsible for food defense. Identifying and documenting known vulnerabilities, keeping records of mitigation activities, and striving for continuous improvement and employee training as the facility personnel and processes change over time increases the likelihood the food defense plan will be effective at mitigating food defense risks.

The food defense management team is responsible for developing, monitoring, and maintaining the FDP. In many instances, obtaining assistance from consultants and other outside experts is necessary to ensure the proper analysis of food defense vulnerabilities and risks, and to develop and implement effective food defense risk mitigation techniques and strategies.

When conducting a vulnerability assessment, or developing or modifying an FDP, it is imperative to select mitigation procedures and technologies that are effective and practical to the characteristics and day-to-day operation of the food production facility, as well as the food facility's physical environmental characteristics. There is not a single solution to food defense. Rather, each food defense mitigation technology or strategy can be viewed as a layer of Swiss cheese, and as layers of Swiss cheese are stacked on top of each other its more difficult for an object to pass through the combined layers of cheese. The more layers of food defense mitigation, the more resilient the food defense system or facility will be to intentional contamination events. Every food facility should have its own unique food defense plans (even those operated by the same company) based upon the types of processes, products, conditions, and different employees present. Finally, it is extremely important to select food defense mitigation strategies and technologies that effectively mitigate intentional contamination likelihood through qualitative and quantitative vulnerability and risk analyses.

## Assessing Food Defense Vulnerabilities

FSMA directs the FDA to require preventive controls against intentional adulteration at vulnerable points in the food system and inside food facilities. Each registered facility is required to have preventive controls in place that guard against the possibility of intentional contamination (a.k.a. intentional adulteration). The necessary controls and mitigation strategies needed to comply with regulations will vary considerably based on the product or commodity produced and the types of processes used in production. The vulnerabilities of each facility and production process need to be assessed to build a preventive control strategy that reduces the ease and probability of intentional contamination. Vulnerability assessments are like the hazard analysis required by the FDA for food safety; however, vulnerability assessments are different since they focus on acts of intentional contamination. Vulnerability assessments also focus on the characteristics of facilities, and the underlying manufacturing processes and systems, rather than the potential for food safety hazards. In the context of food defense, vulnerability is defined as a weakness in the design, implementation, or operation of an asset or system that can be exploited by an adversary. Vulnerability assessment is a process used to identify specific points in food facilities and processes where intentional contamination has the greatest potential to instill fear in consumers, and harm the government, industry, economy, or the public's health. It is a systematic process of identifying and prioritizing the weaknesses (i.e., vulnerabilities) in a food production system or individual food facility. Vulnerability assessments require brainstorming and thinking critically from an adversarial perspective to effectively identify exploitable weaknesses, and to then place effective and cost effective controls or mitigation strategies within the FDP.

FSMA requires privately owned companies and organizations to implement measures to protect against the intentional adulteration of food by bad actors. FSMA requires companies to implement practical food defense measures at specific points in the food

supply chain where intentional contamination has the greatest potential to cause serious harm. The specific points where controls need to be placed can be determined using vulnerability assessment tools like the FDA's CARVER + Shock, Food Defense Plan Builder, red team analysis, systems analysis, physical security assessment, and cyber security assessments. Strategies to prevent intentional adulteration best apply to foods in bulk or batch form that have not been packaged. Foods that are widely distributed in large quantities are potentially the most valuable target to adversaries. Contaminated foods that many people consume in a short time span (e.g., fluid milk), and contain multiple servings, are potentially of highest value to the adversary because of the likelihood of wide spread serious adverse health consequences and death from intentional contamination. The use of industry best practices along with guidance from FDA, United States Department of Agriculture Food Inspection Service (FSIS), and the Department of Homeland Security (DHS) form the basis for vulnerability assessments, which must be incorporated in the Food Defense Plan to be practical and effective. Food companies may use other approaches or modify the approaches that are referenced to suit their organization's needs and available resources.

To begin the process of a vulnerability assessment, the food defense team must identify and record all the food facility's production processes and characteristics. Each of the identified operations, processes, and critical control points must be evaluated to measure and quantify the vulnerabilities, and to create mitigation strategies and implement food defense technologies to reduce the likelihood of intentional contamination. Keep in mind that up until this point that food defense threat has not been discussed. The food defense threat directly comes from people that attempt to intentionally contaminate food products, or indirectly from people that inadvertently provide access to vulnerabilities in food production systems and facilities.

There are many types and examples of vulnerabilities that all derive from people. For example, Employees working at companies with relatively low employee morale, work values, security knowledge, security awareness, and poor attitudes are more vulnerable to attack. Companies where employees are not aware of security and intentional contamination, or are not focused on protecting their employer, the outcomes of their work, or the quality and safety of their products are less likely to engage protective behaviors and are more vulnerable to intentional contamination. Having employees with high employee morale, strong values, security knowledge, security awareness, and positive attitudes greatly reduces the likelihood that an intentional contamination event will be successful. Fostering a security aware environment, and creating incentives to maximize security within the company, may be the best protective measures against intentional contamination.

The insider threat is a difficult problem facing all food production companies. To mitigate the threat and vulnerability, using staff screening at all organizational levels may prevent persons with prior criminal histories from having access to the food system. Employee screening will not eliminate vulnerability to intentional contamination, but may reduce the likelihood of people with a history of poor judgment from having access to the system. Often, terrorists have no criminal background and are well educated, and employee screening typically will not prevent these people from working or gaining access to your facility. Some employees may exhibit suspect behaviors. Caution must be taken of employees that want late night, cleanup, multi-shift, less desirable work schedules, or assignments where they are overqualified. Employees with suspect behaviors should be reassigned to other equal tasks, be closely supervised, and monitored with various security technologies.

## Quantifying Food Defense Risk and Management

The goal for any approach, procedure, or tool used to analyze food defense risk is to facilitate the identification and quantification of food defense vulnerabilities within the facility and determine the risk that the food defense vulnerabilities pose. This is somewhat like the hazard analysis step used in food safety risk analysis and hazard identification, and additional vulnerabilities may be discovered or explored during the assessment. Regardless of similarities, or differences between, different methods of vulnerability and risk assessment, there are multiple valid ways to assess risk in an organization. The method of assessment is not overly important as most assessments have similar outcomes. However, engaging the Food Defense Team in the adversarial mindset, and forcing the team to use critical thinking to mitigate identified food defense risks may be the most beneficial component of the food defense process.

Operational Risk Management (ORM) is a good example of one approach to assessing risk. ORM enhances traditional risk management through a specific six-step process, utilizing analytical tools to optimize risks and mission outcomes. The United States military regulation defines ORM as the systematic the process of identifying hazards, assessing risk, analyzing risk control options and measures, making control decisions, implementing control decisions, accepting residual risks, and supervising/reviewing the activity for effectiveness.

To better understand how to assess the risks associated with intentional contamination, the ORM Step 2 (assess the risk) is outlined below to specifically determine how risks are assessed and ranked for identifying the appropriate mitigation steps.

Step 2 requires the food defense team to assess the risks and hazards, and to analyze the probability of occurrence versus the severity of exposure. This step requires the food defense team to question the impacts to people, materials, facilities, operations, distribution, and the environment. The food defense team may choose from the available impacts' severity categories (catastrophic, critical, moderate, or negligible). The combined analysis of probability and severity is commonly performed in the Risk Assessment Matrix.

			PROBABILITY				
			Frequent	Likely	Occasional	Seldom	Unlikely
			A	B	C	D	E
SEVERITY	Catastrophic	I	1	2	6	8	12
	Critical	II	3	4	7	11	15
	Moderate	III	5	9	10	14	16
	Negligible	IV	13	17	18	19	20
			Risk Levels				

The severity and probability of occurrence are important to fully assess the risks presented by intentional contamination. The severity is generally a more objective measurement whereas the estimation of the probability of occurrence is more subjective. The following gives examples of both severity and probability of occurrence ([http://www.uscert.gov/control\\_systems/satool.html](http://www.uscert.gov/control_systems/satool.html)):

### Severity

- Catastrophic - Death(s) and/or complete business disruption
- Critical - Severe injury and illness and/or major business disruption
- Moderate - Minor injury or illness and/or minor business disruption
- Negligible - Less than minor injury or illness and/or less than minor business disruption

### Probability of Occurrence

- Frequent - Occurs often and population is continuously exposed
- Likely - Occurs several times and population are exposed regularly
- Occasional - Will occur sporadically within a population
- Seldom - Rarely occurs in a population
- Unlikely - So unlikely you can assume it will not occur or rarely occurs in a population

The matrix can be used to review each of the potential vulnerabilities, and can be used to make more informed decisions about severity and frequency. Numbers or values can be assigned to aid in setting priorities for the food defense plan. Lower numbers or values (in the red zone of the matrix) are especially important and are likely at the highest risk.

Other factors can have an influence on the level of risk assigned during the vulnerability assessment. These subjective factors are based on the ability of a specific food or operation to be easily targeted by criminals or terrorists.

For example, intelligence concerning a threat to a food may increase its probability of occurrence. Also, certain characteristics of foods may make them higher risk. These can include: foods made in large batches may reflect a large population of consumers; foods that require or have a uniform mixing process can contaminate all servings in a single batch; short shelf life foods have minimal time to identify problems and intervene, and are rapidly consumed by people; and, ease of access to targets increases their attractiveness.

As part of the vulnerability assessment, the facility must be critically reviewed either by a food defense team walk-through or by an outside consultant. The first step is to develop a facilities diagram illustrating secure and insecure areas. The assessment process can be completed face-to-face or can be assisted via computer software. It should be noted that the Department of Homeland Security can aid with Critical Infrastructure Vulnerability Assessments. DHS's assessment team, site operators, and local law enforcement can examine a facility upon request and complete a physical security vulnerability assessment at any facility. The Department's Protective Security Coordination Division conducts specialized field assessments to identify vulnerabilities, interdependencies, and cascading effects of nationally significant critical infrastructure and key resources (CIKR). These vulnerability assessments are the foundation of the National Infrastructure Protection Plan's risk-based implementation of protective programs designed to prevent, deter, and mitigate the risk of a terrorist attack while enabling timely, efficient response, and restoration in an all-hazards post-event situation. DHS has a team of Protective Security Advisors (PSA). They are highly experienced and well trained to conduct critical infrastructure protection and vulnerability assessments. Because most US critical infrastructure is privately owned, the effectiveness of vulnerability assessments depends upon the voluntary collaboration of private sector owners and operators. DHS facilitates information sharing between the government and individual sites and industry sectors, as well as between facilities and sectors by offering comparative assessment data that supports national-level, cross-sector risk analysis of nationally significant



CIKR. For the DHS's federal, state, and local partners, special reports are available (e.g., Characteristics and Common Vulnerabilities, Potential Indicators of Terrorist Activity, and Protective Measures). These reports identify common critical infrastructure vulnerabilities, sector-specific background information, and the types of terrorist activities that might be successful in exploiting these vulnerabilities.

## Vulnerability and Food Defense Plans

The food defense team is a key element in the strategy and approach to development of a food defense system. One of the primary functions of the team is to develop a functional food defense plan. Typically, persons knowledgeable in food defense and security measures will be the primary developers of the food defense plan. In practice, the format of food defense plan varies per the characteristics of the process and product or based on the needs of the specific industry. A typical Food Defense Plan is organized in four sections: (1) Outside security measures; (2) Inside security measures; (3) Personnel security measures; and, (4) Incident response security measures. The results of the vulnerability assessment will identify the areas of concern and help determine where points of control are needed within the food defense plan. Like with a food safety hazard analysis, the vulnerability assessment should be a written or electronic document that identifies the areas discussed, documents the discussion, and provides a rationale as to the level of risk (including the severity and occurrence). If the identified vulnerabilities or risks need to be mitigated, then this information needs to be included in the food defense plan. Records of the vulnerability assessment and the outcomes need to be part of the facilities overall food defense program and may be subject to review by regulators.

## Summary

Food defense is not a new or even recent problem. Intentional contamination of food and water supplies has existed throughout human history. The only thing that has changed is how society approaches the problem of intentional contamination. Food defense vulnerabilities originate in the design, implementation, or operation of a food production process, critical control point, or system that can be exploited by an adversary. Food defense vulnerability identification is a systematic process used to identify specific points in the food system where intentional contamination has the greatest potential to cause economic or public health harm. The process of identifying and prioritizing the vulnerabilities in a food system can be completed in person, by using specialized computer software to assist in the task, or by outside experts (consultants or others). Vulnerability assessments require brainstorming and critical thinking from an adversarial perspective to effectively conduct the assessment, and to then place the proper controls or mitigation strategies within the food system. The assessment results are used to form the basis for the food defense plan by identifying the points of control where mitigation is appropriate. The Food Defense Team's assessment rationale and results should be documented and be a part of the food defense program records. Due to the complexity and diversity among processing facilities, not all vulnerability assessments will be the same, even those that may be designed for the same food product. Regardless, what matters most is that the team and the organization routinely and systematically engages in the food defense risk analysis and mitigation process.

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# Food Fraud and Adulteration: Where We Stand Today

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## Introduction to Food Fraud

**Food fraud** – including the sub-category of the U.S. FDA defined *economically motivated adulteration* or EMA – is generally defined as illegal deception for economic gain using food (ISO, 2011; Spink and Moyer, 2011; GFSI, 2012; SSAFE, 2012; CRS, 2014; DEFRA, 2014; CFSA, 2015a,b; CODEX, 2017; GFSI, 2017). This has become a separate food industry focus along a continuum that starts with food quality and food safety and now also includes a separate focus on food defense. Addressing food fraud prevention requires a fundamentally different approach since the fraudsters are intelligent adversaries who are clandestine, stealthy, and actively seek to avoid detection.

While the food fraud term has been used for many years, the research on the definition was first published in a 2011 peer-review, refereed journal article “Introducing the Public Health Threat of Food Fraud” (Spink and Moyer, 2011). While there is a continuing evolution of the details and application – such as by the International Standards Organization (ISO), Codex Alimentarius (CODEX), and European Committee for Standardization (CEN), the UK Elliott Review, and others – the basic definition has remained. A key consideration is how food fraud fits into the hierarchy of all other food risks.

The scope of food fraud is beyond food ingredients and adulterant-substances that can cause public health harm to all types of fraud and for all products. The key point is that all types of fraud can create a vulnerability that could become a root-cause of a health hazard. The types of food fraud include: adulterant-substances (dilution, substitution, concealment, unapproved enhancements), mislabeling/misbranding, grey market production/theft/diversion, and counterfeiting (intellectual property rights) (Table 1).

The basic food risks are *food quality* (unintentional act with no public health or economic harm), *food safety* (unintentional act with public health harm), *food defense* (intentional act with intent for public health or economic harm), and food fraud (Spink, 2011). To effectively manage all food risks then all food risks should be assigned to a specific cell, and every incident should be assigned to a specific food risk type. For effective management, there should be a formal incident assignment system that includes

**Table 1** Food fraud types, definitions, and examples

GFSI (1) type of food fraud	Definition from SSAFE (2)	Examples from GFSI FFTT (3)	General type of food fraud
Dilution	The process of mixing a liquid ingredient with high value with a liquid of lower value.	<ul style="list-style-type: none"><li>Watered down products using non-potable/unsafe water</li><li>Olive oil diluted with potentially toxic tea tree oil</li></ul>	Adulterant-substance (Adulterant)
Substitution	The process of replacing an ingredient or part of the product of high value with another ingredient or part of the product of lower value.	<ul style="list-style-type: none"><li>Sunflower oil partially substituted with mineral oil</li><li>Hydrolyzed leather protein in milk</li></ul>	Adulterant-substance or Tampering
Concealment	The process of hiding the low quality of a food ingredients or product.	<ul style="list-style-type: none"><li>Poultry injected with hormones to conceal disease</li><li>Harmful food colouring applied to fresh fruit to cover defects</li></ul>	Adulterant-substance or Tampering
Unapproved enhancements	The process of adding unknown and undeclared materials to food products in order to enhance their quality attributes.	<ul style="list-style-type: none"><li>Melamine added to enhance protein value</li><li>Use of unauthorized additives (Sudan dyes in spices)</li></ul>	Adulterant-substance or Tampering
Mislabelling/Misbranding	The process of placing false claims on packaging for economic gain.	<ul style="list-style-type: none"><li>Expiry, provenance (unsafe origin)</li><li>Toxic Japanese star anise labeled as Chinese star anise</li></ul>	Tampering
Grey market production/theft/diversion	Outside scope of SSAFE tool.	<ul style="list-style-type: none"><li>Mislabeled recycled cooking oil</li><li>Sale of excess unreported product,</li><li>Product allocated for the US market appearing in Korea</li></ul>	Over-run, Theft, or Diversion (4)
Counterfeiting (IPR)	The process of copying the brand name, packaging concept, recipe, processing method, etc. of food products for economic gain.	<ul style="list-style-type: none"><li>Copies of popular foods not produced with acceptable safety assurances</li><li>Counterfeit chocolate bars</li></ul>	Counterfeiting (IPR)

Notes: (1) GFSI – Global Food Safety Initiative; (2) SSAFE – Safe Secure and Affordable Food For Everyone; (3) GFSI FFTT – Global Food Safety Initiative: Food Fraud Think Tank; (4) Gray Market – a market employing irregular but not illegal methods; Theft – something stolen; Diversion/Parallel Trade – the act or an instance of diverting straying from a course, activity, or use.

Adapted from Spink and Moyer (2011), Spink (2013), SSAFE Organization (2015), PWC (2016), GFSI (2017), and Spink et al. (2017).

		<b>Motivation</b>
Food Quality	Food Fraud (1)	Gain : Economic
Food Safety	Food Defense	Harm: Public Health, Economic, or Terror
Unintentional	Intentional	
<b>Action</b>		

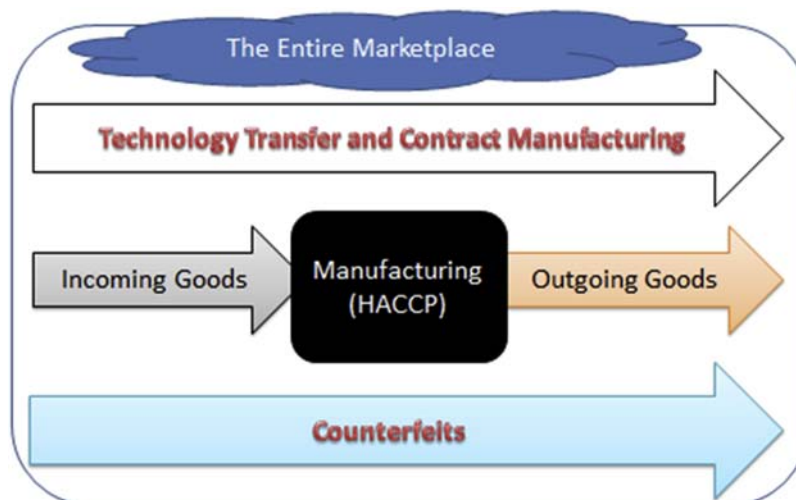
(1) Includes the subcategory of economically motivated adulteration and food counterfeiting

**Figure 1** Food Risk Matrix (Spink, 2011).

clear identification of who is “accountable” for managing the incident. The Food Risk Matrix is an efficient way to present the relationship between the risks (Fig. 1) (Spink, 2011).

The Food Risk Matrix is an efficient way to review all types of food risks and to make sure that everything is covered under the broader concept of “food integrity.” *Food Integrity* has an evolving definition but is generally “ensuring that food which is offered for sale or sold is not only safe and of the nature, substance, and quality expected by the purchaser but also captures other aspects of food production, such as the way it has been sourced, procured and distributed and being honest about those elements to consumers” (DEFRA, 2014). Another related term is *food authenticity* which is generally regarded as the process and confirmation “ensuring that food offered for sale or sold is of the nature, substance, and quality expected by the purchaser” (DEFRA, 2014). In addition, the focus on crimes on food products has led to the use of the term *food crime* which “has two common definitions of (1) incidents involving food that is a violation of a criminal statute and (2) Food Fraud incidents that are conducted on a larger scale” (MSU-FFI, 2018).

Another consideration is the scope of food fraud is where the vulnerabilities occur. The Food Supply Chain Vulnerabilities for the “Entire Marketplace” including the company supply chain of “Incoming Goods” and “Manufacturing.” The manufacturing process is managed by HACCP. The proprietary supply chain focus usually ends when “Outgoing Goods” leave the manufacturing plant. An area that is often not considered is risks in “Technology Transfer and Contract Manufacturing” where someone had legal and authorized access to your products, recipes, patents, trademark, and genuine packaging. Finally, “Counterfeits (IPR)” is often not a focus because the product is completely outside the proprietary supply chain and maybe never even enters the legal and legitimate supply chain. The focus must be on the entire supply chain because a problem at in any vulnerability could lead to a health hazard, economic impact, and a recall (Fig. 2).



**Figure 2** The Food Supply Chain Vulnerabilities (Spink, 2011).

## Food Fraud History and Shift to Prevention

While food fraud has been ongoing since the very beginning of commerce, it has been recorded as far back as 400 BCE. A series of more impactful incidents – combined with increased impact based on improved detection and traceability capabilities – has led to more of a research focus which has led to the refinement of an interdisciplinary approach focused on prevention. A series of escalating food fraud incidents created and had maintained a focus on the topic.

- 2004 – Sudan Red carcinogen colorant in paprika and other spices (adulterant-substance)
- 2007 – Melamine in infant formula and pet food (adulterant-substances)
- 2012 – Horsemeat in beef (adulterant-substance)
- Future – and an ongoing stream of incidents identified as “food fraud”

The increasing public health and economic harms led to specific research that defined food fraud and then the need for a more holistic, all-encompassing focus on the root-cause or a shift from response to prevention. In crime prevention and criminology, once a crime is specifically defined, it can be identified and categorized. For example, the system of Food and Feed Safety Alerts - European Commission (RASFF) now has a category for “fraud and adulteration.” Incidents now can be categorized in this specific category. For example, before this category was established, the melamine incident the assigned “hazard category” would have probably been “chemical contamination” and the horsemeat incident assigned to “Labelling absent/incomplete/incorrect” or “industrial contaminants.” These two incidents now can be assigned to “fraud and adulteration” which can lead to a coordinated focus to detect, deter, and prevent.

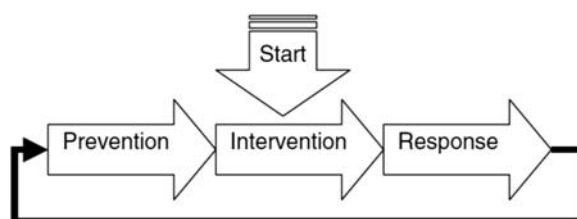
A natural evolution in the research on an emerging threat is a shift from intervention and response back to prevention (Fig. 1) (Spink, 2011). While prevention is the logical starting point, the cycle usually begins when a problem or incident is identified. The concern leads to a focus first on intervention such as identifying melamine as the toxin and then determining detection methods. Next, once melamine is identified as the problem and the detection test is defined, there is a focus on finding and removing the melamine-contaminated products from the marketplace. Finally, once the crisis has been managed, there should be a review of how to prevent this incident – or like problems – from occurring again. This shift to prevention is most efficient when leveraging Criminology theories that focus on reducing the opportunity or vulnerability.

When beginning to focus on prevention, there should be a focus on the root-cause of the problem which, in this case, is not *E. coli* but a human criminal adversary. “If the biological organism in question were a microbe so, we would naturally leverage the discipline of microbiology; for food fraud, the biological organism in question is a human, so it is natural to leverage Social Science and Criminology.” While the field of Criminology is firmly developed the ideas are novel to microbiologists and food scientists. Specifically, the crime prevention focus has been studied since the mid-1970s in Situational Crime Prevention which focused on the physical characteristic that allows a crime to occur. Researching the physical space of crime is “Environmental Criminology” whereas studying the criminal is “Traditional Criminology.” Situational Crime Prevention is based on more basic theories of Rational Choice Theory (criminals believe they will not get caught) and Routine Activities Theory (criminals become aware of opportunities in their daily life). Considering that a company is trying to protect a specific product and a country is trying to defend a border or consumer, for food fraud prevention, the focus on Situational Crime Prevention is immediately actionable and very resource efficient.

These theories are presented in the “Crime Triangle” which has been adapted to the food fraud with elements that include: victim, fraudster, and guardian or hurdle gaps (Fig. 3) (Spink, 2011).

At this point, it is important to review the types of fraudsters and to specifically address the threat of violence or retaliation against a food inspector, purchasing agent, or a manufacturing plant receiving dock worker. The types of *fraudster* include recreational (action for entertainment to amusement), occasional (infrequent, opportunistic), occupational (incidents at their place of employment either as an individual actor in collaboration with the company), or professional (crime fully finances their lifestyle Spink et al., 2013b). It is then crucial to understand the *type of offender organization* including individual/small group, general criminal enterprise, Organized Crime (formal structure and extensive threat not just “crime that is organized”), terrorist organizations (supporters or direct members), foreign government offenders (state-sponsored crime), and others (Spink, 2011).

For example, a “food inspection” becomes a “criminal investigation” when there is a collection of evidence that could criminally convict an individual or group. Many times a food safety inspector unknowingly shifts from a traditional audit (“let’s check the lot codes to see if they match the manifest?”) to a criminal investigation (“how come you said you used 50 pallets of the product but only have invoices for two?”). Considering the threat of violence against that food inspector, the most dangerous situation is against



**Figure 3** Food Protection Plan Progression (Spink, 2011).

a novice criminal who might get anxious and immediately, violently retaliate. A hardened criminal who is well versed in the investigation and prosecution system would not resist and would be non-threatening.

Often the corporate liability insurance for a food safety inspector does not cover criminal investigations. If an inspector is sent into a situation where fraud occurs any harm may not be covered by insurance and – if there is knowledge of actual fraud – the supervisor or company managers may be criminally liable for creating this danger.

### The Role of the Public–Private Partnership and the Role of Food Science and Technology

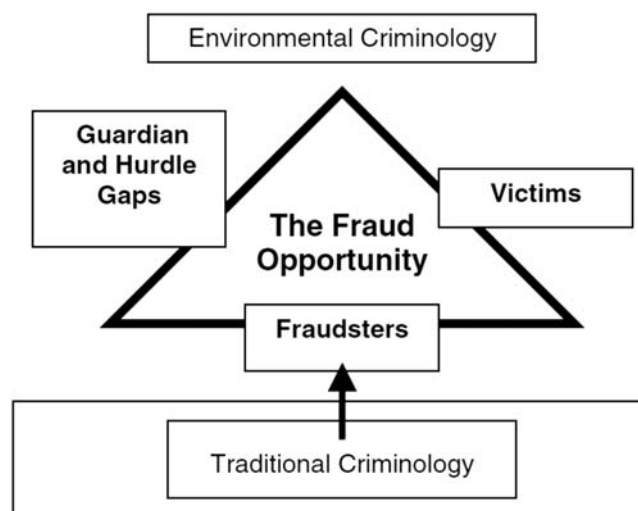
Considering the optimal role of the public-private partnership—the interaction of government, industry, and others such as academics and associations – is especially important when dealing with a complex, inter-disciplinary, inter-agency, and international issue such as food fraud prevention. Among others, entities such as INTERPOL and Europol have led collaborations such as through Operation Opson, the European Commission funded the Food Integrity Project, the World Health Organization (WHO) and Food and Agricultural Organization of the United Nations (FAO) has begun work within Codex Alimentarius (CODEX), and the industry collaboration of the Global Food Safety Initiative (GFSI) has created a harmonized and standardized overall food safety management system.

A key efficiency is that there is currently coordination and communication in the public-private partnership. While the requirements are evolving and formalizing, an efficient and holistic system is developing (Fig. 2). Government agencies are starting to formally recognize the value of “accredited third-party certification” as a valuable and resource-saving system. For example, in the “Third-Party Audits and FSMA” report, the U.S. Food and Drug Administration (U.S. FDA) stated, “FDA contemplates leveraging third-party audits as part of its overall compliance strategy” (FDA, 2017). The formal leveraging of third-party audits has been discussed publicly by a wide-range of international governments. While not a substitute for a formal government inspection the third-party audits are becoming more and more relied upon when governments have increasing confidence in the system and when under pressure of reduced human and financial resources.

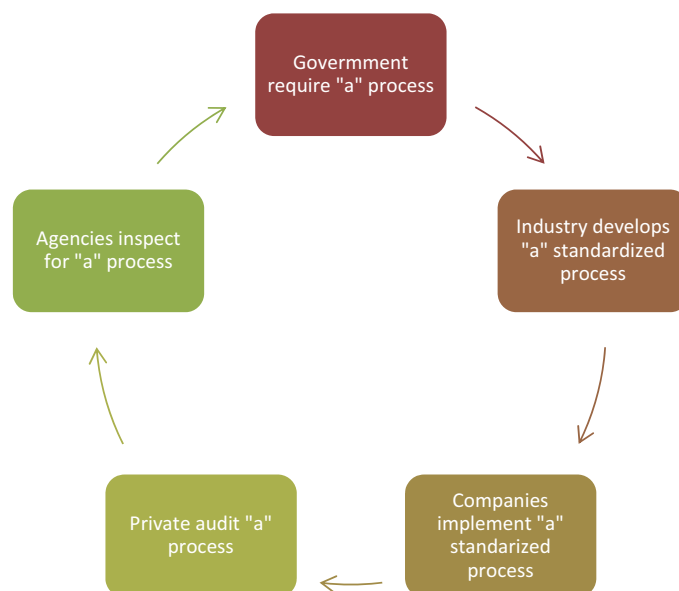
An efficient system is for the government to require “a” process to assess and manage all hazards including those that are economically motivated. This is a specific quoted requirement in laws such as the U.S. Food Safety Modernization Act section on “hazard analysis.” By requiring “a” process – but not specific details – the government makes this a priority but also allows for industry innovation, coordination, and harmonization. The government requirement increases the industry focus on developing and managing systems, companies to fully develop prevention strategies, the private third-party audits to be strong, and then agencies may be confident in relying upon the audits, which end up further protecting the food supply chain which is the ultimate goal of the government food laws (Fig. 4).

Finally, the concepts of defining the term, exploring the scope of the problem, considering crime prevention about the public-private partnership, lead to an efficient and effective consideration of how to address the problem. Addressing the problem includes identifying vulnerabilities, conducting an assessment, and then considering the optimal role of food science and technology.

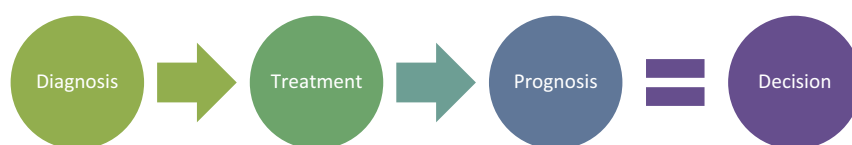
Think about a sick person visiting a medical doctor. Overall there is a process for “diagnosis,” then consider a series of possible “treatments,” and each treatment considers a “prognosis” (Fig. 5). The diagnosis is similar to considering vulnerabilities. The treatments are the countermeasures or control systems which do include “do nothing.” Finally, each diagnosis-treatment option should



**Figure 4** The Crime Triangle applied to Food Fraud (Spink, 2011#1418). Adapted from Felson, M., 1998. Crime and Everyday Life. Pine Forge Press, Thousand Oaks, California.



**Figure 5** Public-Private Partnership Cycle Reinforcing “a” Process.



**Figure 6** Continuum of Diagnosis, Treatment, Prognosis, and the Decision.

consider a prognosis or result of the effort. For example, if fraud is occurring at 1% to 5% of the finished good, then there is no need for a treatment that reduces the sensitivity from 1 part per thousand to 1 part per million (Fig. 6).

**Vulnerabilities:** The vulnerabilities need to be identified from the perspective of the “fraud opportunity” and not just previous adulterant-substance incidents or the application of detection methods. For example, the fraud may not involve an adulterant-substance. For example, they may reduce the amount of a valuable ingredient and replace with a filler ingredient. Also, a fraudster may research the current test methods and figure a way to circumvent or fool the current tests. For example, switching from horse-meat to stolen or lower quality beef.

**Assessment:** The risk assessment should cover all types of fraud and for all products. If the identification of vulnerabilities is not full and the assessment is only focused on specific areas, then the entire company is not protected. A human criminal adversary may perceive the threat and shift their type of crime. This follows the logic from Rational Choice Theory and the Crime Displacement.

**Optimal Role of Science and Technology:** Food science and technology provides the critical role of detection. If the fraud cannot be detected, then the intervention step never starts, and the prevention process is not activated. Detection is the most complex of the countermeasures or control systems but cannot be efficient or optimized without the previous criminology and quality management identification of vulnerabilities and risk assessments. Once the root-cause has been investigated and identified, then a very precise and defined “specification” can be provided for selection or development of countermeasures or control systems (Spink et al., 2013a; IUFoST, 2016). In many cases “more” testing is not needed but just a re-focus of resources or refined communication of information.

In an article co-authored by Professor Elliott from Queen’s University Belfast and Kevin Swoffer who was the former subject matter expert for the Global Food Safety Initiative (GFSI), “Whilst better means of detecting Food Fraud are required, “success” must be measured in terms of how the activities support prevention” (Spink et al., 2013a).

## Conclusion

Food fraud is becoming a unique and specific research area for government, industry, and academics. The focus has expanded to an extremely interdisciplinary task starting with social science and criminology, applying risk management and decision sciences, to include information technology and data analytics science, leveraging supply chain management, before the resource-allocation

decision-making such as from Enterprise Risk Management and COSO managerial accounting practices, before providing food science and technology researchers with a very clear scope and specification of detection requirements. It is important and critical to realize that Social Science and Criminology are – naturally – key “science & technology” parts of the food fraud response.

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## Food Fraud and Vulnerability Assessments

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### Glossary

**Counterfeiting** The process of copying the brand name, packaging concept, recipe, processing method etc. of food products for economic gain.

**Dilution** The process of mixing a liquid ingredient with high value with a liquid of lower value.

**Food fraud mitigation measures** Hard and soft actions that are taken to combat against identified food fraud vulnerabilities.

**Food fraud vulnerability assessment** The process of collection and evaluation of information on potential food fraud risk factors as well as mitigation measures which, when combined, determine the actual fraud vulnerability.

**Food fraud vulnerability** Susceptibility or exposure to a gap or deficiency that could place consumer health at risk and/or have an economic or reputational impact on a food company's operations if not addressed.

**Food fraud** A collective term encompassing the deliberate and intentional substitution, addition, tampering, or misrepresentation of food, food ingredients or food packaging, labelling, product information, or false/misleading statements made about a product for economic gain.

**Food adulteration** The process in which the quality of food is lowered secretly and intentionally either by the addition of inferior quality material or by extraction of valuable ingredients/constituents in order to mislead customers for own economic gain.

**Mislabeled** The process of placing false claims on packaging for economic gain.

**Substitution** The process of replacing an ingredient or part of the product of high value with another ingredient or part of the product of lower value.

**Unapproved enhancement** The process of adding unknown and undeclared materials to food products in order to enhance their quality attributes and conceal fraud.

### Introduction to Food Fraud

Food fraud is the intentional, deceptive misrepresentation of foods for financial gain (Spink et al., 2017). Fraud is a form of criminal behaviour in any definition of crime: whether we consider the legal (acts forbidden by law) or the non-legal definition. The two share two crucial elements: criminal behaviour is harmful and morally reprehensible (Passas, 2005). Food fraud qualifies for both. Food fraud always involves at some stage deliberate dishonesty to obtain an advantage from or cause losses to or harms against individuals (e.g. consumers), groups (e.g. companies, market actors and investors) or the state (e.g. tax and revenue). In this sense, we can distinguish these intentional acts from a range of behaviours and regulatory violations that are often referred to as 'non-compliance' issues, such as carrying out inadequate due diligence on suppliers, or 'lawful but awful' practices (Lord et al., 2017).

Food fraud involves all forms of misrepresentation, i.e. full (counterfeiting/mislabeled) or partial replacement (adulteration) of the original product. Some products may be fully replaced because of their added value due to their brand/type of product (expensive wines, premium brand dairy products) or due to special attributes related to production origin (organic) or geographical origin. Adulteration concerns the removal of ingredients or constituents or their substitution by lower value counterparts. Examples are dilution of high value extra virgin olive oil with refined olive oil, bulking out spices with inferior material, or inflating meat with water. In some cases, it may also concern the apparent enhancement of constituents determining the product's value, such as the protein content of milk. Furthermore, it may concern also concealment which is required to cover up the adulteration practices. Examples are the addition of colourants to spices, or Manuka pollen to diluted Manuka honey to disguise the fraudulent product and deceive the customer and the laboratory checking the product.

Awareness of food fraud and its consequences has raised in the past decade. Cases, such as melamine in infant formula in China in 2008 (Rovina and Siddiquee, 2015) and the substitution of beef by horsemeat in the EU in 2013, set the alarm bells ringing. Food fraud has often been considered an economic issue, but recent cases have highlighted that these practises are detrimental to businesses and economies, but also ruin consumer confidence. After the various incidents in the past decade, both industry and authorities recognized that a single incident can have global market consequences, with broad implications for company brands, industry performance, people's lives, and countries' reputations.

## Impact of Food Fraud

Food fraud impacts seriously on businesses involved, their customers, others in the chain or tier who are painted with the same brush, as well as the consumers. Losses may include social losses and punishments, third party losses (e.g. extra testing), confidence losses, sales losses and over payment, as well as recall losses. The kinds of impact of various fraud cases has been studied by Bindt (2016). Across the board, social losses and punishments are most frequently reported. Sale drops ranged between 13%–80% for the cases studied, stock prices declined by 37%–75%, and confidence/trust in industries was reduced by 7%–64%.

Let's take the melamine incident as an example to list the various kinds of losses. The social losses of the melamine case were enormous: 240,000 infants got ill, 60,000 were hospitalized, and six children died (Dani and Deep, 2010). The Chinese court involved imposed death penalties, imprisonment and fines on the offenders (Cucchiella and Gastaldi, 2006). The scandal resulted in the bankruptcy of the Sanlu Group co, Ltd in 2008, a company that sold the melamine tainted infant formula, leaving a debt of \$160 million. Financial losses reached far beyond the Sanlu Group though. Ten days after disclosure, the sales of the Chinese companies Mengniu and Yili dropped by 80%, and overall sales of the dairy sector in China dropped by 30%–40% (Dani and Deep, 2010; Lu and Tao, 2009). Stock prices of companies dropped between 37%–60% at that time (Evershed and Temple, 2016). Purchase volume of milk decreased to ~20% of normal sales on 22 September 2008 (Qian et al., 2011). The estimated recall costs for the Chinese dairy industry amount \$3 billion, whereas worldwide recall expenses are estimated on \$18 billion (based on 30 affected brands in more than 60 countries) (Xiu and Klein, 2010; Evershed and Temple, 2016). Chinese dairy products were boycotted by 30 EU countries and confidence in the Chinese dairy industry plummeted. When interviewed in that period, 45%–64% of the Chinese consumers perceived at least one of the Chinese dairy products (milk (powder), yoghurt, ice cream) as unsafe (Qiao et al., 2010). The Chinese dairy industry is still recovering from this incident today, nearly a decade later.

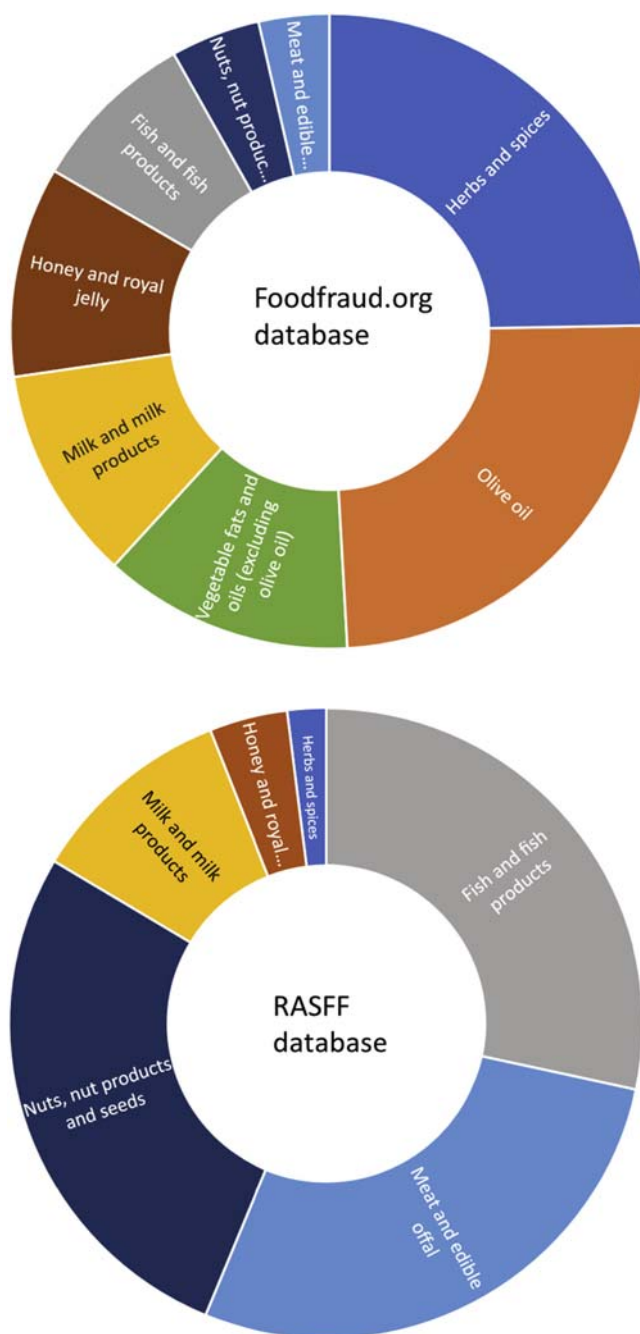
## Fraud Vulnerable Food Product Groups

When people try to manage food fraud risks, one of the first questions asked is which products are more at risk than others? In fact, we do not know because there is no global fraud case report system. However, there are some databases with specific data sources that may shed some light. Some products or chains appear more frequently in these databases than others. This phenomenon was explored by carrying out an inventory of the most frequently reported food product groups in three public databases (Weesepeol and van Ruth, 2015). The Foodfraud.org database (USP, 2017) comprises primarily scientific reports on food fraud. Out of the 615 reports on eight major product groups in the 2008–13 period, the five predominant food product groups are in decreasing order: (1) herbs and spices, (2) olive oils, (3) vegetable oils (not including olive oils), (4) milk and milk products, and (5) honey and royal jelly (Fig. 1, upper graph). The Rapid Alert System for Food and Feed of the European Commission comprises a section on food fraud reports (RASFF, 2017). This section included 201 reports for these eight groups in the 2008–13 period. The five prime food product groups in this database are (1) fish and fish products, (2) meat and edible offal, (3) nut, nut products and seeds, (4) milk and milk products, and (5) honey and royal jelly (Fig. 1, lower graph). In this RASFF database, cases are reported by authorities, but only if there is a safety risk and a concern that other EU member states may be affected as well. The differences in ranking of product groups from the two databases are caused by their very different data sources. Although the ranking differs, generally the same food products appear in the list, except for the oil categories (olive oils and vegetable oils). Although there are numerous food fraud reports on oils, in this period obviously very few were associated with safety risks in the EU.

## Tempted Offenders

But what about the offenders? What drives them, and how do they interact with their environment? There is some popular belief that food fraud is mostly an external threat caused by organized crime groups seeking to permeate the food supply chain. Although politically convenient, it seems more often a problem within the food system itself and committed by legitimate food supply chain actors. Lord et al. (2017) argued that food fraud is better understood as an 'endogenous' phenomenon within the food system where legitimate occupational actors and organizations are in some way necessarily involved rather than being an 'exogenous' phenomenon perpetrated by externally organized (transnational) 'criminal enterprise'. The melamine case is a good example of fraud that was committed by legitimate dairy chain actors.

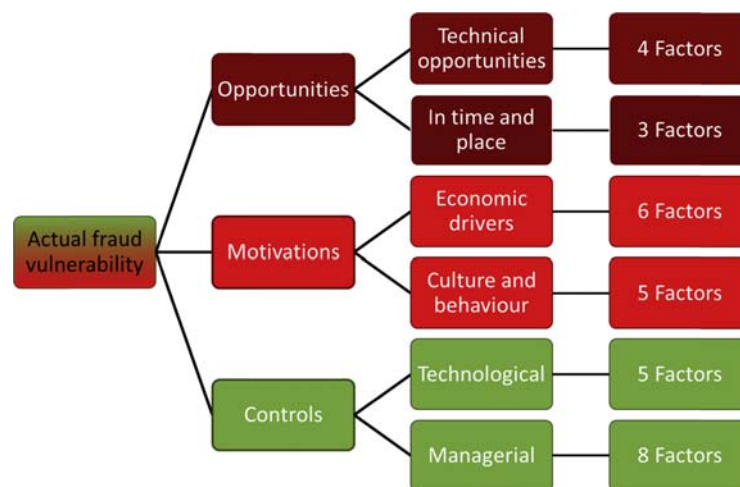
Food fraud is the interplay between the motivated actors, and their (social/criminal) networks, to make the most of fraudulent opportunities that arise (Lord et al., 2017). These opportunities are presented by victims and by those entrusted with controlling risks (Levi, 2012). For example, this may occur within businesses where certain malpractices may be 'acceptable' and within facilitative market and industry structures and cultures. The goal of fraudsters may indeed be to maximize profit but in other cases this may be a secondary outcome as actors in the supply or production chain seek to maintain their business. Some will act out of greed, but many also out of need. If it has become a certain culture to commit fraud in a supply chain network, law-abiding businesses in the same chain are affected as well. In some cases, these businesses are affected to the extent that they must choose between 'being driven out of business' or 'swim with the tide' to survive the severe and unfair competition (Baucus and Near, 1991). Frauds may be of pre-planned, but may be more often a response to a situation, or be more of a slippery-slope nature (Lord et al., 2017).



**Figure 1** Division of the major food product groups of the reports in the [Foodfraud.org](https://www.foodfraud.org) database (upper graph) and the Rapid Alert System for Food and Feed (RASFF) database (lower graph) in the EU in the period 2008–13.

### Fraud Vulnerability

To get insights into the ‘what’ and the ‘who’, but also especially the ‘why’ of food fraud, the criminological routine activity theory (Cohen and Felson, 1979) is quite suitable to describe the various facets of food fraud. Applying the theory, food fraud can be conceptualized as the outcome of the convergence in time and place of 1) motivated offenders and 2) suitable targets (opportunities) in 3) the absence of capable guardians (van Ruth et al., 2017). The vulnerability to fraud results from openings for undesirable events resulting from weaknesses or flaws related to the system (Spink et al., 2017). It can be framed by enablers and drivers existing in the system, as well as counteracting control measures. The actual fraud vulnerability of a company is determined by the key elements opportunities, motivations and control measures. The three key elements can again be subdivided into six sub categories. These categories comprise (1) technical opportunities, (2) opportunities in time and place; (3) economic drivers, (4) culture and behaviour, (5) technical control measures and (6) managerial control measures (visualized in Fig. 2). Each sub category comprises



**Figure 2** Schematic presentation of the food fraud vulnerability concept based on the routine activity theory: the three key elements opportunities, motivations and controls, their sub categories, and number of fraud factors.

of several factors. They are detailed in [Table 1](#). The selection of these fraud factors and its scientific justification has been published previously ([van Ruth et al., 2017](#)). Food fraud vulnerability results from threats from both the external and the internal environment of a business. Therefore, some of the factors need to be considered for the own company as well as for external environmental layers, such as the direct suppliers and customers, the wider chain/industry network, and the (inter)national environment.

## Fraud Vulnerability Assessments

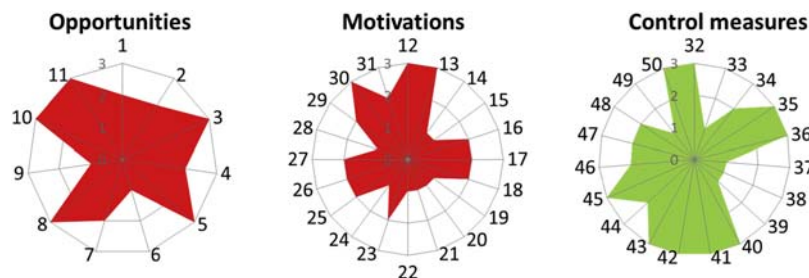
The surfacing fraud cases have increased the need to strengthen companies' ability to combat fraud within their organizations and across their supply chains ([Manning and Soon, 2014](#)). Because of the raised awareness in industry food fraud needs nowadays to be considered in food quality/safety management plans. The Global Food Safety Initiative published a white paper ([GFSI, 2014](#)), in which was announced that two food fraud mitigation steps in the form of two new key elements in the GFSI Guidance Document would be added. These elements required a company (1) to perform a food fraud vulnerability assessment and (2) to have a control plan in place ([GFSI, 2014](#)). Since food fraud vulnerability assessments did not exist yet, several initiatives started to develop protocols. For example, the U.S. Pharmacopeia Convention (USP) developed the USP tool to guide companies to identify the most vulnerable ingredients within their supply chains and to choose valid situation-specific mitigation measures ([USP, 2014](#)). Furthermore, organizations such as the Grocery Manufacturers Association in the US ([GMA, 2016](#)), the British Retail Consortium ([BRC, 2015](#)), the European Spices Association ([ESA, 2017](#)), and the feed industry ([GMP+, 2017](#)) among others developed questionnaires and guidelines to identify fraud vulnerabilities. At the same time, also in response to the GFSI white paper, a large interactive and iterative process with representatives from the global food industry, retail, authorities and the scientific community commenced, which was initiated and coordinated by SSAFE. SSAFE is a non-profit collaboration of various multinational companies. The aim was to develop a practical, but science-based food fraud vulnerability self-assessment tool, a tool which would be based on the theoretical concept described in the section 'Fraud Vulnerability' of this chapter. Through a very extensive process the self-assessment was developed and validated. The self-assessment tool can be downloaded as spreadsheet for free in various languages ([SSAFE, 2017](#)), but has also been made available as a free downloadable app by PwC/SSAFE ([PwC, 2017](#)). The assessment comprises 50 questions and associated answering grids based on the fraud factors, and was tested globally. Nine questions relate to opportunities, 20 to motivations and 21 to control measures. The answering grids present descriptions of low, medium and high vulnerability situations for each question and link to a score system. The scores (1,2,3) can be visualized in spider web diagrams for a first impression of the company's vulnerability. An example for a meat processor is presented in [Fig. 3](#). They show moderate vulnerability in view of opportunities and motivations (some red areas), and partly implementation of control measures but with room for improvement (some green area).

## Fraud Vulnerability in Various Chains

We have seen in the previous sections that the number of fraud reports varies with product groups, but also with data sources. Fraud vulnerability assessments are designed to assess a company's vulnerability to fraud. However, they may also provide insights in the vulnerability in supply chains as well as across supply chains when applied to groups of actors. In a current study various tiers in the fish, meat, milk, olive oil, organic bananas, and spice supply chains were assessed for their fraud vulnerabilities with the SSAFE food

**Table 1** List of food fraud key elements, sub categories and individual fraud factors

<i>Fraud key elements</i>	<i>Sub categories</i>	<i>Fraud factors</i>
Opportunities	Technical opportunities	<ul style="list-style-type: none"> <li>• Simplicity/complexity of adulteration</li> <li>• Simplicity/complexity counterfeiting</li> <li>• Availability of technology and knowledge to adulterate</li> <li>• Availability of detection technology</li> </ul>
	Opportunities in time and place	<ul style="list-style-type: none"> <li>• Accessibility to materials in production/processing</li> <li>• Transparency supply chain network</li> <li>• Historical evidence</li> </ul>
Motivations	Economic drivers	<ul style="list-style-type: none"> <li>• Supply and pricing of materials</li> <li>• Special product attributes or value determining components of materials</li> <li>• Price differences in countries</li> <li>• Economic health business</li> <li>• Level of competition</li> </ul>
	Culture and behaviour	<ul style="list-style-type: none"> <li>• Financial strains imposed on suppliers</li> <li>• Business strategy</li> <li>• Ethical business culture</li> <li>• Previous criminal offences</li> <li>• (Inter)national corruption level</li> <li>• Victimization</li> </ul>
Controls	Technological controls	<ul style="list-style-type: none"> <li>• Specificity and accuracy of fraud monitoring system</li> <li>• Systematics and autonomy of verification of fraud monitoring system</li> <li>• Accuracy information system for mass balance control</li> <li>• Extensiveness tracking and tracing system</li> <li>• Fraud contingency plan</li> </ul>
	Managerial controls	<ul style="list-style-type: none"> <li>• Strictness ethical code of conduct</li> <li>• Application integrity screening</li> <li>• Support whistle blowing system</li> <li>• Contractual requirements suppliers</li> <li>• Social control and transparency across supply chain</li> <li>• Established guidance for fraud prevention across supply chain</li> <li>• Specificity national food policy</li> <li>• Strictness enforcement for fraud prevention regulation/law</li> </ul>

**Figure 3** Spider web diagrams of the fraud vulnerability assessment of a meat processor: opportunities, motivations and control measures. Numbers refer to questions 1–50 in the SSAFE food fraud vulnerability self-assessment tool (van Ruth et al., 2018).

fraud vulnerability assessment tool (van Ruth et al., 2018). Thirteen fraud factors related to opportunities and motivations scored high across all supply chains indicating their importance as general fraud drivers and enablers. They included: availability technology and knowledge to adulterate raw materials/final products; fraud detectability in raw materials/final products; historical evidence of fraud in raw materials/final products/branch of industry; supply and pricing of raw materials; valuable components or attributes of raw materials; corruption level in the country of the supplier; economic conditions of the branch of industry; the level of competition in the branch of industry; and price asymmetries. Use of control measures varied across supply chains and tiers, but technical controls were generally more implemented than managerial controls. Comparing the various chains significant differences in vulnerability for about half of all the fraud factors was observed. Thus, considerable differences between chains existed and could be explained by approximately 50% of the fraud factors. Different tiers (e.g. processor, trader, retailer), only showed significant differences for one out of seven of the fraud factors results. Consequently, the chain seems to be more important for perceived fraud vulnerability than the position (tier) in the chain. When ranking the various chains, it appeared that fraud vulnerability was highest



in the spices chain, which was followed by the olive oil, meat, fish, milk and organic banana chains. Regarding tiers, the wholesale/traders group appeared most vulnerable, and they were followed by retailers and processors. When comparing these results with the [foodfraud.org](http://foodfraud.org) databank results in the previous section, it appears that there is certainly overlap in product groups/chains. Spices and olive oil were reported most frequently in the 2008–2013 period, and scored also high in terms of fraud vulnerability by the actors in the chain. Milk, fish and meat scored lower, both regarding reports in the databank and in terms of fraud vulnerability by the actors in the chain. There is considerably discrepancy with the RASFF databank though, which is probably because fraud cases with a safety impact and with a risk for other EU member states are reported only. On the contrary the fraud vulnerability assessment does not discriminate between vulnerabilities with or without potential safety impact.

## Concluding Remarks

To manage food fraud risks in the food industry, it is pivotal to map fraud vulnerabilities. Various factors need to be considered: those that make products suitable targets as well as those that drive and influence the potential offender. When these vulnerabilities have been mapped and vulnerability appears high, there are two options. The first option is to avoid the situation, which will often mean cease activities. Often that is not acceptable or not even feasible. Alternatively, one may try to mitigate the risk by implementing control measures. Depending on the control measures the fraud vulnerability may be reduced to an acceptable level. Fraud vulnerability assessments allow authorities to discover weak points in the system, which they can use for risk-based, dedicated enforcement of legislation. Finally, these assessments may be used in science to improve the understanding of food fraud occurrence by elucidation of the interaction of fraud drivers, enablers and controls.

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# Food Fraud Vulnerabilities in the Supply Chain: An Industry Perspective

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## Background: Food Fraud and Economically Motivated Adulteration (EMA)

Food fraud is a generic term encompassing a wide range of deliberate fraudulent activities such as counterfeiting (fraudulently passing off inferior goods as established and reputable brands), product tampering, product diversion (sale or distribution of products outside the intended markets), theft, smuggling and others malpractices (Spink et al., 2016a; Global Food Safety Initiative, 2014; US Pharmacopeia appendix XVII, 2015). This generic term needs to be distinguished from the economically motivated adulteration (EMA or economic food adulteration) which is the result of intentional addition, substitution, dilution, unapproved enhancement or concealment performed in food raw materials or finished products using foreign compounds (adulterants) to increase revenue. Economic food adulteration corresponds to the scope that we are going to cover in this chapter (in line with the definition of food fraud in the European Union, see EU, 2014). Moreover, it is worth differentiating food fraud from food defense, where intentional adulteration is ideologically motivated to cause public health harm, economic harm or terror.

## Food Adulteration Is a Criminal Act of Potential Safety Concern

Economically motivated adulteration (EMA) is of increasing concern for society. It deprives the consumer of the quality products they intend to purchase, decreasing consumer trust and leading potentially to significant consequences on human health (Spink and Moyer, 2011; Everstine et al., 2013). To maintain consumer trust, food companies must remain alert to potential adulteration and work actively with their suppliers to identify and mitigate the associated risks (Stadler et al., 2017). This requires industries to implement an effective *food adulteration management system*.

## Food Adulteration Management System

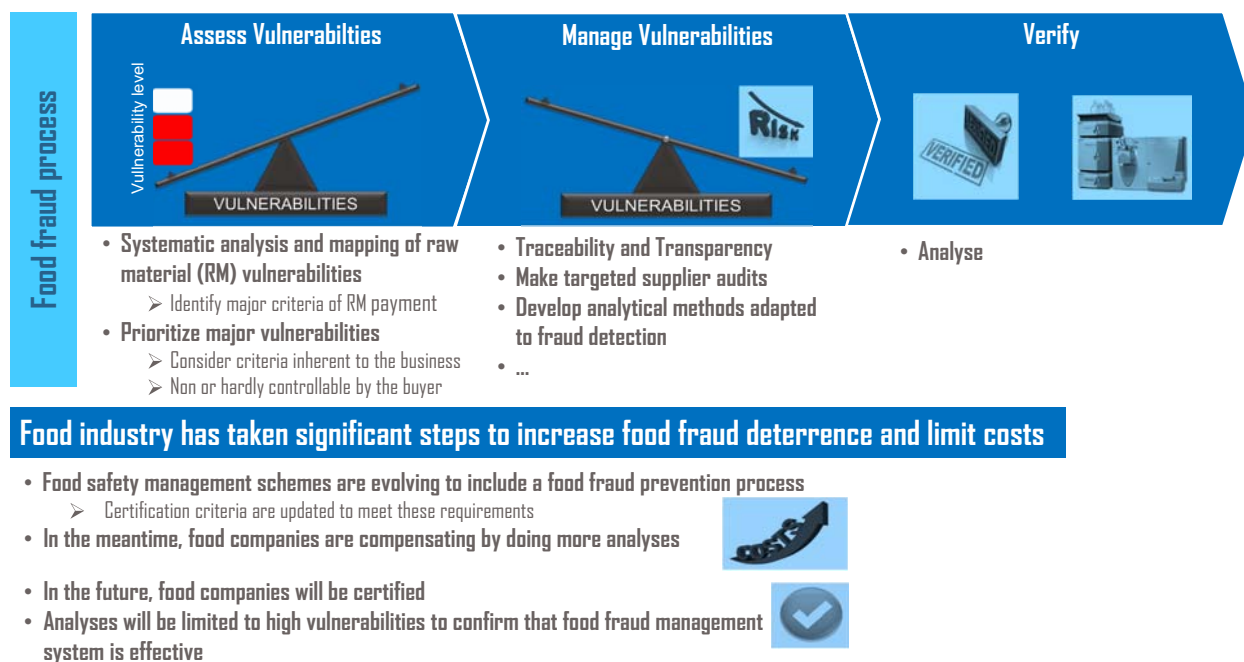
Management of food safety risks often focuses on hazards that occur naturally in foods or come from the environment (e.g. heavy metals) or the food production chain (e.g. food processing, storage and handling). Those hazards are adequately identified and managed. In contrast, in the case of food adulteration, industries are facing unknown adulterants where the traditional risk-based preventive control systems cannot be applied (Stadler et al., 2017; Spink et al., 2017).

The food industry has recently taken significant steps to prevent the risk of food adulteration in the food value chain. The Global Food Safety Initiative (GFSI) is an industry-led initiative within the Consumer Goods Forum (CGF) aiming at mitigating food safety risks across the value chain. GFSI has introduced additional requirements for food safety schemes (e.g. British Retail Consortium (BRC), FSSC 22000) to address the risk of food adulteration. Consequently, the food safety schemes recognized by the food industry at large (e.g. food producers, manufacturers, distributors) have updated their certification criteria to meet the requirements defined by GFSI for food fraud prevention (GFSI, 2014; BRC food issue 7, 2014). These requirements ensure that adequate food safety management schemes include a food fraud prevention process. Such process begins with an evaluation step to identify and characterize vulnerabilities in the food supply chain (vulnerability assessment) and is followed by the design, validation and implementation of mitigation measures (Fig. 1).

## Vulnerability Assessment

It is important to know what drives the fraudsters to better understand vulnerabilities in food adulteration (Van Ruth et al., 2017; Cavin et al., 2016). The term vulnerability is normally applied for economic food adulteration addressing both public health and economic consequences rather than the term risk used in food safety (USP appendix XVII, 2015; Spink et al., 2016b).

Guidance document and self-assessment tools have been developed by a number of organizations (e.g. USP appendix XVII, 2015; SSAFE, 2017; FDF, 2014) to help food companies undertake their own vulnerability assessment and implement appropriate control plans. In general, a higher level of processing will induce a higher vulnerability since it is easier to adulterate a raw material that has been transformed into liquids or powders (e.g. oils or milk powders). Weight is the most common payment criterion but other criteria are often used (protein or fat content, color etc.), giving additional adulteration opportunities and thus, increasing the number of substances that can be used by fraudsters. Materials paid on the basis of multiple payment criteria are often highly vulnerable. Fresh milk, which is paid based on the volume, protein content, fat content and bacteriological quality, is a good example. It is one of the most adulterated raw material worldwide. Many milk adulterants have been identified and can be grouped by classes of adulteration related to the different milk payment criteria (Cavin et al., 2016; Poonia et al., 2017).



**Figure 1** Food fraud management process to assess and manage vulnerabilities and verify that the process is effective. By application of this process and introduction of new requirements for food safety schemes (certification criteria for food fraud prevention), the food industry has taken important initiatives to increase food fraud deterrence and limit the costs of analyses.

Food companies are often facing adulteration vulnerabilities on many raw materials in various food categories, highlighting the need for prioritization. Such prioritization can take into account additional vulnerability criteria inherent to the business such as the raw material volume purchased, average prices, and prices fluctuations that are often hardly controllable by the buyer (e.g. a particularly low market price may indicate a lack of food control/or regulatory enforcement framework in the country of origin or country of transit).

Large-scale economic food adulteration cases are reported in the scientific literature but smaller local incidents are usually identified by web scouting of media sources worldwide. Therefore, the vulnerability assessment is a live process, which needs regular updates for effectiveness (see food fraud mitigation guidance [USP appendix XVII, 2015](#)). Commercially available webscouting tools (e.g. Digimind, Radian6) or online regularly updated databases (e.g. Horizon scan, USP food fraud database) are available. These tools help food industries in their continuous identification of new emerging food vulnerabilities. However, and this is inherent to the nature of economic adulteration, current tools will hardly predict pro-actively new food vulnerabilities potentially associated with economic, regulatory, geopolitic or other rapid changes occurring worldwide. In this context, some industries and internationally funded research projects (e.g. [EU Food Integrity project](#)) are working on automated Early Warning digital approaches for food adulteration. They are looking for abnormalities in characteristic attributes or key drivers of food adulteration and further process them using statistical models to help identifying alerts predicting new vulnerabilities within the supply chain.

### Identification of Adulterants of Safety Concern Based on Past Incidents

While economic food adulteration is not in principle intended to harm consumers, dramatic consequences on human health occurred in the past, often by ignorance of the adulterant toxicity. Therefore, the vulnerability assessment is also aimed at identifying adulterants of major safety concern for which analytical methods are required rapidly (if not available yet). Previous cases help extrapolating the risk to other food raw materials. For example, Sudan dyes and lead oxide addition in spices led to the identification of many illegal dyes and inorganic pigments of significant health concern for humans ([Ramesh et al., 2005](#); [EFSA, 2005](#)). Similarly, the identification of ground peanut shells and almonds proteins in cumin and paprika highlighted the risk of allergenicity associated with spent materials that can easily be added as bulking agents in raw material powders ([FDA consumer advice, 2017](#)). It also helped to identify other practices linked to allergens such as the addition of cow milk in coconut milk. Moreover, toxic plant varieties such as the Japanese star anise containing high level of anisatin versus the traditional Chinese plant led to further research of toxic adulterants which could be used by substitution with other plant varieties from the same family ([Moore et al., 2012](#)). As a result, several toxic compounds have been identified in spices, herbs, oils and even indirectly in fish following substitution of tuna by bonito (*Sarda sarda*) from tropical area contaminated with toxic algae (ciguatera). In addition, past adulteration cases

demonstrated that fraudsters are mainly opportunists and may not care of potential health issues. The use of protein hydrolysates extracted from leather residues that have been previously treated and colored with chemicals is a good example. Those residues contain dangerous substances including possible residual levels of Chromium (VI), a highly toxic element for humans (Chaudhary and Pati, 2016). Another example is refined cooking oils collected from restaurants or even gutters (Ng et al., 2015). In addition to residual toxic contaminants such as acrolein, gutter oils could be of allergenicity concern if used previously for cooking seafoods (shrimps, calamari) or other food allergens (fish) and partially refined.

### Strength and Limitation of the Vulnerability Assessment

The initial assessment step is also required to identify common types of vulnerabilities between different food categories, such as sugar addition (e.g. sucrose, inulin, invert sugars from various plants such as beet, cane or corn, high fructose syrups from maltose, isomaltose) in fruit juices and honey, where a similar analytical strategy can be applied. Vulnerabilities common to different raw materials and categories is considered in the prioritization of the development and implementation of new mitigation measures. The substitution of species origin, the addition of bulking agents in powders, the enhancement of protein content in raw materials rich in protein and the concealment of raw materials using preservatives, bleaching agents or dyes are examples of major classes of vulnerabilities that will impact many raw materials and food categories and can be used to prioritize new developments.

The example of cardboard found recently in beef in the meat substitution crisis in Brazil or the addition of paper pulp in milk before drying demonstrated the limitation of the vulnerability assessment in the identification of unexpected adulterants. Therefore, it is important that new analytical methods for the detection of food adulteration take this unpredictability into account. This essential aspect will be further discussed later in the analytical evolution for food fraud detection. However, a special focus will be made initially on the analytical requirements for two main classes of vulnerabilities that have been the subject of recent food crises: A) Substitution of species origin and B) Enhancement or substitution of protein.

Beside the European horsemeat scandal in 2013, cases of meat substitution (minced or specialties such as sausages) have been identified for a very long time and are still identified regularly worldwide. In addition, mislabeling of fish and seafood has been the subject of different surveys from retail outlets and supermarkets showing that 25% (but up to 70% according to fish species) were regularly substituted (Warner et al., 2013). Two recent surveys testing oregano authenticity also identified a high percentage of adulterated samples (24% in UK and 60% in Australia) being substituted with up to 70% of foreign leaves (e.g. myrtle, olive, sumac or cistus) (Black et al., 2016a,b; Choice Australia, 2017). These are just some examples showing the need for rapid detection of species origin across food categories.

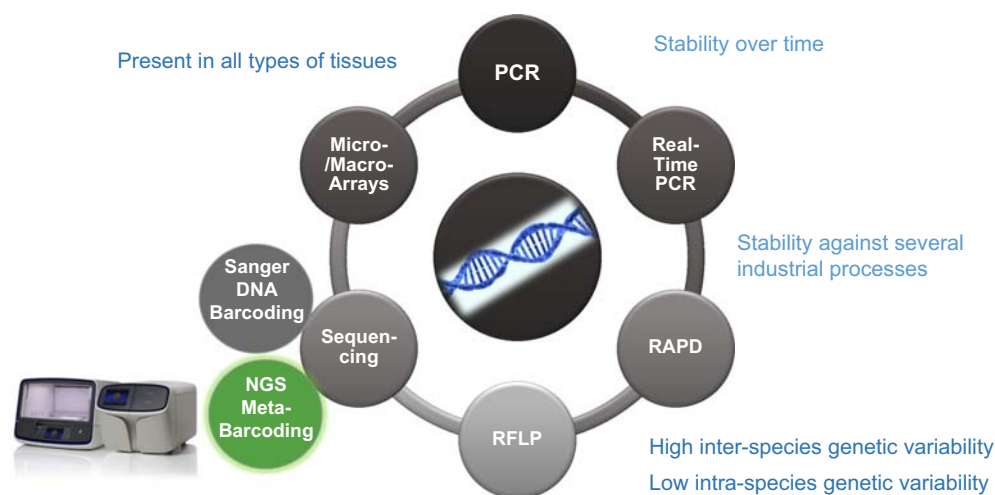
Protein content is a key payment criterion for raw materials under the form of powders (flours, concentrates, isolates) across many food categories (e.g. fish, meat, dairy, soya, leguminous seed, cereals and others). Therefore, the risk of substitution by cheap proteins from plant, animal or insect origin or by hydrolyzed protein produced from animal residues (leather, feather, horn) is significant. In addition, the impressive list of nitrogen-rich compounds (N-rich) calls for new analytical methods or potentially even other solutions that will be mentioned below. This category is critical because of the potential dramatic health consequences as those seen during the melamine crisis (Ingelfinger, 2008; Xin and Stone, 2008), where melamine was added artificially to increase the protein content in pet food and infant formula.

### Evolution of DNA-Based Approaches for Identification of Species Substitution

DNA-based methods are considered as the reference approach for species identification, and have been extensively applied during the EU horsemeat scandal. Compared to proteins, DNA is known to be more stable over time, more resistant to heat and industrial treatments, and present in all tissue types. Several DNA-based methods have been described to identify species from meat, fish and plants, generally all based on Polymerase Chain Reaction (PCR) (Kumar et al., 2015). Classical PCR and real-time PCR methods have been extensively published and are very specific to their targets. They are regularly applied by official control laboratories and service laboratories. Restriction Fragment Length Polymorphism (RFLP) and Random Amplification of Polymorphic DNA (RAPD) have been described as more flexible approaches than targeted PCR (Fajardo et al., 2010), but could lead to ambiguous results on closely related species. These approaches are unfortunately very targeted and often detect only the most common species, whereas more exotic species can be used for substitution; therefore detection methods must cover a broader range of species (Fig. 2).

Recently, the improvement of hybridization and (nano-)fluidic systems has allowed the development of macro- and micro-arrays. For species identification, they are usually based on PCR followed by hybridization of amplicons on arrays spotted with (many) different species-specific probes. A simple macro-array has recently been developed, allowing the detection of 32 meat species, including exotic species such as camel, kudu, cat, dog, fox and rat (Cottenet et al., 2016). The method allowed to detect these species down to 1% (w/w) which complies with the European requirement for meat adulteration. This macro-array is well adapted to meat species identification, but its application to fish reaches its limit knowing that salmon, tuna and white fish largely exceed 32 species. Similarly, this approach can not be applied on plant species identification with more than 300,000 plant species described by botanists.

While all these approaches are targeting a limited number of species, untargeted methods are more and more being developed and used. For DNA-based methods, DNA sequencing is the untargeted approach. Classical Sanger DNA sequencing has been shown to be highly reliable in identifying species. This technology is the basis of the International Barcode of Life initiative (iBOL) which



**Figure 2** Overview of the main DNA-based methods for species identification. With DNA molecule being well-conserved, stable over time, and present in all tissue types, various technologies have been developed over the past years, with NGS being the newest one and expected to be the next reference approach.

developed an identification system for eukaryotic organisms using DNA sequence variability (DNA barcodes). DNA barcoding is now frequently used to detect species substitution in fish and meat, and showed cases of substitution in United States close to 30% and 20% of fish and meat samples, respectively (Staffen et al., 2017; Quinto et al., 2016). In addition to meat and fish, DNA barcoding is also used for plant species identification. However, this technology is applicable to pure/single pieces and not to processed samples (e.g. ground or powder) susceptible to contain several species. If several species are present in the sample, the sequencing spectra from each species overlap and the final chromatogram is unreadable. In addition DNA barcoding usually targets long DNA fragments close to 600 base pairs (bp) preventing its application to processed samples where DNA could be degraded in shorter fragments of 100–200 bp. Mini-barcoding methods have been described to amplify shorter DNA fragments and detect degraded DNA, but they are not considered as the reference approach yet.

Evolution of Sanger DNA sequencing to Next Generation Sequencing (NGS) and meta-barcoding has allowed to reliably identifying species not only in pure/single species but also on mixtures. NGS is a DNA sequencing technology in which all DNA fragments are individually sequenced and recorded. Like classical DNA barcoding, meta-barcoding also starts with a PCR step to amplify consensus DNA fragments from meat, fish or plant. All individual PCR amplicons are then sequenced by NGS. Since all amplicons are sequenced and numerated, identification of species in a mixture is not only possible, but quantification could also be considered. DNA meta-barcoding is usually based on mini-barcodes and is thus more applicable to processed samples than DNA barcoding. In addition NGS presents the possibility to pool the tested samples altogether which can drastically reduce the analytical costs and lead to an affordable technology.

NGS DNA meta-barcoding has already been described on meat species identification and showed reliable species identification not only on classical species but also on more exotic ones (Staats et al., 2016). Some of them have been spiked down to 1% (w/w) and were successfully detected. Application on fish species identification also showed satisfactory results. Similarly, meta-barcoding allowed reliable species identification, even on very close-related species like the various tuna species, or the wide Salmonidae family (Pacific salmon species, Atlantic salmon species, Trouts etc.). NGS has also been shown to reliably identify plant species in spices, herbs and botanical supplements which are well known to be vulnerable to species substitution (Staats et al., 2016). With more than 300,000 plant species, identification of closely related species can be less precise and can be limited to the genus or family level in some cases, and depending on the developed method.

With its ability to identify thousands of species, and its applicability to mixtures and processed samples, NGS is expected to be the reference species identification tool in the coming years. It can be applied for meat, fish, and plant species identification. In addition, application to insects has already been described (Shokralla et al., 2015), knowing that insect can be considered as a hygienic issue in the food supply chain, but also as a protein-rich food ingredient, certainly prone to some adulteration once more commercialized.

### Analytical Detection of Protein Enhancement

Protein content in foods is commonly measured by determining the amount of nitrogen (N) and converting it into protein using a specific nitrogen-to-protein conversion factor (Nitrogen Conversion Factor or NCF). Nitrogen is measured by digestion (Kjeldahl) or combustion (Dumas). Those two methods are widely accepted for protein quantification although the accuracy of the conversion factors is regularly questioned. While those methods are able to quantify nitrogen in an extensive variety of matrices, they can be deceived by addition of N-rich compounds and are unable to detect protein substitution (Moore et al., 2010).

### Adulteration by Nitrogen-Rich Compounds

Targeted analyses such as high-performance liquid chromatography - ultraviolet detection (HPLC-UV) or liquid chromatography - tandem mass spectrometry (LC-MS/MS) remain limited because of their targeted nature; they require a knowledge of the potential adulterants and are, by definition, blind to novel substances. In contrast, untargeted analytical methods such as near-infrared (NIR) and Principal Component Analysis (PCA) can be used to detect deviations from reference models (e.g. detection of adulterants in milk) (Balabin and Smirnov, 2011; Santos et al., 2013). This approach requires a sufficient level of homogeneity within the target food category to allow the creation of a pertinent model. Reference models have to be developed for each raw material at risk and need to be maintained on a continuous basis to monitor and correct for natural deviations.

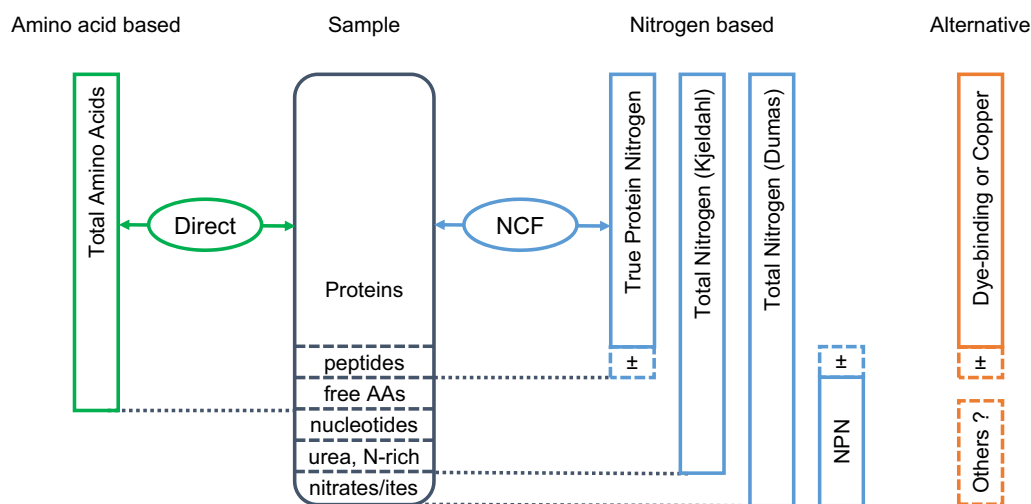
Another way to tackle adulteration by N-rich compounds is to assess protein content in a more specific manner (true protein content). In milk, proteins can be precipitated to measure (true) protein nitrogen, a value not impacted by adulterants such as melamine. Comparison of true and total protein nitrogen can reveal N-rich compounds addition (USP FCC, 2016). However, this approach is currently only validated in milk and cannot be applied to foods with a high variability of non-protein nitrogen content or to adulterants that precipitate under similar conditions.

In the long term, a payment scheme based on true protein content would remove the incentive for adulteration by N-rich compounds altogether. Amino acid profiling is the only method able to directly measure true protein content but is more complex than nitrogen-based methods; it requires two distinct sample hydrolyses and a comprehensive quantification of all amino acids that is prone to cumulative errors (which has however to be weighed against the vagueness of the NCFs used in nitrogen-based methods). Amino acid profiling methods are likely to further improve in the near future and could be used to establish reference values for a large variety of raw materials. Of note, amino acid profiling also measures free amino acids and small peptides, and the definition of true protein content will require an alignment between all the stakeholders to re-define globally accepted payment schemes.

Rapid copper-based (biuret, Lowry or BCA) or dye-binding (anionic dyes or Bradford) methods have also been assessed for their ability to measure true protein content. While those methods are not strictly specific for proteins and can be deceived by chemical substances, they could be calibrated against a reference value such as that obtained by amino acid profiling (Fig. 3).

### Adulteration by Protein Substitution or Addition

Apart from addition of N-rich compounds, protein adulteration can also be achieved by substitution with cheaper protein sources or dilution with bulking agent and addition of cheaper protein sources. There, most methods described above cannot be applied because the adulterants share many properties with the food matrices to which they are added. Amino acid profiling might be used to detect foreign protein addition provided the profile of the adulterant is radically different from that of the target food matrix, and provided the amino acid profile of the food product is well established. Infrared spectroscopy has been shown to be able to detect addition of soymilk into bovine milk (Jaiswal et al., 2015).



**Figure 3** Schematic measures of protein quantification. Sample nitrogen is distributed between various compartments (proteins, peptides, urea, nitrates, etc.). Different protein quantification methods based on nitrogen (blue) differ in their selectivity: combustion (Dumas) measures all nitrogen and Kjeldahl measures all organic nitrogen plus ammonia. To measure true protein nitrogen, proteins are precipitated before establishing their nitrogen content by Kjeldahl or Dumas. Non-protein nitrogen (NPN) can either be directly measured from the non-precipitated material or obtained by difference between total and true protein nitrogen. Peptides are found in both protein and non-protein nitrogen fractions (±) because the cutoff between peptides and proteins is blurry. To convert nitrogen into protein content, nitrogen-based methods rely on Nitrogen Conversion Factors (NCFs) whose accuracies are regularly questioned. In absence of separation, amino acid quantification (green) directly measures the sum of proteins, peptides and free amino acids (AAs) without the need for conversion factors. Alternative methods should be calibrated against a reference method and could be influenced by other compounds that are not related to nitrogen.



In principle, powder protein samples (concentrates, isolates ...) contain enough DNA to be detected by NGS (see previous section on DNA-based approaches for species identification). Therefore, NGS could in principle also be used as a screening tool to detect the presence of added protein. Of note, NGS cannot be used when the adulterant comes from a different part of the same organism (i.e. offals in meat), or when the adulterant is derived from the target food matrix (i.e. whey in milk ...). In those cases, specific methods and/or appropriate surrogate markers have to be developed for each particular vulnerability.

By-products such as protein hydrolysates (leather, feather ...) possess particular characteristics that differ from standard protein samples, notably with respect to polypeptide size distribution and number of free C- and N-termini. Those characteristics can be used to detect added hydrolyzed products either by methods that are sensitive to polypeptide length (i.e. non-protein nitrogen, Bradford) or that react with particular chemical groups (i.e. free amino groups). The nature, heterogeneity, and degree of hydrolysis of the adulterant will profoundly impact the efficacy of those methods for the detection of added hydrolysates.

When the source of adulteration cannot be identified by NGS, or when a direct identification of the foreign protein source is needed, peptides generated by proteolytic digestion of the samples can be analysed by LC-MS/MS to identify the corresponding proteins. Hydrolyzed samples can be analysed similarly without need for prior proteolysis.

## Analytical Evolution for Food Adulteration Detection

A number of technologies have already been applied successfully to verify the authenticity of raw materials with regard to their composition, processing or origin. They include molecular methods (DNA-based approach), rapid spectroscopic methods (e.g. near- and mid-infrared spectroscopy, Fourier transform Raman (FT-Raman) or nuclear magnetic resonance) or spectrometric methods (e.g. LC-MS/MS, MALDI-MS and others).

Application of analytical platforms other than genomic (DNA-based methodologies described above for species substitution) have been shown to provide good complementary solutions. Publications and applications related to food adulteration detection using spectroscopy is increasing very fast. Near- or mid-infrared spectroscopy and, more recently, Raman technology propose several advantages in terms of ease of use, speed (high sample throughput), and minimal sample preparation (Ewing and Kazarian, 2017). Applications of these spectroscopic technologies combined with chemometric analyses have been shown as powerful tools (Qu et al., 2015; Zhang et al., 2011; Cozzolino, 2012). Moreover, significant technical advancements in miniaturization have been achieved using new technologies for generating optical mechanical functions in the near- or mid-infrared spectroscopy but also with regard to high resolution spectrometers. These various degree of sophistication (from handheld to benchtop spectrometer) make them valuable for raw materials inspection by industries e.g. in the field or milk collection centers, at raw material reception areas or in specific laboratories requiring more sophisticated facilities. These techniques have a wide range of applications from solid, semi-solid and liquid samples and thus have been applied to many food matrices for food adulteration detection. They are highly efficient to discriminate species origin, geographical origin in parallel to qualitative and quantitative analysis of specific ingredients and physical properties (e.g. proximates and freezing point in raw milk, phenols, alpha-farnesene in apples) (Huck et al., 2016; Wang et al., 2017; Wang and Yu, 2015). Moreover, NIR and MIR spectroscopic technologies are widely used in the dairy industry to analyse proximates such as protein, fat, at the same time as performing a rapid untargeted screening of potential adulterants (Coitinho et al., 2017; Botelho et al., 2015; Poonia et al., 2017). Miniaturization is still ongoing with the apparition of handheld infrared spectroscopic devices which are promising (e.g. for fish, milk, meat) (Grassi et al., 2018; Nieuwoudt et al., 2016; Schmutzler et al., 2015). When combined with immunoassay and sensors these new technologies will become more and more critical for rapid adulteration detection.

Food adulteration detection also often requires more specialised laboratory analyses (such as spectrometric technologies and more sophisticated spectroscopy technologies such as nuclear magnetic resonance (NMR). The SNIF-NMR technique (site specific natural isotope fractionation-NMR) along with chemometric data treatment has been widely applied for classification of wine origin and identification of substitution with cheaper ones (Charlton et al., 2010). This technique has been successfully extended to fruit juices and honey adulteration with sugar (e.g. beet sucrose or modified sugars). Specific companies have further developed screening applications for over 3000 fruit juices. Detection of foreign sugar addition in fruits juices and honey has been complemented by the analysis of stable isotope in food (isotope ratio mass spectrometry, IRMS). This technology is also widely applied to determine the geographical origin of many raw materials and the identification of synthetic molecules in ethanol, vinegar or flavorings as well as for the differentiation of organic versus conventional farming methods (Rossier et al., 2016; Manning and Soon, 2014).

As described by Ellis et al. (2016), recent metabolomics studies appear to encompass a diverse range of applications for adulteration detection. For example, nuclear magnetic resonance (NMR) metabolite fingerprinting has been used for the determination of the country of origin of coffee, detection of saffron dilution from cheaper plants, and fully automated analysis of wine authenticity (Spraul et al., 2015). Others have used metabolomics (gas chromatography mass spectrometry (GC-MS) based multimarker profiling) to elucidate discriminant markers for the authentication of expensive raw material (e.g. the expensive coffee Kopi Luwak). In some cases, a combination of methodologies using metabolic approaches is necessary. It is the case for example for the detection and quantification of oregano adulteration with foreign leaves where FT-IR and LC-MS-based metabolic profiling has been used (Black et al., 2016a,b).

For speed and simplicity, efforts have recently been made towards the development of ambient mass spectrometry (AMS) technologies. Their new ionization technique allows samples to be analysed directly with no (or limited) sample preparation with

a high resulting sample throughput (Black et al., 2016a,b). The rapid growth with over 30 different techniques available have shown interesting applications for food fraud detection. Interestingly, a number of them (e.g. direct analysis in real time (DART), easy ambient sonic spray ionization (EASI), liquid extraction surface analysis (LESA), rapid evaporative ionization (REIMS)) have demonstrated not only qualitative but also quantitative applications. Detection of hazelnut oil in extra virgin oil down to 6% and adulteration of soft cheese with vegetable oils at 1% (w/w) were shown using DART technology (Vaclavik et al., 2009; Hrbek et al., 2014). Surface extraction and direct ambient MS analysis of cooked meat species showed detection of 5% (w/w) chicken in beef samples using LESA (Montowska et al., 2014). The use of REIMS for rapid characterization of meat products showed possible detection of meat mixtures at a limit of 5% (w/w) (Balog et al., 2016). These new technologies are producing fast and accurate results comparable to those obtained from conventional techniques such as LC-MS or NMR. Identification of white fish species using REIMS showed nearly 99% correct classification with species identification obtained within a few seconds unlike any other form of food fraud analysis (Black et al., 2017).

## Conclusions

Food adulteration deterrence is complex because of its unpredictable nature. Significant efforts have been made recently in many aspects by food companies, authorities and academics. Beside finding new analytical approaches, food manufacturers are also improving the knowledge of their supply chain in term of length (number of tiers), complexity and vulnerability. Food chain transparency and full raw material traceability is primordial for an effective food fraud prevention system. A consortium regrouping different organizations has been created to work on new platforms designed to be compatible between all partners, such as food manufacturers, raw material suppliers, and even authorities. Ideally, the system should provide supply chain transparency in real-time, with end-to-end product traceability. New emerging technologies such as the blockchain should further help to reach this goal, increasing accountability and thus providing to suppliers, regulators and consumers higher trust on food integrity. Recent alliances have been formed with a computer organization to create a standard-based method of collecting data about the origin, safety and authenticity of food. Initial application showed that the blockchain technology could reduce the time to trace a package of specific raw materials (e.g. fruits) from the farm to the store from days to few seconds.

Mapping supply chain vulnerabilities is leading to the identification of the most vulnerable chains where confidence to suppliers may be lower (e.g. raw materials are purchased through traders that may have multiple vendors and procure through auctions). Enhanced attention (e.g. regular vendor audits, addition of new parameters to prevent food adulteration in supplier purchasing specifications) are being performed on the most vulnerable raw materials in order to increase confidence to suppliers, in addition to regular monitoring. Analytical technologies and methodologies are evolving to be better adapted to food adulteration detection. Untargeted analytical methodologies, which are often required for rapid detection of raw material abnormalities, are easier to implement today and can be used to detect unknown adulterants. Instrument suppliers are more and more integrating specific software in their hardware to help building and running chemometric models. DNA-based approaches are also much more performant such as NGS with the possibilities to detect not only single species but mixtures via sequencing and associated databases. Miniaturization of instruments is an additional significant technical advancement for direct verification of raw materials early in the chain at low cost. More sophisticated methods are also evolving rapidly to get simpler with the development of ambient technologies requiring minimum sample preparation. This results in diverse applications in food fraud detection with a decrease of cost related to raw material surveillance. As shown in this document, food manufacturers are implementing many preventive actions all along the supply chain to increase significantly the food adulteration deterrence.

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# Food Chemistry and Analysis for the Purpose of Kosher and Halal

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## Introduction

The objective of this chapter is to introduce the kosher and halal laws and as this is done, the author will try to indicate where food chemistry, particularly food analysis, might be challenged to provide analyses that will aid in insuring the integrity of the process. In some cases these can be quite challenging. It should be noted that on-site inspection by religious personnel remains the main way to insure the integrity of the system and laboratory testing is a way to back-up the inspectors as most food manufacturing facilities do not have continuous inspection.

## The Kosher and Halal Laws

Before looking into the laws, it may be helpful to understand the religious significance of these dietary laws for Jews and Muslims. The kosher (kashrus) dietary laws determine which foods are “fit or proper” for consumption by Jewish consumers who observe these laws. The halal dietary laws determine which foods are “lawful” or permitted for Muslims. For both religions, the food laws are a small part of a comprehensive system of religious laws cover many aspects of daily life. How an individual Jew or Muslim will live their life is a personal matter often influenced by the standards of the local community. Although these variations may at times be frustrating making products for these markets. But it is also a great strength internally to be able to respect within group diversity.

Why do Jews and Muslims follow dietary laws? The explanation by Rabbi I. Grunfeld provides an explanation that really speaks on behalf of both religions although it is clearly of Jewish origin ([Grunfeld, 1972](#)).

And ye shall be men of a holy calling unto Me, and ye shall not eat any meat that is torn in the field

Exodus XXII:30.

Holiness or self-sanctification is a moral term; it is identical with ... moral freedom or moral autonomy. Its aim is the complete self-mastery of man.

To the superficial observer it seems that men who do not obey the law are freer than law-abiding men, because they can follow their own inclinations. In reality, however, such men are subject to the most cruel bondage; they are slaves of their own instincts, impulses and desires. The first step towards emancipation from the tyranny of animal inclinations in man is, therefore, a voluntary submission to the moral law. The constraint of law is the beginning of human freedom ....

The three strongest natural instincts in man are the impulses of food, sex, and acquisition. Judaism does not aim at the destruction of these impulses, but at their control and indeed their sanctification. It is the law which spiritualizes these instincts and transfigures them into legitimate joys of life.

## The Kosher and Halal Market

Both kosher and halal are important specialty markets. Briefly, the deliberate consumers of kosher food, i.e., those people who specifically look for the kosher mark, are estimated to be over 12 million Americans (of which 2/3 are not Jewish) and they are purchasing almost 15 billion dollars of kosher products ([Lubicom Marketing Consulting, 2012](#)). Others include consumers who may find kosher products helpful in meeting their own dietary needs such as Muslims, Seventh Day Adventists, vegetarians, vegans, people with various types of allergies – particularly to dairy, grains, and legumes – and general consumers who for one reason or another prefer kosher products. By undertaking kosher certification, companies incrementally expand their market, i.e., increased incremental sales.

On a global scale, the halal market is a significant component of trade in foods. The number of Muslims is estimated to be between 1.3 and 1.8 billion people (i.e., 20% to 25% of the world's population!) and inter-country trade in halal products continues to grow. In many countries halal certifications acceptable to the national government have become necessary for products to be allowed to be imported.

### The Kosher Dietary Laws With Reference to Halal Dietary Laws

The laws predominantly deal with three issues, all focused on the animal kingdom with a fourth set of laws dealing with the holiday of Passover that are added to the regular laws but focus more on the plant kingdom.

There are also many additional laws dealing with special issues such as grape juice, wine, and alcohol derived from grape products; Jewish supervision of milk; Jewish cooking, cheese making, and baking; equipment kosherization; purchasing new equipment from non-Jews; and old and new flour. Those with potentially challenging analytical needs will be discussed.

#### Allowed Animals

Ruminants with split hoofs that chew their cud (ruminants), the traditional domestic birds (e.g., chicken, turkey, duck and geese), and fish with fins and removable scales (i.e., visible and they do not tear the skin; cycloid and ctenoid, all fish with scales have fins) are generally permitted.

Pigs (which have often become the symbol of both kosher and halal observance) and camels (a ruminant without a split hoof) are not permitted although the latter is halal.

Among commercially available birds, the ostrich family is prohibited for kosher but is accepted as halal. Animals that spend their entire life in the water are permitted by some in the halal community although there is a great deal of divergence with respect to the acceptability of marine life.

Most insects are not kosher. A few types of grasshoppers/locust are kosher and halal. Modern IPM (integrated pest management) programs that increase the level of insect infestation in fruits and vegetables can cause problems for the kosher consumer, who must inspect produce suspected of having insects and then remove any insects found. The red pigments carmine and cochineal are not permitted by most rabbinical supervisors, although a few do permit it because it comes from the inedible shell. The Muslim community also debates this topic although many of the Muslim schools of thought prohibit it. However, bee's honey and shellac (lac resin), an insect exudate are permitted by most kosher and halal authorities.

#### Analytical Challenges

Can one detect the presence of any material from a non-kosher animal? If the adulterant still contains DNA, the testing protocols are being developed. However, this is more difficult for certain animal-based derivatives without DNA such as fats and oils and its derivatives (e.g., mono- and di-glycerides). Can one use the fatty acid profile to determine relatively small amounts of contamination? Another important animal ingredient, gelatin (and collagen) can be obtained from a number of different fish and animals. As the use of insects for food increases, new analytical challenges will need to be addressed. The challenge in many cases is not only to determine their presence when one suspects a particular adulterant might be present, but to do so without knowing which adulterant one might be looking for, which is often the greatest challenge for analytical chemists. And some of these issues are also of concern to the broader community because of the issue of mislabeling.

#### Prohibition of Blood

Ruminants and fowl must be slaughtered according to Jewish law by a specially trained religious slaughterman (shochet) using a special knife designed for the purpose (chalef). The knife must be razor sharp and totally free of nicks. It must have a very straight blade that is at least twice the diameter of the neck of the animal to be slaughtered. This knife is checked after each slaughter. The cut is done horizontally across the neck in a specifically designated region. Both carotid arteries and both jugular veins along with the windpipe and esophagus are cut. When done right on calm animals, this leads to strong bleeding which leads to rapid unconsciousness of the animal in a calm fashion. The Muslim community uses a similar slaughter along with a blessing. They highlight not sharpening the knife in front of the animal, not allowing another animal see the slaughter, and having the animal facing Mecca. Some Muslim scholars permit pre-slaughter stunning of an animal and some rabbis permit post-slaughter stunning.

#### Analytical Challenges

The importance of blood removal is central to the process for both Muslims and Jews. The use of blood derived ingredients is prohibited. Can one determine if such compounds were used as opposed to the natural post-bleed-out blood found in the animal? Can one tell after the fact if an intervention was used in addition to the traditional slaughter?

Slaughtered animals for kosher are subsequently inspected for visible internal organ defects by rabbinically-trained inspectors, with a special emphasis on the lungs. Meat and poultry must be further prepared by properly removing certain veins, arteries, prohibited fats, blood, and the sciatic nerve. To further remove the prohibited blood, red meat and poultry must then be soaked, salted and rinsed within 72 h of slaughter. Livers and hearts will be handled separately. If this is not possible, then according to some Rabbis meat may be specially washed (begissing, a real wash and not just a light spray) and this wash procedure may be repeated for up to two more times, each time within 72 h of the previous washing. This is done to make sure the blood and meat surfaces remain moist.

The soaking is done for half an hour in cool water (not cold), thereafter, the salting is done for 1 h with all surfaces, including cut surfaces and the inside the cavity of a chicken or turkey. After the hour of salting, the meat is then rinsed three times. The salted meat must be able to drain throughout and all the blood being removed must flow away freely. The special crystal size used for this process is called kosher salt (crystals large enough to not dissolve in an hour, but small enough to adhere and totally cover surfaces).



### Analytical Challenges

Some fraudulent operators have soaked the meat in brine to obtain more easily “salt” the meat. Can one use changes in the muscle or the blood of the meat to determine whether proper salting with salt crystals has been done?

Some concern has been raised about the salt level in kosher meat. Note that only the surfaces are salted, generally using primal cuts (i.e., 20–40 pound pieces of meat), and that the penetration of the salt is less than a half centimeter in red meat (NY Department of Agriculture and Markets, personal communication). Many pieces of meat, as consumed, have therefore not been directly subjected to the salt treatment. If salt content in a diet is a very important consideration, then one should cut off all surfaces and not use any of the drippings that come out during cooking as much of the salt that goes into the meat at the surface is cooked out with the drippings.

Any ingredients or materials that might be derived from animal sources are generally available in a kosher and/or halal form derived from plant oils. A possible exception might be normative mainstream gelatin, which is now being produced from kosher beef hides or fish. Also some kosher rennet, the cheese-coagulating enzyme, is obtained from the dried fourth stomach of a kosher-slaughtered milk-fed calf although most commercial cheese today is made using microbial enzymes that come either from genetically modified bacteria or other microbes whose coagulating enzyme(s) can be successfully used. Microorganisms must be produced in kosher or halal systems (i.e., all ingredients and the equipment are kosher or halal).

### Prohibition of Mixing of Milk and Meat

Thou shalt not seeth the kid in its mother's milk.

Exodus XXIII:19, Exodus XXXIV:26, Deuteronomy XIV:21

This passage appears three times in the Torah (the first five books of the Hebrew Scripture) and is therefore considered a very serious admonition. As a result, the law cannot be violated even for non-food uses of such mixtures such as pet food. Neither can one derive benefit from such a mixture. The meat side of the equation has been rabbinically extended to include poultry (but not fish) as both meat and poultry need to be inspected, deveined, salted, and soaked. The dairy side includes all milk derivatives.

Keeping meat and milk separate in accordance with kosher law requires that the processing and handling of all materials and products fall into one of three categories:

1. A meat product
2. A dairy product
3. A neutral product called “pareve”.

### Analytical Challenges

How does one detect a dairy product in small amounts, recognizing that modern dairy products include materials like caseinates, whey, lactose, dairy protein hydrolysates, and derivatives of whey including whey alcohol and citric acid (which if derived from whey would according to most authorities remain dairy).

The pareve category includes all non-meat or non-dairy products and includes all plant products along with eggs, fish, honey, and lac resin (shellac). To assure the complete separation of milk and meat, all equipment, utensils, pipes, steam, etc. must be kept in the properly designated category.

Pareve ingredient or product must be produced on equipment that has undergone equipment kosherization (see Section on **Equipment Kosherization**).

### Other Kosher Issues

#### Grape Products

To be kosher, all grape juice-based products can only be handled by observant Jews from grape-pressing to final processing. If the juice is heated to at least 140 °F (or 60 °C), then it can be handled by any worker, as an ordinary kosher ingredient. Some rabbis require a higher temperature.

### Analytical Challenge

Can one after the fact determine the approximate temperature of heating?

#### Jewish Cheese (*gevinas yisrael*)

Most normative mainstream kosher supervision organizations require that a Sabbath-observing Jew add the coagulating agent, i.e., the agent such as chymosin that causes curd formation. Any ripened cheese that does not meet this requirement is unacceptable. This rule does not apply to fresh cheeses.

Kosher whey can be created more easily than kosher cheese. If all the ingredients and equipment used during cheese making are kosher, the whey will be kosher as long as the curds and whey have not been heated together above 120 °F (49 °C) before the whey is drained off. This is true even if a rabbi has not added the coagulant.

**Analytical Challenge**

Can one determine the maximum temperature received by the whey?

**Cholev Yisroel**

Some kosher-observant Jews are concerned about possible adulteration of milk with the milk of non-kosher animals, such as mare's milk or camel's milk, and therefore require that the milk be watched from the time of milking (i.e., on the farm). "Cholev yisroel" milk is required by some of the stricter kosher supervision agencies for all dairy ingredients. Rabbis who accept non-"cholev yisroel" milk in the United States do so for two reasons. First, they believe that the laws in the United States and many other developed countries are strong enough to assure that adulteration does not occur. Second, the non-kosher milks are worth more money than kosher milks, so there is no incentive to add the non-kosher milk.

**Analytical Challenge**

Can one detect the presence of a non-kosher milk or milk ingredients?

**Passover**

The Passover holiday occurs in spring and requires observant Jews to avoid eating the usual products made from the five Biblically prohibited grains: wheat, rye, oats, barley, and spelt (Hebrew: chometz). One can only eat the special unleavened holiday bread from wheat (Hebrew: matzos). Some matzos are made to a stricter standard with rabbinical inspection beginning in the field. For others supervision does not start until flour milling. Matzo made from oats and spelt are now available for consumers with wheat allergies.

In the middle ages, the rabbis of Europe also made products derived from corn, rice, legumes, mustard seed, buckwheat, and some other plants (Hebrew: kitnyos) prohibited for Passover that they believed could be made into flour and might be mistaken for chometz. In addition to the actual "flours" of these materials, many contemporary rabbis also prohibit derivatives such as corn syrup, corn starch, and corn starch derivatives such as citric acid. A small number of rabbis permit the oil from kitnyos materials, or liquid kitnyos products and their derivatives such as corn syrup. The major source of sweeteners and starches used for production of "sweet" Passover items are either real sugar or potato or cassava (tapioca) starches.

**Analytical Challenge**

Can one determine if any prohibited materials have been used?

**Kosher: Other Processing Issues****Equipment Kosherization**

There are three ways to make equipment kosher or to change its status back to pareve. Rabbis generally frown on regularly going from meat to dairy or vice-versa. Most conversions are from dairy to pareve or from treife to one of the categories of kosher. There is a range of processing procedures to be considered, depending on the equipment's prior production history.

After a plant, or a processing line, has been used to produce kosher pareve products, it can be switched to either kosher dairy or kosher meat without a special equipment kosherization step. It can also subsequently be used for halal production (from pareve or dairy lines, not always from meat lines), and then, finally, for non-kosher products. In many cases, a mashgiach (the rabbinically approved kosher supervisor), is needed on site for equipment kosherization, so it normally is beneficial to minimize the number of changeovers from one status to another. Muslims also have a procedure for changing equipment that generally involves a supervised washing.

The simplest equipment kosherization occurs with equipment that has only been used for cold products. This requires a good liquid caustic/soap cleaning – i.e., the type of cleaning done normally in most food plants. Some plants do not normally do a wet clean up between runs (e.g., a dry powder packing plant or a chocolate line), and these would need to seek specific rabbinical guidance for the change-over if permitted. Materials such as ceramics, rubber, earthenware, and porcelain cannot be koshered.

Most food processing equipment is operated at cooking temperatures, generally above 120 °F (49 °C), the temperature that is usually rabbinically defined as "cooking." However, the exact temperature for "cooking" depends on the individual rabbi and may also be product specific (range 110–140 °F (~43–60 °C)) with 120 °F (49 °C) being agreed upon in the US for dairy products. Such equipment must be thoroughly cleaned with liquid caustic/soap. The equipment must then be left idle for 24 h, after which it is "flooded" with boiling water in the presence of a kosher supervisor. The details depend on the equipment being kosherized. In some cases, particularly foodservice establishments, a "pogem" (bittering agent, oftentimes ammonia) is used in the boiling water in lieu of the 24 h wait. The absolutely clean equipment (e.g., silverware) is put into the pogem containing boiling water followed by a second boiling with clean water.

In the case of ovens or other equipment that uses "fire," or dry heat, kosherization involves heating the metal until it glows. Again, the supervising rabbi is generally present while this process is taking place. Sometimes the same oven can be used sequentially for pareve and dairy baking. The details are beyond the scope of this chapter and require a sophisticated rabbinical analysis. If kosherization of an oven is needed, this often requires the use of a blow torch to heat surfaces to "red hot."

## Kosher and Allergies

Many consumers use the kosher markings as a guideline to determine whether food products might meet their special needs including allergies. There are, however, limitations that the particularly sensitive allergic consumer needs to keep in mind. The kosherization procedure may not yield 100% removal of previous materials run on the equipment.

In a few instances where pareve products contain small amounts of fish (such as anchovies in Worcestershire sauce), this ingredient may (but not always) be marked as part of the kosher supervision symbol as fish cannot be used directly with meat. Many certifications do not specifically mark this if the fish in the initial material is less than 1/60 (a special amount used by the rabbis to determine if a process of nullification can be used after the fact). Someone who is allergic should always carefully read the ingredient label including the allergy warning. Kosher should be used only for a first screening of products.

## Additional Information About the Halal Dietary Laws

### Halal Dietary Laws

The halal dietary laws define food products as halal (permitted), haram (prohibited) and put a few items go into the category of makrooh (questionable to detestable), which is often the most controversial. The law deals with the following five issues; all but the last are in the animal kingdom.

### Prohibited and Permitted Animals

As previously mentioned, pork is prohibited, as are the carnivorous animals and birds of prey. Again the ruminants, including the camel is permitted, but also the rabbit. However, most Muslims consider horsemeat to be makrooh. Birds are specifically those that do not use their claws to hold down food.

The animals fed unclean or filthy feed (e.g., formulated with biosolids (sewage) or protein from tankage), must be quarantined and placed on clean feed for a period varying from three to forty days before slaughter to cleanse their systems.

### Analytical Challenge

Besides previous questions, can one determine what an animal ate just before slaughter? With the internal organs available and more challenging without them.

Food from the sea, namely, fish and seafood, are the most controversial among the various denominations of Muslims. Certain groups, particularly Shiia, only accept the kosher fish, while others consider as halal everything that lives in the water all the time. But others may consider some of these makrooh and avoid them. Animals that live both in water and on land (amphibians) are not consumed by the majority of observant Muslims.

The status of insects is uncertain except that locust is specifically mentioned as halal. However, from legal discussions, it seems that both helpful insects like bees, ants, and spiders, and harmful or dirty creatures like lice, flies, and mosquitoes are all haram.

Eggs and milk from permitted animals are also permitted for Muslim consumption. Unlike kosher, there is no restriction on mixing meat and milk.

### Prohibition of Blood

According to the Quranic verses, blood that pours forth is prohibited for consumption. There is general agreement among Muslim scholars that anything made from blood is unacceptable.

### Analytical Challenge

Can one determine the presence of blood or blood derivatives such as fibrin/fibrinogen in foods?

### Proper Slaughtering of Permitted Animals

In addition to those discussed earlier, there is the rule that only after the blood is allowed to drain completely from the animal and the animal has become lifeless can the dismemberment begin.

### Prohibition of Alcohol and Intoxicants

Consumption of alcoholic drinks and other intoxicants is prohibited.

Although there is no allowance for added alcohol in any beverage like soft drinks, small amounts of alcohol contributed naturally from food ingredients may be considered an impurity and hence ignored. Synthetic or grain alcohol may be used in food processing for extraction, precipitation, dissolving and other reasons, as long as the amount of alcohol remaining in the final product is very small, generally below 0.1%. Each importing country may have its own guidelines.

### Analytical Challenge

Can the source of the alcohol be determined, i.e., beverage alcohol or industrial alcohol? Also for kosher can the source of the alcohol (or vinegar) be determined in terms of both grape based alcohol and grain/kitnyos alcohol?

## Both Kosher and Halal

### Biotechnology

Rabbis and Islamic scholars currently accept products made by simple genetic engineering; e.g., chymosin (rennin) was accepted by the rabbis about a half a year before it was accepted by the US FDA. The basis for this decision involves the idea that the gene isolated from a non-kosher source was not “visible.” Subsequently, it is copied many times *in vitro* and then eventually injected into a host where it is then reproduced many times. Thus, the original source of the “gene” is essentially totally lost by the time the food product appears. The production conditions in the fermenters must still be kosher or halal, i.e., the ingredients and the fermenter, and any subsequent processing must use kosher or halal equipment and ingredients. A product produced in a dairy medium would be kosher dairy. Mainstream rabbis may approve porcine lipase made through biotechnology when it becomes available, if all the other conditions are kosher. Islamic scholars are still considering the issue of products with a porcine gene; although a final ruling has not been established, the leaning seems to be towards rejecting such materials.

If the gene for a porcine product were synthesized (i.e., it did not come directly from the pig), Islamic scholars still need to determine if they will accept it. Again the leaning seems to be negative. Because the religious leaders of both communities have not yet determined the status of more complex genetic manipulations; such a discussion is premature.

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### Additional Resources

- Kashrus Magazine - The Online Source for Kosher Information. <http://www.kashrusmagazine.com>.
- Kashrut.com - The Premier Kosher Information Source on the Internet. <http://www.kashrut.com>.
- Food Marketing Institute. <http://www.fmi.org>.
- American Meat Institute. <http://www.meatami.org>.

# Modern Concepts in Chemical Risk Assessment

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## Glossary

**ALARA** As low as reasonably achievable  
**BMD** Benchmark Dose  
**BMDL** Benchmark Dose; Lower confidence interval  
**BMDL<sub>10</sub>** Benchmark Dose at 10% effect; Lower confidence interval  
**EFSA** European Food Safety Authority  
**MoA** Mode of Action  
**MOE** Margin of Exposure  
**NOAEL** No Observed Adverse Effect Level  
**POD** Point of Departure  
**TFI** Tolerable Daily Intake

## Introduction

The presence of contaminants in food is non-intended in most cases, being due to certain events or steps during the process of production of the food product including all raw materials or ingredients. They can be detected in the final food product intended for consumer use (Schrenk and Cartus, 2017). Thus, contaminants differ from additives, ingredients etc. which were added on purpose, e.g. for technological or other reasons. Furthermore, compounds that did not enter the food process from outside but were generated during the process (e.g. thermal treatment) such as acrylamide or furan are also considered as contaminants, sometimes also specified as ‘process contaminants’. As an example, Table 1 shows a list of contaminants assessed by the CONTAM-Panel of the European Food Safety Authority (EFSA) since January 2014.

Furthermore, it is evident that sometimes contaminants may also originate from illegal or criminal addition of chemicals during the food production process.

In contrast to contaminants, residues are ‘left-overs’ from the intended use of plant protection chemicals (in food of plant origin) or veterinary drugs (in food of animal origin) in agricultural production processes. In cases where such chemicals were factually not used but nevertheless found in the product, contamination e.g. via drift of pesticides from crop fields in the vicinity etc. may have occurred, however, i.e., such findings could also be classified as contaminants.

Food of animal origin, coffee or cereal products are examples of foods that were under discussion in recent years because of the detection of chemical contaminants.

**Table 1** Selected Scientific Opinions published by the European Food Safety Authority (EFSA), CONTAM Panel, 2014–2017 ([www.efsa.europa.eu](http://www.efsa.europa.eu))

Contaminant(s)	References (EFSA Journal)
Chromium in food and drinking water	12(3), 3595
Beauvericin and enniatins in food and feed	12(8), 3802
Perchlorate in food, in particular fruits and vegetables	12(10), 3869
Chloramphenicol in food and feed	12(11), 3907
Modified forms of certain mycotoxins in food and feed	12(12), 3916
Nickel in food and drinking water	13(2), 4002
Nickel in feed	13(4), 4074
Acrylamide in food	13(6), 4104
Clorate in food	13(6), 4135
Nitrofurans	13(6), 4140
Tetrahydrocannabinol (THC) in milk and other food of animal origin	13(6), 4141
Erucic acid in food and feed	14 (11), 4953
Furan and methylfurans in food	15 (10), 5005

## Data Selection and Use in Toxicological Risk Assessment

The toxicological risk assessment of chemical compounds starts by describing the structural and physico-chemical features of the compound as detailed as possible. Furthermore, the pathways of generation or synthesis and methods of quantitative analysis of the compound should be known and be discussed. Another cornerstone in risk assessment is the analysis of levels (contents) in various categories and types of food. At this stage, it is often required to take into account other sources or levels, e.g. in the environment, at the workplace etc.

On the basis of occurrence data of this type, the assessment of human exposure follows. It considers databases for regional, national or international consumption of certain food categories and types. Since marked variations in consumption occur, e.g. between nations, probabilistic methods are applied which make use of the distribution of consumption data e.g. for fish, cereals, meat and meat products etc. Multiplying these data with levels of the contaminant in the respective food category for average or high (95th percentile) consumers of this category yields estimates for exposure. The data then can be refined for various cohorts, age groups etc. taking into account average body weights in these groups.

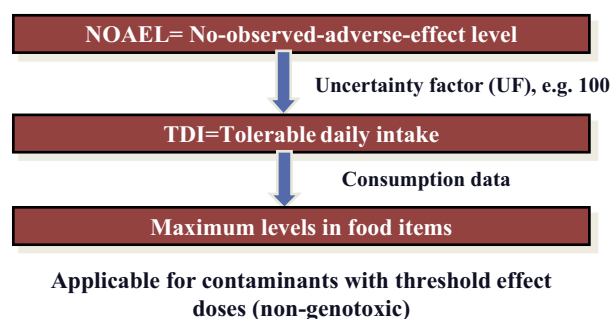
In the next step, facts on the fate and effects of the compound of interest in relevant biological systems are gathered. Such relevant systems comprise animal (mammalian) models such as experimental animals, isolated organs, cells, subcellular fractions or purified enzymes. Usually, human studies are not available for ethical reasons with the exception of certain test data on mild skin irritation or similar. Nevertheless, studies in human cells, subcellular organelles (e.g. mitochondria), enzymes, receptors etc. are possible. It has to be noted, however, that data from human permanent cells, which are quite popular among scientists, are of limited value in quantitative risk assessment. In vivo and in vitro studies try to provide reliable estimates on absorption e.g. in the gastrointestinal tract, metabolism, distribution and elimination from the body. Since the extrapolation of such in vitro data to the in vivo situation has become more and more reliable in the last years, a reduction of animal testing in toxicological risk assessment appears feasible in the future.

The next step in risk assessment is the selection and analysis of data about the toxic (adverse) effects of the compound. Also in this case most data are usually derived from experimental animals, since they reflect the complexity of an intact mammalian organism. These comprise particularly data from acute, subacute or chronic feeding or gavage studies, i.e., the test compound was mixed in to the feed or given intra-gastrically. A special requirement for quantitative risk analysis is the use of different dose levels, some of them showing pronounced effects, others no effects. The latter are used for the derivation of a non-toxic dose level (NOAEL = No-observed-adverse-effect level) as shown in Fig. 1. In this scenario, a sufficient number of animals and a thorough documentation of effects including a critical review of any findings also in untreated (negative control) animals are needed. Finally, the critical toxic effect, the so-called 'toxic endpoint', should be a clearly defined easy to measure effect with obvious adversity. The effect should become worse with increasing dose levels. It should be biologically plausible and statistically relevant (different from negative controls).

Ideally, mechanistic findings are available which allow an understanding of the compound's Mode of Action (MoA). Under these conditions, the confidence in the risk assessment process is strongly improved, in particular if similar MoAs were reported for structurally related compounds.

An appropriate understanding of the MoA is crucial in several respects. First, the extrapolation of findings from animal experiments to humans can be evaluated much more effectively, if the MoA is known. In many cases an identical MoA is considered to be operative or at least feasible in humans. While the physiology of rodents can differ considerably from that in primates, certain MoAs are known not to occur in humans thus making the respective findings in an animal (e.g. rodent) species irrelevant for human risk assessment.

Frequently, chemicals can affect the body's functions attacking more than one target. Thus they elicit a spectrum of different symptoms that not necessarily originate from the same MoA. Cadmium is a good example since it can affect both kidney function and bone growth both effects not sharing the same MoA. In such cases, the more sensitive endpoint is usually chosen since protective risk management measures protecting the most sensitive target will also protect other targets. The situation becomes more complicated if carcinogenicity (tumor formation) is one of the critical endpoints. Here, the characterization of the MoA as genotoxic vs. non-genotoxic plays a decisive role.



**Figure 1** Derivation of a Tolerable Daily Intake (TDI) and of maximum levels in food from a No-observed-adverse-effect level (NOAEL).



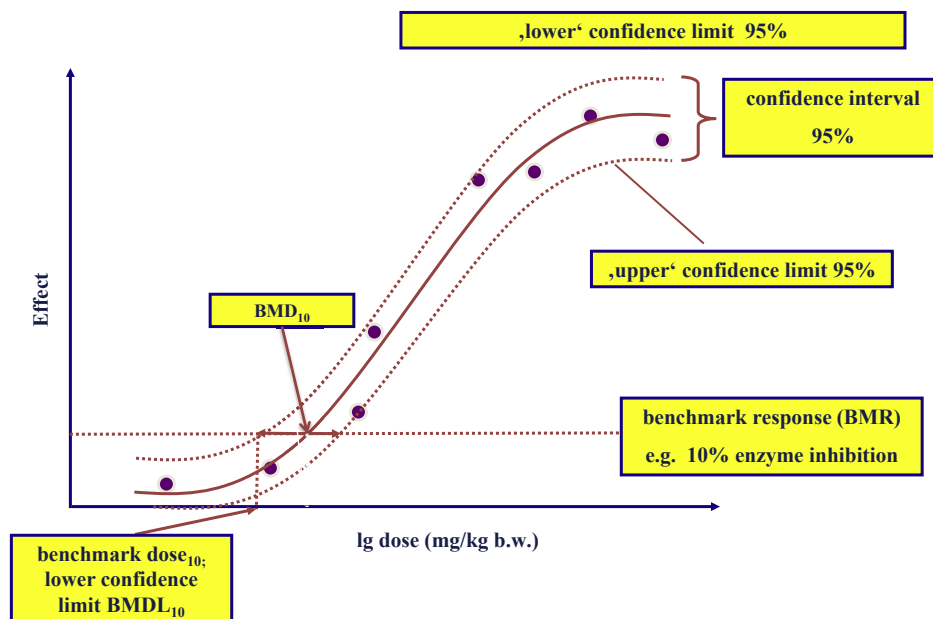
## Characterization of the MoA as Genotoxic/Carcinogenic Using In Vivo and In Vitro Methods

Investigations using in vitro models can be of great value in order to characterize the MoA. They often have been initiated by findings from in vivo experiments and are aimed at elucidating the molecular MoA in more detail. A widely used method is the use of primary cells, i.e., cells freshly isolated from animal or human tissue. It is a major advantage of these cells that they reflect the response of normal cells in the body in a highly authentic manner. Unfortunately, after prolonged culture the cells lose many of their specific properties, making their further use less valuable. For these reasons, the isolation of such cells usually requires the sacrifice of animals or the availability of specimens from humans. Permanent cell lines sometimes are a good alternative, although they differ substantially in many aspects from their original counterparts in the body. In most instances, permanent cell lines were originally isolated from tumors making them even less indicative for effects in normal cells. In general the value of such in vitro models for the prediction of a broad pattern of adverse effects in animals and humans is overestimated. They can be important sources of information, however, if clearly defined mechanistic issues shall be addressed. For this purpose it has to be established that the model responds in a way similar to normal cells in the intact organism. Examples are the activation or blockage of receptors, the triggering of apoptosis, cell division etc.

A particularly successful application of in vitro cell culture models is the issue of genotoxicity of chemicals. Here, a variety of in vitro tests in bacteria (Ames test) or mammalian cells is used. A positive outcome indicates a (direct or indirect) effect of the test compound or its metabolite(s) on the DNA, e.g. via direct chemical interaction or by effects on DNA processing factors in the cell. As a result a permanent alteration in the base sequence and/or the chromatin structure may occur even leading to a complete loss of parts of the genome. Such effects could be correlated with an increase in tumor incidence (carcinogenicity) although such in vitro findings do not allow an absolutely reliable prognosis on the carcinogenic potential of the compound in vivo. In a typical case, the compound would then be tested in a chronic animal study. If it turns out to be carcinogenic, the evaluation of in vitro data can be very helpful in understanding the MoA. In a few cases, however, the in vitro models are inadequate to predict in vivo genotoxicity. The opposite case is more frequent, i.e., an in vitro positive finding of genotoxicity cannot be confirmed by animal testing.

## Risk Characterization and Recommendations for a Health-Based Guidance Value

If the data on toxicity are considered sufficient, a selection is made among the various non-neoplastic endpoints including neuro-, immuno- and reproductive toxicity. The data are then used for the derivation of quantitative dose-response relationships. These are usually established by applying mathematical models (best fit models). The modeled function usually has statistical confidence bands reflecting the variability of the data/the estimate. Since the critical effect height has been defined prior to the experiment (e.g. 10% inhibition of thyroid hormone production), the dose causing this effect can be derived. The lower limit of its 95%-confidence interval is termed BMDL<sub>10</sub> (Benchmark Dose 10%, lower confidence limit; Fig. 2). Alternatively, a NOAEL can be derived (see above). Both BMDL and NOAEL are called 'Point of Departure' (POD), i.e., a starting point which may allow the calculation of



**Figure 2** Benchmark-dose-modeling and derivation of a BMD<sub>10</sub>- and a BMDL<sub>10</sub>-value.

a health-based guidance value by applying a safety (or uncertainty) factor (usually 100). A typically guidance value is a 'Tolerable Daily Intake' (TDI), i.e., a daily exposure level per kg body weight which is considered safe (without adverse effects) for humans. For chemicals accumulating in the body due to a long elimination half-life (such as cadmium or dioxins) the tolerable dose for a period longer than one day is usually derived.

The situation is fundamentally different when it comes to genotoxic carcinogens. It is a current paradigm in toxicology that for (directly acting) genotoxic carcinogens no ineffective (safe) threshold exists. This assumption is based on the idea that a single base modification in the DNA could lead to a critical mutation eventually becoming the precursor of a cancer cell. In fact this assumption appears highly unlikely since the genome undergoes multiple damage events on a daily basis without transformation into cancer. Such factors include sunlight, other ionizing irradiation or genotoxic natural compounds (e.g. in food). Since many chemicals under scrutiny by toxicologists are 'man-made' they appear to be avoidable which may also nourish the idea of a 'zero tolerance'. In reality, food contaminants of this type, as all environmental carcinogens, cannot be avoided completely. Likewise the presence of polycyclic aromatic hydrocarbons has been documented in ancient samples dating many thousand years back (Isosaari et al., 2002). The presence of acrylamide in food cannot be avoided if food is heated which will remain the fundamental procedure in our type of food preparation.

These considerations illustrate a severe communication problem. In the past dose-response data of carcinogenicity in animals were extrapolated to the (usually much lower) exposure levels in human cohorts. Linear extrapolation thus allowed the calculation of 'hypothetical cancer victims'. Sometimes such calculations resulted in dramatic numbers. There was a 'general agreement' that a hypothetical (extra) risk of one in a million is acceptable, leading to the result that the associated dose is called 'virtually safe' (Vainio and Hemminki, 1989). Therefore, it is also called the 'acceptable risk model'. The concept is hampered, however, by several drawbacks, one being the high uncertainty resulting from a linear extrapolation over several orders of magnitude. The extrapolation is unavoidable since lower doses, relevant for human exposure, would not result in additional tumor-bearing animals because of the low efficacy of low doses. The only way to address the low efficacy would be an enormous increase in the number of test animals per group, a completely unrealistic scenario for several reasons.

Furthermore, the predicted cancer cases in the 'acceptable risk model' cannot be verified since the risk is calculated for a uniform exposure level identical for all members of the cohort. Thus the incidence of cancer is considered as a stochastic event not related to a particularly high individual exposure. For these reasons this model is no longer in use.

It has been replaced by the Margin-of-Exposure (MoE) model (EFSA Scientific Committee) which is particularly used for substances which are genotoxic and carcinogenic. It starts from a POD for carcinogenic effects, usually a BMDL<sub>10</sub> from animal experiments and compares it to an average or high exposure in a given human cohort (e.g. consumers in the EU) taking into account various age groups (Benford et al., 2010). If this comparison reveals a difference of 10,000-fold or higher, the situation is classified as 'of low concern' while in case of a lower MoE it is considered as of concern (Table 2). Likewise, the average exposure towards acrylamide in all age groups (European consumers) or the exposure with pyrrolizidine alkaloids in high-end consumers among toddlers were classified by EFSA as 'of concern' (EFSA BIOCONTAM Panel, 2015, 2011). The classification is thought to provide a basis for management decisions by authorities, in parliaments etc. It can be used to give recommendations or to set maximum values in food items etc. An example for maximum values for genotoxic carcinogens are aflatoxins (European Commission, 2006). Their maximum values were set, however, based on the ALARA principle (*As Low As Reasonably Achievable*) which is based on an expert agreement taking into account feasible measures to reduce the level of contamination. Since this principle is not based on pure scientific toxicological considerations, there have been suspicions that it might reflect a 'half-hearted deal' with industry. The future must show if the practical implication of the MoE concept can provide a better protection of the consumer than the ALARA principle.

Furthermore, it should be noted that the value of animal data for risk assessment of contaminants is more or more under discussion. Since in vitro experiments in most cases do not provide quantitative data useful for risk assessment, epidemiological studies (in humans) are envisaged as a better solution. According to most risk assessment experts, however, such data are useful in a few special cases only. Reliable correlations are typically found under 'strong' influences such as smoking, alcohol or drug abuse, malnutrition etc. For food contaminants, the relatively low level of exposure in consumers and the presence of many variable factors such as individual diet etc. make the data very heterogenous. In contrast to studies at the workplace where in 'simple' cases a single chemical prevails in causing adverse effects, or where a certain type of chemical mixture is consumed at a relatively high dosage on

**Table 2** Classification of Margin-of-Exposure values for genotoxic and carcinogenic contaminants by the European Food Safety Authority (Isosaari et al., 2002)

<i>Margin of exposure (MoE)</i>	<i>Classification</i>
<10,000	of concern
10,000–1,000,000	of low concern
>1,000,000	of no concern

a regular basis over a long period of time, a focused study on symptoms and diseases at common exposure to widespread contaminants appears extremely cumbersome if not useless.

## Summary

Chemical contaminants can enter the food chain at all levels, i.e., during production, harvest, manufacturing including packaging and storage. Their sources are manifold including infections by toxin-generating organisms, environmental contamination, process-related contaminants and migration from packaging materials etc. The toxicological risk assessment of these compounds comprises a series of steps starting from a thorough description of the chemical including its generation and reliable analysis, its sources and levels in the environment and in food, exposure assessment, and characterization of its toxicity. The latter includes dossiers on the toxicokinetics, the mode of action, the adverse effects in animals and humans and the final assessment of the consumer's risk. For this purpose, various tools have been developed which start from a classification of the contaminant as genotoxic or non-genotoxic. While for non-genotoxic compounds, maximum 'thresholded' exposure levels can be considered as safe, a Margin-of-Exposure approach describes the 'distance of safety' between overt carcinogenic doses in experimental animals and the actual exposure levels in humans.

Producers are confronted with the challenge to understand and possibly apply the principles of toxicological risk assessment. Therefore, it is mandatory that all food manufacturers, traders etc. as well as regulatory agencies have a solid knowledge on the principles mentioned above. A general defensive strategy against 'more stringent regulations' is not timely and will not address consumers' concerns in an adequate way.

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## Emerging Food Safety Risks

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### Glossary

**Emerging risk** A risk to human, animal or plant health resulting from a new source or increased susceptibility or exposure to an existing source.

**Hazard** A substance or activity which has the potential to cause adverse effects to living organisms or environments.

**Risk assessment** A specialised field of applied science that involves reviewing scientific data and studies in order to evaluate risks associated with certain hazards. It involves four steps hazard identification, hazard characterisation, exposure assessment and risk characterisation.

**Risk management** The management of risks which have been identified by risk assessment. It includes the planning, implementation and evaluation of any resulting actions taken to protect consumers, animals and the environment.

**Risk-benefit analysis** A method for weighing up the likely risks (in terms of the incidence and severity) associated with exposure to a substance versus the likely benefits.

**HACCP** A hazard analysis and critical control point (HACCP) is a system that identifies, evaluates and controls hazards to food safety. It is implemented by food businesses to ensure safe production, storage and transport of food.

**Food contact material** Any material, typically packaging or kitchen equipment, designed to come into contact with foodstuffs.

### Nomenclature

List of nomenclature used including all definitions and units.

#### Abbreviation

MRLs	Maximum Residue Levels
WHO	World Health Organisation
FAO	Joint Food and Agriculture Organisation of the United Nations
JECFA	Expert Committee on Food Additives
TDI	Tolerable Daily Intake
ADI	Acceptable Daily Intake
BfR	German Federal Institute for Risk Assessment
ANSES	French Agency for Food, Environmental and Occupational Health & Safety
EFSA	European Food Safety Agency
FSA	Food Standard Agency
HACCP	Hazard Analysis Critical Control Points
TACCP	Threat Analysis Critical Control Points

## What are Emerging Food Safety Risks

If history repeats itself, and the unexpected always happen how incapable must man be of learning from experience

Goerge Bernard Shaw (1856–1950)

The term emerging food safety risks refers to issues, that present a risk to human health, arising from either unknown hazards or known hazards that are subject to new influences. The hazards maybe microbial, chemical residues, environmental contaminants, physical hazards and infestation, all of which can be detrimental to human health. Simply put, an emerging food safety risk is a new and unexpected hazard or threat. A re-emerging risk is a known hazard or threat which is increasing in frequency of occurrence or severity. Hazards maybe subjected to new influences such as new scientific insights on a hazard or new routes of human exposure from changing consumer habits, leading to the re-emergence of food safety issue.

For example, chlorate a known biocidal molecule that has been in use for decades for purification of drinking water and sanitation became an emerging food safety issue in 2014 following development of analytical methods in food matrixes at low limits of

quantification (CVUA, 2014). The detection of chlorate in foods prompted toxicological exposure assessments raising concerns beyond what had been known, challenging decades old sanitation practices. Alkaloids in food have emerged due to changing dietary habits for consumption of natural or organic foods and new trends for vegetarian foods. More alkaloids and their derivatives are now being identified as having food safety concerns (EFSA, 2013; EFSA, 2017a). Other notable emerging contaminants are methyl mercury, halogenated flame retardants, musks and UV filters in seafood resulting from both new scientific influences, changes in dietary habits and globalisation (Marques et al., 2017).

The identification of emerging food safety risks is paramount for protecting public health and maintaining consumer confidence in the food chain. Additionally emerging food safety issues can have huge negative impacts on the agri-food industry therefore is important to identify and prevent these impacts. The emergence of the link between BSE and the fatal human disease in 1995 resulted in suspension of all export markets for British beef impacting an estimated \$1.7b industry (Craven and Johnson 1999). Beyond human health, the impact of such a food safety risks has economic consequences not only on industry and its brands but also at national and regional levels. This chapter focusses on how food safety risks emerge and gives a framework for anticipation and identification in order manage them.

## Drivers of Emerging Risks

Emerging food safety risks arise due to various influences both scientific and socio-economic factors. These factors are like drivers bringing change into how hazards are perceived and therefore managed. Some of these drivers are shown in Table 1 with examples of food safety risks. Globalisation has brought about interconnectedness and complexity to the food chain making it increasingly difficult to ascertain and isolate causal factors which give rise to emerging food safety issues (SAFE-FOOD, 2013).

### New Science: the More We Know the More We Have Solve

Science is a key driver of emerging food safety issues. The application of scientific and technological advances to food production, measures of analysis and control is a major source of detecting and identifying emerging food safety issues.

New scientific information enables the assessment and establishment of toxicological end points and the setting of thresholds of concern. The main safety factors defining acceptable levels of exposure have been determined for many chemicals. These are the Acceptable Daily Intake (ADI) which pertains to residues of chemicals deliberately used in food production such as additives, pesticides and veterinary medicines, and the Tolerable Daily Intake (TDI) which pertains to chemical contamination of food from the environment. These safety factors are an indication of the amount of a chemical in food or drinking water that is not considered harmful if ingested every day over a lifetime (WHO). More recently the margin of exposure (MOE) is a tool used in risk assessment to explore safety concerns arising from the presence of a potentially toxic substance in food or animal feed. Also in use is the threshold of toxicological concern (TTC), a screening tool that provides conservative exposure limits in the absence of sufficient chemical-specific toxicological data. TTC is a science-based approach for prioritising chemicals with low-level exposures that require more data over those that can be presumed to present no appreciable human health risk (EFSA Glossary). In most cases the new knowledge generated leads to lowering of these safety factors and subsequently re-emergence food safety concern.

**Table 1** Drivers of emerging food safety risks

<i>Driver</i>	<i>Example of emerging food safety risk</i>
New Science and research	Acrylamide Monochloropropane-1,2-diol and Glycidyl Esters
Improved Analytical capabilities	Chlorate
Environment	Mycotoxins Heavy metals
Globalization	Marine biotoxins Biological hazards
Fraud	Melanie Horsemeat Scandal
Regulatory Loopholes	Dioxins Differing risk assessments positions for plant protection products
Novel foods and Technologies	Genetically Modified Organisms Nanoparticles
Consumer trends	Tropane alkaloids in organic Food Isoflavones in Soy based products

The increasing body of science linking the toxic effects of acrylamide, a process contaminant that has been in existence since man began cooking food, to cancer is a good example of how new science is a driver for emerging food safety issues. Animal studies had led to the classification of acrylamide as neurotoxic, genotoxic and as a probable human carcinogen (World Health Organization, April 1999), the key study in 2002, from the Swedish National Food Administration and researchers from Stockholm University reported significant levels of acrylamide in carbohydrate-rich foods that had been prepared/cooked by frying, grilling, baking or toasting brought acrylamide into the spotlight as an emerging food safety risk (Tareke et al., 2002). Risk and exposure assessments by both JECFA and EFSA have concluded that dietary exposure levels of acrylamide are indeed a concern for human health and advised continued efforts to reduce acrylamide in food (World Health Organization, 2005, EFSA, 2015a,b). Food industry has as a result invested in finding ways to reduce this process contaminant and have developed a Toolbox for reduction of acrylamide in food (Food Drink Europe: Acrylamide Toolbox, 2013).

Whereas industry has in the last century worked on ensuring food is adequately heated and cooked for prevention of microbial hazards and spoilage, today new scientific insights show that heating regimes need to also take into account prevention of heat process contaminants. Linked to acrylamide is furan and its methyl derivatives with possible human health impacts (Seok et al., 2015; EFSA, 2017b). Other heat process contaminants gradually becoming a concern are carboxy methyl Lysine (Nguyen et al., 2014), hydroxymethylfurfural (Morales, 2008; Abraham et al., 2011) and advanced glycation endproducts (Poulsen et al., 2013).

Another science driven food safety issue generating continuous debate is genetically modified technology (GMO). Contradicting scientific reports and risk assessments continue to fuel the GMO debate leading to a lack of consumer trust in the technology (Gaskell et al., 2005; World Health Organisation, 2014; EFSA, 2017c). As such consumer pressure groups press for GMO labelling of food products adding to the complexities for food industry, who have to manage global food supply chains.

Food safety concerns of nanoparticles have emerged with nanotechnology, the engineering of functional systems using very small particles that are less than 100 nm in size (He and Hwang, 2016). Nanotechnology is fast advancing in its use for example as nanosensors built into food packaging or nanocomposites in food containers to minimise carbon dioxide leakage out of carbonated beverages, or prevent oxygen inflow, moisture outflow, or the growth of bacteria to extend product shelf-life and sensory attributes (Jain et al., 2018). Health concerns arise on how nanoparticles behave in human systems, how the needle like structures may interact with human cells and potential to disrupt DNA structures. These nanotoxic attributes are linked to the small size and large surface area of nanomaterials, which enable easy dispersion and invasion of anatomical barriers in human body (He and Hwang, 2016) and also how nanoparticles may illicit allergy (Syed et al., 2013). Reports have started to emerge following new analytical capabilities to detect nanoparticles in foods putting focus on traditionally trusted and natural food additives such as tricalcium phosphate and calcium carbonate (Schoepf et al., 2017). In the next few years it can be anticipated that various nanoparticles beyond the ones commonly used and known today will be major food safety concern (Food and Beverage, 2017; Schoepf et al., 2017).

Studies on packaging and food contact materials have highlighted food safety concerns with mineral oil hydrocarbons, Bis phenols and phthalates. The toxicological impacts to human health continue to raise concerns in spite of the numerous attempts to prove their safety. Furthermore, there are increasing concerns with regards to the presence of non-intentionally added substances (NIASs) inherent to the food contact materials and end-up in food. NIAS substances are often difficult and in some cases not possible to identify and measure making it difficult to evaluate their safety upon exposure (Leeman and Lisette Krul, 2015). Ongoing efforts are underway to develop pragmatic, scientifically valid safety assessment evaluation of NIAS substances.

Endocrine disruption as a toxicological endpoint is an emerging topic that is more and more affecting many compounds: pesticides, biocides, veterinary drugs, and many food contact materials alike (Kabir et al., 2015).

### Improvements in Analytical Capability

Advances in analytical science have seen a lowering of the detection and quantitation limits for a multitude of chemical compounds in food, as well as the development of screening techniques which can simultaneously test for multiple chemical compounds. Enhanced analytical capability has highlighted the presence of hazards at low levels of concern, presence of chemical derivatives or breakdown substances. The simultaneous detection of the presence of multiple hazards has prompted questions on potential synergistic effects. New analytical tools have permitted the assessment of existing known hazards or threats bringing new insights but also show shortcomings in analytical capability.

Following improved analytical capacity and subsequent expansion of the toxicological database, ethyl carbamate known since the 1980s as probable genotoxic carcinogenic, became a food safety risk to human health prompting stricter regulations for food and drink (Dennis et al., 1989; Zimmerli and Schlatter, 1991). Chlorates have now been detected in all food products where chlorinated drinking water is used as a result of an improvement in the analytical method to quantify chlorate and perchlorate at levels as low as 0.005 mg/kg and 0.002 mg/kg respectively for these breakdown compounds of chlorine based agents (CVUA, 2014; EFSA, 2015a,b; Kettlitz et al., 2016). Food safety concerns from heat reaction products (acrylamide, furan, and other Maillard reaction products) are all arising today due to our ability to i) detect these compounds at low levels and, ii) ability to analyse and quantify their effects even at very low concentrations and diverse food matrices.

Sensitive and rapid screening methods for both targeted and non targeted chemical contaminants of concern; microorganisms using metagenomic sequencing techniques, screening for antibiotics (such as chloramphenicol and sulphonamides) and marine toxins (azaspiracids and tetrodotoxin), plant alkaloids and toxins (tropane alkaloids, pyrrolizidine alkaloids) will give rise to an



increase of food safety issues in the coming years. As rapid methods become affordable for screening large numbers of samples by different endusers, such as food safety and environmental agencies, research and monitoring laboratories, and consumer organisations, the food industry needs to be prepared to deal with the flood of emerging food safety issues (Marques et al., 2017; ANSES, 2017).

### Emerging Microbial Food Safety Risks

The Centers for Disease Control and Prevention (CDC) estimates there are 76 million cases of foodborne illness each year, resulting in 325,000 hospitalizations and 5000 deaths (IFT). There are more than 200 known diseases caused by food related pathogens, their toxins, or other substances. However a cause has not been identified for more than half of all recognized foodborne disease outbreaks, indicating that the real number of disease-causing agents is likely to be much larger than 200. The key microbiological agents of foodborne illness include viruses, bacteria, parasites, and toxins produced by algae and fungi (IFT). *Campylobacter*, *Salmonella*, verocytotoxigenic *Escherichia coli* (VTEC) and *Listeria* remain the main causal bacterial species of foodborne disease.

Whole genome sequencing of bacterial strains and environmental samples begins to take center stage in linking infections to food and food production facilities. As such, recent cases of *Salmonella agona* infections in France have been linked to infections in Spain and Sweden. Whole genome sequencing of historical samples has also given insights into this pathogens survival and potential re-emergence in food production facilities (EFSA and ECDC, 2018). Beyond whole genome sequencing it can be anticipated that the whole suite of genomic applications (metagenomics, transcriptomics, metabolomics) will become available to generating new knowledge that will impact the food industry and how we manage known and the many unknown microbiological hazards that will be identified and reported via these methods.

Emerging microbiological hazards are a results of new microorganisms or new virulence of know microorganisms, as new methods become available to identify and link a pathogen to food and illness. In this regard bacterial gene transfer has huge consequences for food safety, the *E. coli* O104:H4 outbreak in 2011 is an example of such a transfer of pathogenic or hyper virulent variants (EFSA and ECDC, 2011).

Antimicrobial resistance resulting from the extensive use of antibiotics in food production. Food-related bacteria and pathogens become resistant to antibiotics by mutations in their DNA or acquisition of antibiotic-resistant genes from their environment. The last decade has observed an emergence of antibiotic resistant zoonotic bacteria transmitted to humans via the food chain, and increased incidence of treatment failure and severity of disease, with negative impacts on public health (ECDC). Antimicrobial resistance has been observed in food related pathogenic *Salmonella typhi*, *Typhimurium* and *Enteritidis*, having resistance to ciprofloxacin, nalidixic acid and resistance to third and fourth generation cephalosporins. Meat and poultry are now know as key route for human exposure to fluoroquinolone and cephalosporin resistance. It is these food production systems that require particular attention to prevent the spread of resistance from these sources (EFSA, 2008). Risk assessments of antimicrobial resistance for specific food-bacterium combinations that are being undertaken will shed light on what food supply chains, and stakeholders, have to focus on to reduce and prevent public exposure.

Food borne viruses are transmitted by contaminated food and water. Viral food borne infections by hepatitis A, Norwalk virus and poliomyelitis virus remain the most common foodborne pathogens, causing over 60% of food-related illnesses in the Western world compared to about 10%–14% from bacterial infections of *Salmonella* and *Campylobacter* (Mead et al., 1999; Gould et al., 2013; CDC, 2018). There is a growing need to have systematic inspection and virological control criteria for the presence of viruses in the food. This can be aided today by exploiting the growing knowledge on viruses and their transmission coming from next generation sequencing tools enabling surveillance and detection of foodborne viruses outbreaks at an early stage.

### Impacts of Climate Change

Climate change cannot be ignored with regards to its impact on food safety. Several issues have been identified by Miraglia et al. (2009) such as:

- i) increase in mycotoxins formed on plant products in fields or during storage;
- ii) residues of pesticides in plant products affected by increases in pest infestations;
- iii) trace elements and/or heavy metals in plant products depending on changes in their abundance and availability in soils and due to shifting rainfall patterns (for example lead and arsenic);
- iv) polycyclic aromatic hydrocarbons in foods resulting from changes in long-range atmospheric transport and deposition in the environment;
- v) marine biotoxins in seafood from production of phycotoxins by harmful algal blooms;
- vi) and the presence of pathogenic bacteria in foods following more frequent extreme weather conditions, such as flooding and heat waves.

Mycotoxins for example are associated with different disease states in humans. The importance of proactively identifying the potential health and economic impacts of mycotoxins influenced by climate change has been emphasised including the need to monitor these toxic compounds as an emerging risk (Paterson and Lima, 2011; Baranyi et al., 2015). Similarly marine biotoxins have been observed as a significant and expanding food hazard throughout the world and have been associated with mass killings of marine life and illness and mortality in humans (FAO, 2004). Biotoxin poisoning events from Ciguatera fish were once confined

in tropical regions but have now been observed in other regions. Marine phycotoxins are now an emerging issue impacting the European shellfish and pelagic fish industries (SAFEFOOD, 2013).

### Emerging Consumer Trends Come With New Food Safety Risks

Firstly the challenge is for industry to ensure that food safety is not compromised in meeting consumer demands for minimally processed foods or more natural food products. Secondly the introduction of novel foods, novel food processing technologies and ready to eat convenient foods are watchout areas for emerging food safety risks (Kelsey et al., 2017). Thirdly the demand for organic foods and plant based alternatives of animal proteins come with emerging issues and food safety concerns that have to be mitigated.

The increased demand for organic crops and less modified crop varieties like sorghum, millet and buckwheat has lead to increased exposure to plant toxins like tropane alkaloids (EFSA, 2013; Adamse et al., 2014; Mulder et al., 2015), leading to regulations being put in place in 2016 to protect human health. Consequently ongoing research to understand plant toxins and their derivatives can be expected to shed light on more food safety risks. Pyrrolizidine alkaloids in tea and honey products are an example (EFSA, 2017a). Deoxynivalenol and its derivatives will likely re-emerge as a key concern once studies shed light on the modes of action and their interactions (EFSA, 2017d).

Use of novel ingredients with for example nanoparticles to give free flow functionalities (titanium dioxide and silicon dioxide) or as active agents to prevent spoilage have raised food safety concerns (Nanogenotox, 2013; Rapport d'étude, 2016; Wyser et al., 2016). As consumers seek alternatives for animal protein, it is important to note that there are food safety concerns that cannot be ignored. Recently the French food safety authorities pointed out concerns linked to isoflavone exposure from increased soya-based product consumption (ANSES, 2017). Concerns are also identified for insect proteins with regards to allergies, possible spread of microbiological and parasitic hazards and also potential exposure to pesticide and chemical contaminants used to control insects (Belluco et al., 2013).

### Economically Driven Emerging Food Safety Risk

Opportunities for economic gain via fraudulent practice in the food chain have lead to various food safety risks with grave consequences. Notable is the melamine scandal in infant milk formula which resulted in deaths and illness of children (WHO, 2009; Guan et al., 2009). Economically motivated food safety risks may come from adulteration of food to enhance quality properties (use of sudan red in herbs and spices Petrakis et al., 2017), substitution of a product or ingredient by an inferior alternative (horsemeat scandal Premanandh, 2013), and extension of product shelf life which may expose consumers to microbiological risks.

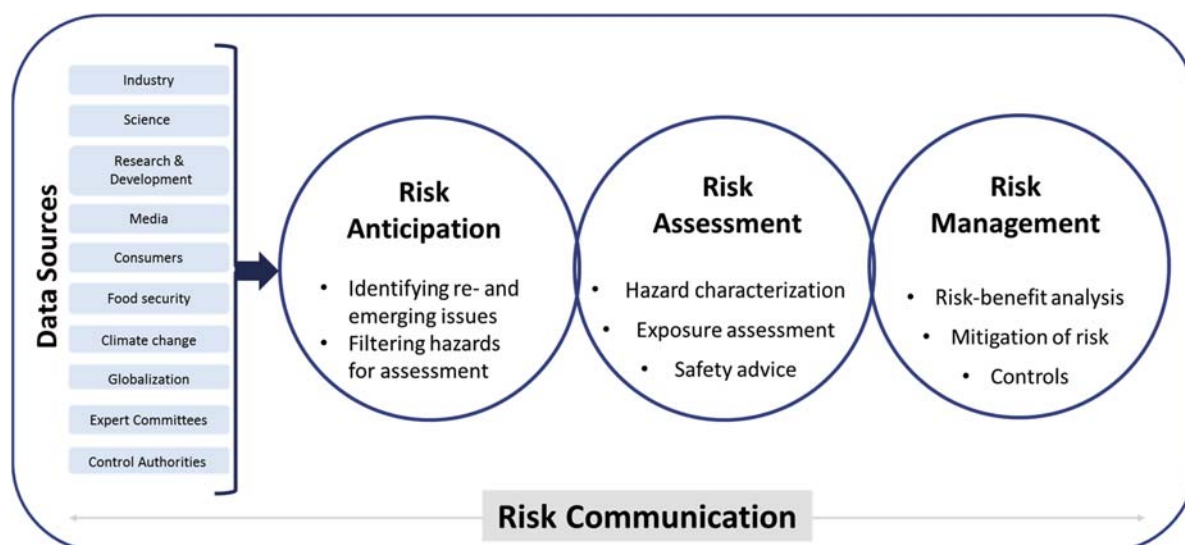
It is important to note here that high value products are more susceptible and also that malpractices are transferrable between industries. Counterfeiting is another route by which food safety risks are introduced in the food chain, often due to use of inferior materials, to maximize the gain. In this regard in order to anticipate potential economically driven emerging food safety issues, threat analysis critical control points (TACCPs) approach is used (Wei-En, 2016). TACCP like Hazard Analysis Critical Control Points (HACCPs) systems encourages systematic assessment of threats, evaluation of processes to identify points of vulnerability, and implementation of preventive actions to improve resilience against malicious attacks. Food fraud and food defense management systems are being implemented by food industry to anticipate on these food safety risks.

### Other Factors Driving Emerging Food Safety

SAFEFOOD (2013) and EFSA emerging risk exchange network. have summarised other influencing factors as being as social, economic, political, legislative, and food security. Indeed changes in legislative regulatory context can lead to an emergence of food safety issues. For example the lack of clarity or harmony in the European legislation on pesticides and biocides has lead to food safety concerns especially for substances with multiple uses. For example there are conflicts in the application of a default pesticide maximum residue levels to substances which are used within Good Manufacturing Practice to assure food safety and good microbiological quality. This is the case for commonly used biocides such as quaternary ammonium compounds. Risk assessment indicate that higher levels can be tolerated from the biocidal use of these substances, however concerns have arisen because that quaternary ammonium compounds have had also pesticide applications for which precautionary default maximum residue limits are enforced in EU (EFSA, 2014; Food Standard Agency, 2016). Thereby such compounds that are otherwise globally accepted worldwide as safe for sanitisation of food processing facilities have become a perceived emerging food safety issue.

### Detection of Emerging in Risk Analysis

Successful detection of emerging risks requires three key components: reliable data sources and skilled human intervention and an intelligence strategy that can be executed in the risk analysis cycle (Fig. 1). Detecting emerging risks is fundamentally a deliberate extension of risk assessment aimed at risk anticipation from the many sources and drivers of emerging food safety risks.



**Figure 1** Framework for anticipation and managing emerging risks.

### Reliable Data Analysis and Trending

Sources of data include: National Food Safety agencies (Bfr, ANSES, ANZ), International scientific committees (JEFCA, EFSA, US FDA), Scientific research, Trade and market data (Table 2). Equally important is media data and information on consumer patterns. The ability to bring together food supply chain stakeholder data including information from industry, primary production, non-governmental organisations and consumers is of utmost importance for early detection of emerging risks. The enormous volume of data requires powerful computing algorithms to analyse and visualize trends in the data to show baselines of typical hazard behaviours, detect peaks of abnormal or unusual events, and identify completely new issues. Meta analysis of data can help to link the abnormal or emerging issues to specific events that lead to their occurrence.

### Skilled Human Intervention

Data interpretation requires vigilance of human expertise and judgement. Several networks exist including Scientific and Governmental Panels and Units, Emerging risks networks, stakeholders consultations involving the whole end to end food supply chain.

**Table 2** Sources of data for detecting emerging risks

Source	Type of data	Impact for food safety
Research	<ul style="list-style-type: none"> <li>- Scientific knowledge on hazards, food safety threats, consumer patterns</li> <li>- Analytical methods</li> <li>- Meta-analysis of data</li> </ul>	<ul style="list-style-type: none"> <li>- New insights on known issues</li> <li>- Identification of emerging issues</li> </ul>
Food Safety Agencies FAO, JEFCA, EFSA, FDA (– US, China ANZ etc) (National and international)	<ul style="list-style-type: none"> <li>- Risk assessment</li> <li>- Exposure assessment</li> <li>- Evaluation of existing data</li> </ul>	<ul style="list-style-type: none"> <li>- Safety factors (TDI, ADI, MOE etc)</li> <li>- Advice for risk management</li> <li>- Facilitation of Information exchange</li> </ul>
Governments and Control Authorities	<ul style="list-style-type: none"> <li>- Alerts RASFF</li> <li>- Surveillance and monitoring data</li> <li>- Trends from controls</li> <li>- Trade and economic data</li> </ul>	<ul style="list-style-type: none"> <li>- Food safety policy and controls</li> <li>- Consumer education</li> </ul>
Industry and food supply chain	<ul style="list-style-type: none"> <li>- Scientific knowledge from research and development and innovation</li> <li>- Risk analysis in food safety management</li> <li>- Data on food ingredients, packaging materials, processing and production environments</li> </ul>	<ul style="list-style-type: none"> <li>- New insights on known issues</li> <li>- Identification of emerging issues</li> <li>- Management of food safety risks</li> <li>- Raising consumer awareness</li> </ul>
Media and Consumer	<ul style="list-style-type: none"> <li>- Food safety conversations on social media</li> <li>- Consumer insights on food preferences</li> <li>- Changing dietary habits</li> <li>- Global food trends</li> </ul>	<ul style="list-style-type: none"> <li>- Perception of food safety</li> <li>- Prevention of consumer panic</li> <li>- Awareness to enable informed choice</li> </ul>

Expertise is required for data analysis, signal detection and filtering and exchange of information for decision making from the many signals that are picked up in risk anticipation and management.

### An Intelligence Strategy

The steps in risk anticipation strategy include:

- i. Profiling and filtering potential risks that need further risk assessment. Analytics such as heatmaps and trend scorecards can be used to prioritise
- ii. Generating further data needed complete risk assessments. This often means that analytical methods have to be improved or developed for investigating hazards across different food matrixes and at multiple stages of production chain.
- iii. Reviewing new results and information taking into account the global food supply chain, not only isolated cases, to capture patterns and indicators that emerge in order to quantify the risks.
- iv. Making recommendations on how to control or change processes to reduce the likelihood of an emerging food safety risk impacting human health.
- v. Communicating and educating stakeholders, consumers and public to assure food is safe.

Emerging food safety issues, and their potential for evolving into real risks is complex and impacts beyond specific geographic, societal and organisational boundaries (Beck, 2007; Smith and Fischbacher, 2009). Therefore, strategies at identifying, and subsequently managing, emerging food safety issues must take into account the globalisation and interconnectedness of modern food chains and rapid transfer of information.

### Perspective

It is a fact that many foods are generally accepted as safe based on their history of safe consumption. However, emerging food safety risk have arisen as a result of new science and technology, new ways for bulk production to feed growing urban populations with changing consumer awareness and perceptions to food safety. Food safety management has improved over the years developing systems that ensure care is taken during development, primary production, processing, storage, distribution, handling and preparation of food. Emerging food safety risks need to be subjected to the risk analysis process to assess potential impacts and, if necessary, to develop approaches to manage the risks (WHO). Risk analysis involves: risk assessment, risk management, risk communication and risk-benefit analysis to guide the decision-making concerning food safety.

Systems such as HACCP and TACCP have been developed ensure comprehensive and systematic approaches for risk analysis in food industry (Wei-En, 2016). Whereas HACCP looks to find key points and implement management measures to prevent known hazards at those points in food production TACCP looks at points of threats or weakness in food supply chain that might lead to potential food safety issues. These two approaches of risk analysis for the whole food supply chain are crucial for anticipation and detection of re- and emerging risks and need to be incorporated in food safety management systems.

Food industry, control authorities, risk assessors and research need to setup processes to anticipate and prepare for emerging food safety issues. Additionally the risk analysis process should ensure that consumers are appropriately and adequately informed to avoid panic, detrimental changes to dietary habits and also unnecessary disruption to trade when food safety risks emerge.

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## Managing Chemical Hazards in HACCP

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### Glossary

**Accidental food (product) contact material.** It is a material that is not in direct contact but not separated from the food (product) by barrier.

**Control measure** Any action or activity that can be used to prevent or to eliminate food safety hazards, or to reduce them to an acceptable level ([International Standard, 2005](#)).

**Declaration of compliance (DOC)** A document issued by a competent authority of the supplier to certify that the delivered good or service meets the required specifications set by customer. It focuses on declaration of legal compliance and disclosure of chemical list required by law. It is also called certificate of compliance, certificate of conformance, or certificate of conformity.

**Design Qualification (DQ)** It aims to determine whether the design of machine & equipment is according to the URS.

**Direct food (product) contact material.** It is a material that is contacting, touching or in very close proximity to the food (product).

**Food safety hazard** Biological, chemical, or physical agent in food, or condition of food, with the potential to cause an adverse health effect ([International Standard, 2005](#)).

**Hygienic design and engineering** Design and engineering of equipment and premises assuring the food is safe and suitable for human consumption ([EHEDG, 2013](#)).

**Indirect food (product) contact material.** It is a material that is in direct contact due to separation by barrier.

**Installation Qualification (IQ)** It aims to determine whether the installation of machine & equipment were delivered correctly in an environment suitable for required operation based on the URS.

**Operational Qualification (OQ)** It aims to determine whether the machine & equipment performs as expected throughout its entire operating range based on the URS.

**Performance Qualification (PQ)** It aims to determine whether the machine & equipment performs as expected during routine operational range and worst case conditions based on the URS.

**Prerequisite Programs (PRPs)** Basic conditions and activities that are necessary to maintain a hygienic environment throughout the food chain suitable for the production, handling, and provision of safe end product and safe food for human consumption ([International Standard, 2005](#)).

**Product contact surface** A surface which is exposed intentionally or unintentionally to the product and surface from which splashed product, condensate, liquids or material may drain, drop, diffuse or be draw into the product or onto product contact surfaces or surfaces that come into contact with product surfaces of packaging materials ([EHEDG, 2013](#)).

**Qualification** Documented verification that determines whether all necessary requirements are met.

**Risk** A function of the probability of an adverse health effect and the severity of that effect when exposed to a specified hazards ([International Standard, 2005](#)).

**User Requirements Specification (URS)** A list detailing all essential requirements related to design, installation, operation, and performance of the unit to deliver expected performance.

### Introduction

The Hazard Analysis Critical Control Point (HACCP) system was pioneered by The Pillsbury Company with the National Aeronautics and Space Administration (NASA) and the US army laboratories to ensure the safety of foods consumed during spatial missions. It is a preventative system based on the Failure, Mode and Effect Analysis (FMEA). It is a systematic and science-based approach to identify specific hazards, assess risk and define control of food safety hazards. HACCP is a tool that focuses on prevention rather than relying on the end-product testing ([Mortimore and Wallace, 1998](#)). HACCP has been widely recognized and accepted as an effective tool to manage food safety by international organizations e.g. CODEX, ISO and WTO. It was initially used to ensure an absence of microbiological hazards in food. Later its application has been broaden to include physical and chemical hazards. This article describes how to manage chemical hazards in food following the HACCP principles. It emphasizes implementation of PRPs that support the chemical hazards analysis, how to identify potential chemical hazards, how to conduct a hazard analysis, and how to determine the control measures to control the chemical hazards in food manufacturing.

## Hazards Analysis

Hazards analysis aims to determine if the hazards are significant to food safety and identify the control measures for the significant hazards. The significant hazards are concluded based on the likelihood of occurrence (probability) and severity of adverse health effect. The hazards analysis involves identifying list of potential hazards in the materials and processing steps, defining severity of adverse health effects and likelihood of the identified hazards in order to conclude the significance.

The challenge in chemical hazards analysis is the fact that all substances can be poisonous if it is consumed in a high dose. Exposure to substances above an official agreed safe level e.g. ADI, PTWI, etc. according to intended food use can pose potential health concerns, which takes into account not only the amount in an individual product, but also the amount totally consumed by complete diet. Questions posted to individual food producer when perform hazards analysis is how to estimate the true level of contamination and how to define the total diet pattern of their consumer. Availability of following information is very important to perform chemical hazards analysis.

- Chemical hazards characteristic and/or scientific literature from reliable sources e.g. US FDA, EFSA, JECFA, Codex Alimentarius etc.
- Defined criteria for evaluation of significant hazard.
- How (and where) the ingredients and packaging materials are produced, controlled and transported to factory.
- Regulatory requirements related to ingredients, packaging materials, and finished product of the selling countries.
- Food safety incidents related to chemical hazards e.g. US FDA Recalls & Alerts, RASFF Food and feed safety alerts etc.
- History of non-conformities e.g. finished product out of specification and consumer complaints, etc.
- Information concerning PRPs implemented at suppliers and in own facilities including the verification results.
- Availability and capability of analytical methods.

Prerequisite programs (PRPs) are foundation of HACCP system and it must be implemented in all food manufacturing. Many people might interpret PRPs relate with hygiene and cleaning practices which primarily aim to prevent microbiological contamination. In fact majority of the hazards can be managed by well established PRPs, while the HACCP procedure focuses on the remaining hazards which will be managed in the critical processing steps in the production. In this article, PRPs cover conditions and activities that are necessary for producing safe product. PRPs are designed based on nature of the food, segment of the production, and type of hazards to be controlled. In some simple processes with low-risk operation e.g. refilling or repacking of dry beverages drinks, all hazards may be controlled by PRPs. More complicated production with higher-risk e.g. milk powder, UHT, and aseptic products, may require implementation of PRPs and HACCP plan to manage all hazards.

## Chemical Hazards

Food safety hazard is a biological, chemical, or physical agent in, or condition of, food with the potential to cause an adverse health effects, either by causing illness or injuries. The hazard is often confused with the term risk, which is a function of the probability of an adverse health effect and the severity of that effect, consequential to a hazards (s) in food (CODEX, 2003).

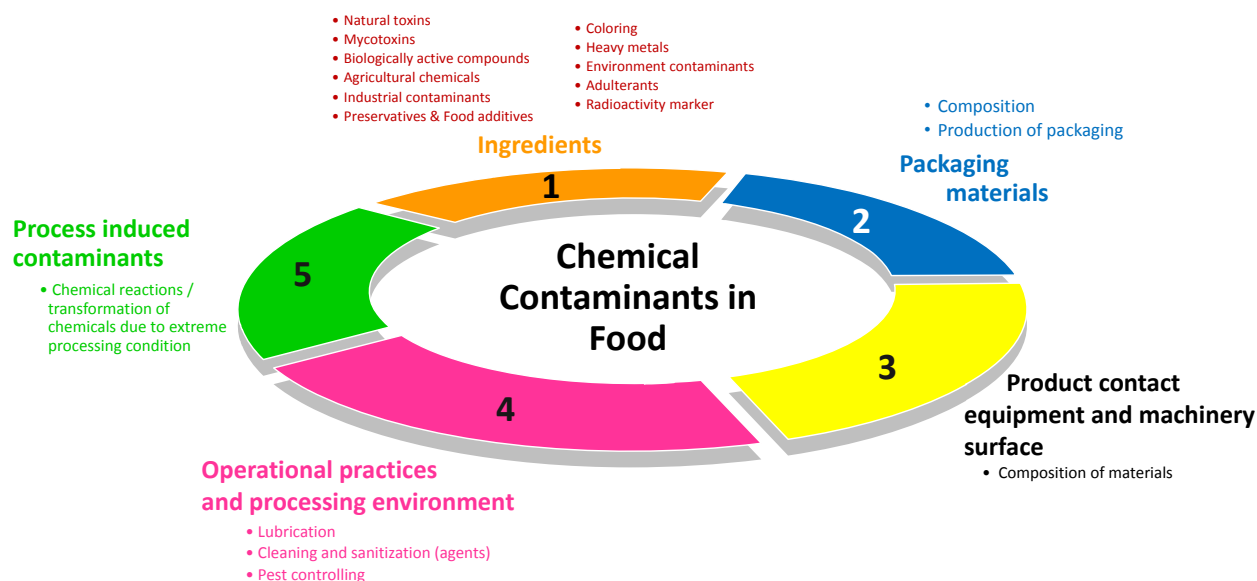
The chemical hazard is any chemical agent in food that has potential to produce adverse health effects or having inherent properties to cause harm. According to CODEX and ISO22000:2005, the chemical hazards broadly cover chemical contaminants and other chemicals that might be present in, or contaminate into ingredients and packaging materials, chemicals intentionally used or unintentionally used in the production and environment in manufacturing facilities, agents in food such as proteins and peptides that cause allergic reaction or hypersensitivity to minority group of consumer, under dose and overdose of nutrients resulting in nutritional deficiency and bacterial toxins (mycotoxins) produced by some groups of bacteria such as Aflatoxin, Ochratoxin A, fumonisins etc. The discussion in this article limits to chemical hazards excluding allergens and nutrition, which require different methodology to perform hazard analysis and define control measures.

Chemical hazards may include (i) chemical substance, a combination of various chemical compounds that can be present or contaminated in the product with high amount, sometimes visible e.g. lubricant, cleaning agents etc. and (ii) chemical contaminant, a single compound that can be present or contaminated in the product with a trace amount quantified through analysis e.g. Cadmium, Aflatoxin M1 etc.

Chemical hazards may come from various sources and may be introduced into food at different points of food chain from farm to fork. They may be broadly categorized according to how they are introduced in food production as following:

- Ingredients
- Packaging materials
- Product contact equipment and machine surface
- Operational practices and processing environment
- Process induced contaminants

Fig. 1 below illustrates origins of the chemical hazards. The hazards analysis and control measures of each type are discussed in later Sections.



**Figure 1** Chemical contaminants in food production.

### Severity of Adverse Health Effects

It is health effects that can be caused by the hazards if consumer consumes hazardous agent exceeding acceptable limits. Severity of the adverse health effect is hazard-specific. Biological and physical hazards usually pose short term health consequences, where chemical hazards potentially found in food may pose both short term and long term health problems depending on its toxicity and dose. It is highly substance-specific. This article classifies severity of adverse health effects for chemical hazards into two sets according to medical terminologies as follows:

- An acute condition is severe and rapid onset and/or a short course. It typically starts suddenly and is short lived. Some acute illnesses can be treated in a short time, while some can be cured by more sophisticated treatment. This condition may be caused by high quantity of chemical substance or chemical contaminant reached level of immediate health effect e.g. consuming 250 g of NaCl, 700 g of Ethanol or 2 g of Vitamin A etc. in one serve.
- A chronic condition is symptom that develops gradually and worsens over an extended period of time. It is persistent or otherwise long-lasting in its effects. The term chronic is usually applied when the course of the disease lasts for more than three months. This condition mostly refers to consumption of food contaminated with low amount of chemical contaminants over a long period of time and eventually results in illness.

The severity of adverse health effects can be ranked from mild to very severe effects e.g. fatality. **Table 1** below demonstrate examples of acute and chronic health effects.

**Table 1** Severity of adverse health effects

<i>Acute health effects</i>		<i>Chronic health effects<sup>a</sup></i>	
<i>Severity</i>	<i>Definition</i>	<i>Severity</i>	<i>Definition</i>
A	Can cause fatality	Alfa ( $\alpha$ )	proven to be mutagenic (no threshold effect) to humans
B	Can lead to serious illness	Beta ( $\beta$ )	proven to have a permanent effect on a vital organ to humans
C	Can cause illness	Gama ( $\gamma$ )	proven to have a mild transient physiological effect to humans
D	Can cause inconvenience	Delta ( $\delta$ )	Might have a mild transient physiological effect to humans
E	Almost of no significance		

<sup>a</sup>T. Stroheker et al. (2017)

## Likelihood of Hazards Occurring

The probability of hazard is qualitative and/or quantitative evaluation or estimation of frequency that hazard is potentially presented or contaminated in the specific ingredient, material or processing step. This becomes a challenge for chemical hazard analysis to properly evaluate the probability corresponding to type of chemical hazards and its health effects. This article describes 2 types of likelihood used in hazard analysis depending on type of chemical hazards. Following description and Table 2 are examples of the likelihood and its ranking:

- **Likelihood of occurrence:** It is a rough estimation of frequency that the chemical could possibly be present or contaminated in the ingredients or in the processing steps. The likelihood of occurrence is used mostly for presence of chemical substances which contaminates in the product in high amount. Evaluation or estimation of the likelihood of occurrence may be obtained through statistical information such as historical analysis or verification data. Following are examples of the likelihood of occurrence ranking:
  - Very unlikely or unlikely ( $<12.5\%$  probability of presence).
  - Rare ( $12.5 < X < 37.5\%$  probability of presence).
  - Could occur ( $37.5 < X < 62.5\%$  probability of presence).
  - Likely ( $62.5 < X < 87.5\%$  probability of presence).
  - Frequent ( $87.5 < X < 100\%$  probability of presence).
- **Likelihood to cause harm:** This likelihood does not only consider the likelihood of presence of the chemical hazards but also considers the impact of overall exposure to the consumer. The likelihood to cause harm is used for chemical contaminants which pose chronic health risk. The evaluation or estimation of the likelihood to cause harm is complicated taking into account the dose and exposure level. Example, the likelihood to cause harm may be categorized into 4 categories referring to 'A new global scientific tool for the assessment and prioritization of chemical hazards in food raw materials' (Stroheker et al., 2017); Negligible, Low, Medium, and High.

Two level of information are usually considered to quantify the likelihood of hazards in ingredients and packaging materials.

- In case there is insufficient information on how the ingredients and packaging materials is made. It is limited resources to collect own data, HACCP team may use information collected by externally recognized organization or historical incidents reported by authorities to estimate the likelihood of hazards. This '**intrinsic information**' does not relate to any specific source or origin of materials.
- Another level of information relates to specific source or origin of materials such as detail preventive programs and historical testing data obtained from various batches supplied by specific supplier. This '**local information**' reflects effectiveness of controls established at specific supplier.

For example generally the intrinsic likelihood of presence of antibiotics in raw milk is high, however in New Zealand the local likelihood is lower due to strong regulatory enforcement and Good Agricultural Practices (GAPs) at farm level. The specific supplier tests representative antibiotics of every incoming lot of raw milk to ensure that no contamination of antibiotic in the received milk.

One may use available data published by externally recognized organization to estimate the intrinsic likelihood while building up local data. Once the local data is sufficient, the local likelihood can be evaluated and used for further decision making.

## Hazards Assessment

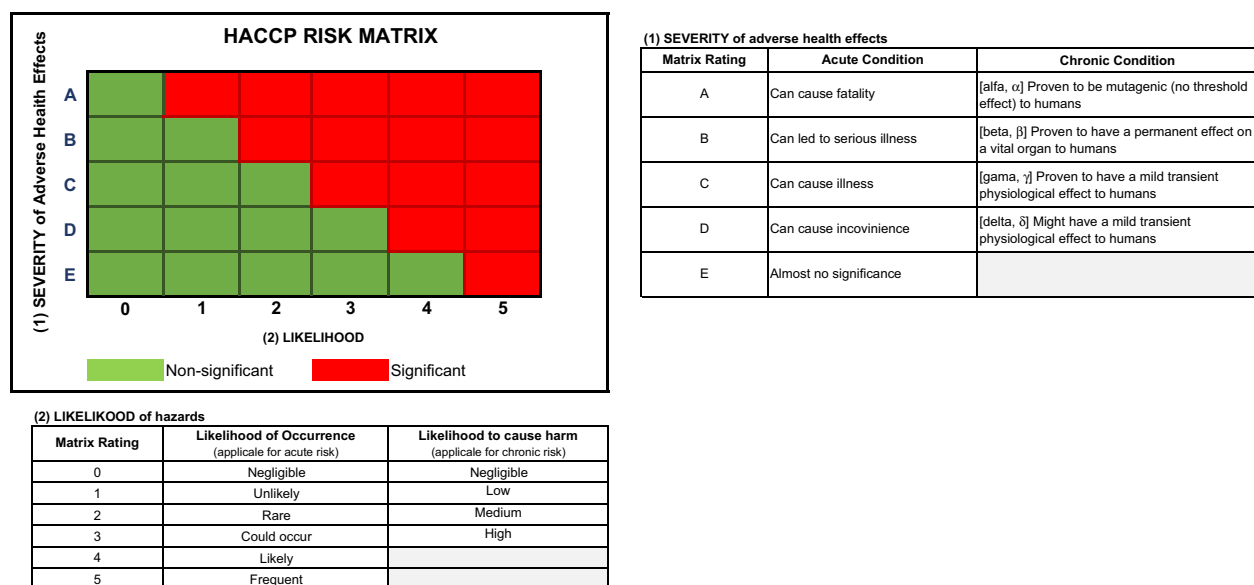
A hazard assessment is conducted to determine whether its control is required additionally from the established PRPs. Following two parameters should be taken into consideration when conducting hazards assessment:

- Severity of adverse health effect
- Probability of occurrence of hazards

The HACCP team may use hazard assessment table to relate severity of adverse health effects, likelihood of hazards, classify and prioritize the significant hazards from non-significant hazards. Fig. 2 below is example of the hazards assessment table.

**Table 2** Examples of ratings used for likelihood of occurrence and likelihood to cause harm

<i>Matrix rating</i>	<i>Likelihood of occurrence (applicable for acute risk)</i>	<i>Likelihood to cause harm (applicable for chronic risk)</i>
0	Negligible	Negligible
1	Unlikely	Low
2	Rare	Medium
3	Could occur	High
4	Likely	
5	Frequent	



**Figure 2** Example of hazards assessment table.

Once the potential chemical hazards are identified, severity of health effect (chronic or acute) and likelihood of hazards (likelihood of occurrence or likelihood to cause harm) are assessed, the significant hazards can be concluded. The non-significant hazards are controlled by existing PRPs. The significant hazard has to be managed through specific control measure which will be further categorized as CCPs or OPRPs. In some cases when the identified control measures are incapable to manage the significant hazards, modification is required which includes modification of materials source or origin, adjusting product recipe or formulation, improving existing control measures or establishing additional control measures.

## Chemical Hazards Analysis of Ingredients

### Prerequisite Programs for Managing Chemical Hazards in Ingredients

When the materials are contaminated with chemical hazards from upstream supply chain e.g. from supplier and during transportation to factory, there is no possibility to eliminate or reduce the contamination in downstream process. Following PRPs are important to ensure good quality of ingredients.

- Establishing material sourcing and approval program including food safety assessment process to ensure that supplier implements necessary control measures for chemical hazards e.g. Good Manufacturing Practices (GMPs), Good Agricultural Practices (GAPs), Good Warehouse Practices (GWPs) etc.
- Material purchasing specification to include food safety and regulatory requirements especially chemical contaminants.
- Establishing a material incoming check procedure prior to utilization of material in the production. Review of testing results stated in certificate of analysis (COA) may be acceptable as an incoming check, provided that sampling plan and analytical method used by the supplier is reliable.
- Establishing risk-based verification (analytical testing) program to verify the controls at supplier

### Potential and Likelihood of Chemical Hazards in Ingredients

Most of the chemical hazards present in food are chemical contaminants derived from ingredients, either they are naturally present in ingredient itself such as natural toxin like lectins, or generated during processing of the ingredient such as acrylamide in starch compounds that had been cooked in high temperature, or contaminated into the ingredients from manufacturing equipment and environment such as dioxins (Edwards, 2010). Following Table 3 are typical types of chemical hazards that may potentially be found in food ingredients.

In terms of likelihood of hazards, it is unlikely that ingredients are free from all undesirable chemical contaminants. But it does not mean that all chemical contaminants present in the ingredients will result in immediate adverse health effects to the consumer. The 'likelihood to cause harm' principle is used to estimate the contribution of contamination in the ingredients to total diet of the consumer and categorize chemical contaminants risk of the ingredients.

**Table 3** Types of chemical hazards and examples in food ingredients

<i>Types</i>	<i>Examples</i>
1. Natural toxins	Lectins, glucosinolates, hydrogen cyanide
2. Mycotoxins	Aflatoxins, Ochratoxin (OTA), Patulin, Trichothecenes (DON), Fumonisin, Zearalenone
3. Biologically active compounds	Agaric acid, aloin, berberine
4. Agricultural chemicals	Pesticides, veterinary drugs (antibiotics, growth promoters, hormones)
5. Industrial & environmental contaminants	Lubricants, sanitizers, oils, paints, dioxins, polychlorinated biphenyls (PCBs), Polycyclic aromatic hydrocarbons (PAHs), Benzo(a)pyrene, Nitrates
6. Processed induced compounds	Acrylamide, 3-MCPD, Ethyl carbamate, Heterocyclic amines
7. Preservatives, Food additives and coloring	Benzoic acid, Sulphur dioxide, Illegal colors e.g. Sudan dye,
8. Heavy metals	Cadmium, arsenic, lead, copper, mercury, aluminium
9. Food adulterants	Melamine, cyanuric acid, urea, food colors
10. Radioactivity marker	Trace of radiation

### Control Measures for Significant Chemical Hazards Originated From Ingredients

In principle, food manufacturer establishes PRPs and relies on control measures upstream at supplier to manage chemical contaminants in ingredients. An expected outcome from hazards analysis (especially with local information) of ingredients is all chemical contaminants are not significant. Thus the PRPs is maintained as part of routine. In some rare cases chemical contaminants in ingredients may be significant due to ineffective upstream controls. This is indicated in supplier audit report and verification testing results. In that situation, it is necessary to review and strengthen the PRPs at supplier. Alternatively, it might be necessary to change origin of ingredients or modify product recipe. Provided that analytical facility is available, contamination is homogeneous, and sampling plan is achievable, screening of incoming materials by chemical analysis before use in production might be an option while improving PRPs upstream. The chemical testing for release of materials could eventually become a CCP or an OPRP.

## Chemical Hazards Analysis of Packaging Materials

### Prerequisite Programs for Chemical Hazards Originated From Packaging Materials

Chemical hazards of concern in packaging materials are mostly chemical contaminants. The PRPs for packaging materials are comparable with the PRPs set for ingredients with additional of followings:

- Obtaining a declaration of compliance (DOC) from supplier to assure they supply material complying with defined specification.
- Establishing a risk-based packaging material development process to ensure that harmful or non-compliance substances are not used in production of packaging materials and that regulatory limits are respected for chemicals allowed to be used with restrictions.

### Potential and Likelihood of Chemical Hazards in Packaging Materials

The concerns in packaging materials are mainly chemical contaminants derived from materials coming into contact with food. It includes auxiliary items which could potentially constitute chemical hazards and transfer it in to food. Sources of chemical hazards are material compositions and processing aids used during packaging material production.

Typical examples of chemical hazards could potentially be found in packaging materials are:

- Toxic metals e.g. lead, cadmium, mercury and hexavalent chromium
- Bisphenol A (BPA) can be found in phenolic-epoxy can coating.
- Plasticisers e.g. *ortho*-phthalates (commonly referred to as phthalates), ESBO etc.
- Acrylonitrile in plastic, adhesives and synthetic rubber.
- Styrene (monomer) in polystyrene plastics and resins.



### Control Measures for Significant Chemical Hazards Originated From Packaging Materials

The risk-based packaging material development process ensures harmful substances including chemical contaminants are not used in packaging material production and that regulatory limits are respected for chemicals allowed to be used with restrictions. Therefore the chemical contaminants do not become significant hazards in packaging materials. In case the significant chemical contaminants hazards is addressed, it is necessary to review and/or modify the packaging material design and compositions, specification, and DOC.

### Chemical Hazards Analysis of Product Contact Equipment and Machine Surface

#### Prerequisite Programs for Chemical Hazards Originated from Product Contact Equipment and Machine Surface

The chemical hazards concerned in product contact equipment and machine surface are chemical contaminants. The PRPs set for product contact equipment and machine surface aims to minimize chemical contaminants migration from product contact surfaces to product. Hygienic engineering design, proper qualification, and acquiring the machine and equipment from reliable supplier are key to prevent chemical contamination to the product. Following PRPs are commonly suggesting to minimize chemical migration in food manufacturing.

- Establishing valid User Requirement Specification (URS) when purchasing new machine and equipment. The requirements include: (i) internal and regulatory requirements related to chemical contaminants, (ii) material component, and (iii) hygienic engineering requirements. There are several hygienic design and construction standards which widely recognized and it can be used as reference to create the URS such as: European Hygienic Design Group (EHEDG), National Sanitation Foundation (NSF) International, 3A Sanitary Standards Inc etc.
- Purchasing of machine and equipment from credible supplier e.g. supplier certified under recognized food safety schemes or Standards.
- Establishing prospective or retrospective qualification consists of Design Qualification (DQ), Installation Qualification (IQ), Operational Qualification (OQ), and Performance Qualification (PQ).
- Obtaining a declaration of compliance (DOC) from supplier declared that machine and equipment complying with recognized regulatory requirements or Standards related to product contact and accidental product contact parts.

#### Potential and Likelihood of Chemical Hazards in Product Contact Equipment and Machine Surface

Metals, alloys, plastics, silicones and rubbers are widely applied as food contact materials or accidental product contact materials in processing equipment in food industry. Therefore they are potential sources of chemical contamination to food, both from main components and unforeseen impurities (Karamfilova, 2016). The concerns related to chemical hazards in machine and equipment are mostly chemical contaminants similarly to packaging materials. Additional concerns are heavy metals include cadmium, inorganic tin, lead and mercury, hexavalent chromium, antimony and nickel (Cederberg et al., 2015; Edwards, 2010) which could be constituted in metals and alloys used in food contact materials in processing equipment.

As a general rule, migration of substances from food contact materials including processing equipment to food must not occur in amounts that endanger human health. Some food safety organization e.g. the Council of Europe has suggested specific release limits (SRLs) for most metals potentially used for processing equipment in contact with product (Cederberg et al., 2015).

#### Control Measures for Significant Chemical Hazards Originated From Product Contact Equipment and Machine Surface

Chemical contaminants might be identified as potential hazards in product contact equipment and machine surface, but it should not be significant supposing the PRPs are well implemented. The product contact surface specification is clearly defined and agreed by supplier, and later confirmed through the qualification process. The supplier complies with international recognized manufacturing standard and able to provide evidences of compliance. The challenge often relates to verification whether testing of certain chemical contaminants is necessary. It is mostly depends on product type and its specific regulatory requirements. In some case it requires a testing report of exact constructing material of the product contact surface such as in pharmaceutical requirements but it does not require any testing in other regulations. Similarly with the packaging materials, in case the significant chemical contaminants is addressed, the machine and equipment is no longer suitable to use in contact with food and it certainly requires modification or replacement.

### Chemical Hazards Analysis of Operational Practices and Processing Environment

#### Prerequisite Programs for Chemical Hazards Originated from Operational Practices and Processing Environment

Implementation of Good Manufacturing Practices (GMPs), sourcing and controlling of chemical utilization ensure minimizing risk of chemical hazards contamination due to inappropriate operational practices and inadequate processing environment.

- Generally the chemical which is intentionally or unintentionally used or applied onto food contact surfaces must be food-grade or compliant with well-recognized regulations or local regulation e.g. US FDA 21 CFR, Food Chemicals Codex (FCC). It means the chemical is acceptable to be used in product contact surface. This is managed through clear specification, supplier selection, review of certificate of analysis (COA) at chemical reception.
- Lubrication could cause chemical contamination to the product if it is not managed well. It is important to establish a lubrication procedure, define right frequency, use of food grade lubricant (e.g. NSF H1 grade) for accidental product contact surfaces, and establish storage control and traceability system of lubricants. This is to prevent excessive dosing and misuse of non-food grade lubricants.
- Cleaning and sanitization of production line and/or packaging materials: pay attention to selection of the chemicals used for cleaning and sanitizing to ensure the chemicals do not contain toxic or legally banned substances. Application of the chemicals must follow the recommended procedure defined by supplier especially the dosage and post treatment such as rinsing with water or drying with hot air etc.
- Pest management involves understanding of the nature of pests, prevent of entry, minimizing of their food source in the production which related to effective cleaning. In the case of infestation, it might require utilization of pesticides and fumigations. In such case, the treatment must be carefully planned and strictly controlled especially fumigation, it must only be done by licensed and trained individuals in full accordance with manufacturer instructions and subject to all local legal provisions.

### Potential and Likelihood of Chemical Hazards Related to Operational Practices and Processing Environment

The concerns related to operational practices and processing environment are mainly chemical substances which may occur in the production due to inappropriate operation practices and inadequate processing environment. It is mainly related to lubrication of moving parts, cleaning, sanitization of line and packaging materials, water treatment, and pest management. [Table 4](#) below presents examples of potential chemical hazards caused by inappropriate operation practices and inadequate processing environment.

Likelihood of occurrence (described in section [Likelihood of Hazards Occurring](#)) is used to estimate the likelihood of hazards related to operational practices and processing environment. Due the nature of operational related data, the rating criteria can be redescribed to suite its application as follow:

Rating 1 - Unlikely (there is no possibility of contamination to product or the contamination is not detectable).

Rating 2 - Rare (contamination is under controlled by PRPs and it might be detectable but below the regulatory or internal requirements)

Rating 3 - Could occur (contamination is being controlled by PRPs, however there is deviation observed in the verification program exiting the regulatory or internal requirements).

Rating 4 - Likely (contamination level is not technically being controlled under current PRPs.)

Rating 5 - Frequent (contamination is happening and able to detect by visual inspection).

### Control Measures for Significant Chemical Hazards Related to Operational Practices and Processing Environment

The chemical contaminants should not be of concern providing that all chemicals used or applied onto food contact surfaces must be food-grade or compliant with well-recognized regulations or local regulations. The chemical substances might be identified as potential hazards. However in principle, it should not be significant because there is no possible control measure to eliminate and reduce the contamination in the product. In case there is a significant chemical substance, it requires thorough review of material specification, supplier selection program, revisit the COA review procedure, chemical application procedure, cleaning procedure and pest management program.

## Chemical Hazards Analysis of Process Induced Contaminants

### Prerequisite Programs for Chemical Hazards Originated from Processing Condition

Managing the process induced contaminants starts in the product development stage where the processing condition is designed and optimized to prevent formation of undesirable chemical hazards.

### Potential and Likelihood of Chemical Hazards Generated From Processing Condition

The formation of undesirable chemicals during production of food has been confirmed through several scientific studies in past decade. Thermal heat treatment such as frying, baking, roasting, fermentation, drying, or extraction are known that can result in the formation of some chemical hazards in some food. Some examples of known contaminants:

- Acrylamide is formed from the reaction between asparagine and reducing sugars under heat treatment (high temperature) such as baking, frying, and roasting.
- Chloropropanols is a group of chemical contaminants, 3-Monochloropropane-1, 2-diol (3-MCPD) is the most notable of the group. It is formed in a reaction between hydrochloric acid and lipids in foods processed at high temperature

**Table 4** Potential chemical hazards and sources related to operational practices and processing environment

<i>Activity</i>	<i>Chemical use</i>	<i>Potential chemical hazards</i>	<i>Origin/source</i>	<i>Type of chemical hazards</i>
Lubrication (food contact and indirect contact)	Grease, oil	<ul style="list-style-type: none"> <li>• Lubricants e.g. grease or oil</li> <li>• PAHs</li> <li>• Prohibited (banned) chemicals.</li> <li>• Heavy metals (Cd, As, Pb, Hg, Ni)</li> </ul>	Excessive lubrication Presence in the chemical use for lubrication	Chemical substance Chemical contaminants
Cleaning and sanitization	CIP solutions (Sodium hydroxide, nitric acid and CIP aids).	<ul style="list-style-type: none"> <li>• Cleaning solution e.g. NaOH, Nitric acid.</li> <li>• Sanitizers</li> <li>• Chlorophenol.</li> <li>• Prohibited (banned) chemicals.</li> <li>• Perchlorate.</li> <li>• Heavy metals (Cd, As, Pb, Hg, Ni)</li> </ul>	Insufficient rinsing with water after cleaning with chemicals. Presence in the chemical use for CIP	Chemical substance Chemical contaminants
	Aseptic filling line - packaging and filling machine sanitization	<ul style="list-style-type: none"> <li>• Cleaning solution e.g. peracetic acid, hydrogen peroxide, sodium hypochlorite</li> <li>• Heavy metals (Cd, As, Pb, Hg, Ni)</li> </ul>	Remaining residue or insufficient rinsing with water after cleaning with chemicals.	Chemical substance
Steam making	Corrosion inhibitor/deposit control agent	<ul style="list-style-type: none"> <li>• Sodium bisulphite</li> <li>• Sodium sulfite</li> <li>• Sodium hydroxide etc.</li> <li>• Prohibited (banned) chemicals.</li> <li>• Heavy metals (Cd, As, Pb, Hg, Ni)</li> </ul>	Presence in the chemical use for sanitization Remaining residue of the used chemicals	Chemical contaminants Chemical substance
Water treatment	Chlorination	<ul style="list-style-type: none"> <li>• Chlorine (to form trihalomethanes – THMs)<sup>a</sup></li> <li>• Heavy metals (Cd, As, Pb, Hg, Ni)</li> </ul>	Remaining residue from chlorination Presence in the chemical use for chlorination	Chemical substance Chemical contaminants
Cooling water	Corrosion inhibitor/deposit control agent	<ul style="list-style-type: none"> <li>• Sodium hydroxide</li> <li>• Ethylenediamine tetraacetic acid</li> <li>• Tetrasodium salt</li> <li>• Sodium molybdate</li> <li>• Sodium bisulphite etc.</li> </ul>	Remaining residue from water treatment	Chemical substance
	Microbial growth inhibitors	<ul style="list-style-type: none"> <li>• Biocides e.g. alkyl polyglycoside, ammonium chloride</li> <li>• Chlorophenol.</li> <li>• Banned chemicals.</li> <li>• Perchlorate.</li> <li>• Heavy metals (Cd, As, Pb, Hg, Ni)</li> </ul>	Presence in the chemical use for cooling water	Chemical contaminants Chemical substance
Pest management	Infestation treatment by chemical e.g. pesticides or insecticides etc.	<ul style="list-style-type: none"> <li>• Methyl bromide</li> <li>• Phosphine</li> <li>• Chloropicrin</li> <li>• Ethyl formate</li> <li>• Sulfuryl Fluoride etc.</li> </ul>	Pesticides and fumigants have not been executed properly and inadequately ventilated.	Chemical substance

<sup>a</sup>Dorworth E.L. (1998)

- Furan can be formed through multiple pathways such as thermal degradation/Maillard reaction of reducing sugars alone or in the presence of amino acids, thermal degradation of amino acids, and thermal oxidation of ascorbic acid, poly-unsaturated fatty acids, and carotenoids.
- Heterocyclic aromatic amines are formed during the heat processing of protein-rich mixtures.

Most undesired chemicals are chemical contaminants. Likelihood of hazards should be assessed during new product development stage considering product composition, possibility of chemical reactions or by-product formation and confirmed hypothesis through product technical trial etc. It requires sufficient product knowledge, technical competence, and in some case might involve statistical calculation.

### Control Measures for Significant Chemical Hazards Related to Process Induced Contaminants

At the product development stage, if significant formation of undesirable chemical contaminants is expected, the most sustainable solution is reformulating the recipe or modifying processing condition to prevent the unwanted chemical reaction. As a result, the concerned contaminants are no longer an issue. However in some cases, it might not be possible to absolutely prevent formation but possible to minimize the contamination level. In such cases, optimum processing condition must be defined, validated, controlled, and monitored during production. Controlling of specific processing condition such as frying of noodle, baking of wafer etc., could eventually become a CCP or an OPRP.

### Conclusion

HACCP is a tool to assist in identifying hazards, prioritizing and determining how best to minimize the identified hazards. It will not result in elimination of all hazards (Van Schothorst, 2004). Therefore quality of the HACCP system depends mostly on competence of the HACCP team to utilize the tool and available information to establish practical and effective control measures. Chemical hazards analysis requires in-depth scientific knowledge about hazards characteristic, origins, health implication and probability of occurrence. Prerequisite programs is essential element of HACCP implementation especially for chemical hazards management where there is no downstream mitigation steps. Food manufacturer needs to allocate sufficient resources to ensure establishing of appropriate prerequisite programs upstream at supplier and in own manufacturing line to prevent contamination of chemical hazards.

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# The Legislative Landscape in the EU: Challenges Faced by the Food Industry

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## Glossary

‘competent authorities’ means the member state organisation(s) that have the legally delegated authority to enforce food safety legislation within the EU.

‘HACCP’ is an acronym for Hazard Analysis and Critical Control Point, a management system which identifies, evaluates, and controls hazards which are significant for food safety;

‘member state’ refers to a country which is member of the European Union (EU), a political and economic union of 28 European countries;

‘risk’ means a function of the probability of an adverse health effect and the severity of that effect, consequential to a hazard;

‘risk assessment’ means a scientifically based process consisting of four steps: hazard identification, hazard characterization, exposure assessment and risk characterization;

‘risk management’ means the process, distinct from risk assessment, of weighing policy alternatives in consultation with interested parties, considering risk assessment and other legitimate factors, and, if need be, selecting appropriate prevention and control options.

## EU Regulatory Framework

The European Regulation which sets out Community procedures for contaminants in food ([Council Regulation \(EEC\) No 315/93, 1993](#)) defines a contaminant as any substance not intentionally added to food which is present in such food because of the production (including operations carried out in crop husbandry, animal husbandry and veterinary medicine), manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food, or as a result of environmental contamination (NB: The text specifically excludes extraneous matter such as insect fragments, animal hair, etc.).

The regulation also states the general principle that food containing a contaminant in an amount which is unacceptable from the public health viewpoint, and in particular at a toxicological level, shall not be placed on the market. According to the regulation contaminant levels shall be kept as low as can reasonably be achieved by following good practices at all the stages of production, and where necessary, maximum levels for specific contaminants shall be established.

Absolute responsibility for food safety is placed upon food businesses by [Regulation \(EC\) No 178/2002](#) (commonly known as ‘The General Food Law’). This states that food businesses are directly responsible for ensuring that no food is placed upon the market if it is unsafe i.e. if it is considered to be ‘injurious to health’, or else unfit for human consumption.

The regulation states that in considering whether any food is injurious to health, regard shall be had:

- (a) not only to the probable immediate and/or short-term and/or long-term effects of that food on the health of a person consuming it, but also on subsequent generations;
- (b) to the probable cumulative toxic effects;
- (c) to the particular health sensitivities of a specific category of consumers where the food is intended for that category of consumers ([Regulation \(EC\) No 178/2002](#)).

Additionally, under- the Food Hygiene Regulation ([Regulation \(EC\) No 853/2004](#)), most EU food businesses have a legal obligation to put in place, implement and maintain a permanent procedure or procedures based on the HACCP principles. These principles include identifying any hazards related to the food and its production, and ensuring that these are either prevented, eliminated or reduced to acceptable levels within the finished product and for its intended shelf life ([Regulation \(EC\) No 853/2004, 2004](#)). In this sense hazard means a biological, chemical or physical agent in, or condition of, food or feed with the potential to cause an adverse health effect.

The General Food Law and the Food Hygiene Regulation, therefore act as umbrella regulations, under which there are a series of Europe-wide provisions covering specific aspects of food safety and contamination. Most of these regulations align with the principle set out in Council Regulation (EEC) No 315/93 and they set specific legal limits for contaminants on the basis of risk assessment and taking into account good practice.

Such regulations include microbiological criteria ([Commission Regulation \(EC\) No 2073/2005, 2005](#)) for bacteria and viruses, specific and overall migration limits for food contact materials ([Regulation \(EC\) No 1935/2004, 2004](#)), and maximum residue limits for plant protection products ([Regulation \(EC\) No 1107/2009, 2009](#)) and for veterinary medicinal products ([Commission Regulation \(EU\) No 37/2010, 2009](#)). Comprehensive and complimentary legislation also exist which regulates and limits the use of certain food additives, flavourings, enzymes, and extraction solvents used in the production of food ([Regulation \(EC\) No 1333/2008, 2008](#)), including on a food safety basis (e.g. setting maximum usage levels and criteria for impurities/contaminants within

individual additives), and which regulates the placing on the market of novel foods ([Regulation \(EC\) No 258/97, 1997](#)) or foods which are derived from non-authorised genetic modification ([Regulation \(EC\) No 1829/2003, 2003](#)), and also irradiated food ([Directive, 1999/2/EC, 1999](#)).

For those chemical contaminants which are not covered under these specific legislative requirements, and which are the focus of this chapter, the EU has again followed the principle established under Council Regulation (EEC) No 315/93 and in many cases, has established maximum levels for specific foodstuffs. Many of these are currently listed within [Regulation \(EC\) No 1881/2006 \(2006\)](#) (as amended). Alternative approaches to managing chemical contaminants do exist, most notably with contaminants which may form during processing. There has been some tentative exploration of legislation in this area, most recently with the Commission's regulation on establishing mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food ([Regulation \(EU\) No 2017/2158](#)).

Food business should also be aware that although the food safety legislation is seemingly segmented into these convenient categories, on occasion the regulations do crossover each other and when this happens resolution is not always clear cut. For example, maximum levels may exist for a specific contaminant in food and should therefore, theoretically, be managed via [Regulation \(EC\) No 1881/2006](#), however the same contaminant may also have a specific or overall migration limit for use in food contact materials and might feasibly therefore be managed by [Regulation \(EC\) No 1935/2004](#), or the contaminant may even be authorised as a plant protection product by [Regulation \(EC\) No 1107/2009](#) and may have a specific maximum residue limit. The question for the regulator is what is the source of the contamination? Is the contaminant derived from illegal use of a plant protection product or is the contamination adventitious? Where these conflicts do occur, they can create significant considerable problems as businesses strive to provide evidence of the safety of their food and full compliance with the legislation.

Lastly it is also worth noting that despite the existence of specific provisions and legal limits, and the legal obligations placed on food businesses to ensure that unsafe food is not placed upon the market, competent authorities in EU member states still retain the ability to impose restrictions and the ability to force a withdrawal from the market ([Regulation \(EC\) No 178/2002](#)) where they themselves believe a food to be unsafe.

## **Regulatory Enforcement of Maximum Levels for Chemical Contaminants, Monitoring and Reporting**

Whilst food businesses have a legal responsibility to ensure that the food they place on the market is safe, member states themselves have a legal responsibility to monitor for chemical contaminants and to enforce legislation.

At the time of writing, major revisions to the EU legislation on official controls have been agreed and have been published within the Official Journal of the European Union. The requirements will be gradually phased in and some of the new requirements will not apply until December 14, 2019 ([Regulation \(EU\) No 2017/625, 2017](#)). In the meantime, many similar requirements for enforcement and monitoring are actually set out within [Regulation \(EC\) No 882/2004](#) (as amended).

These requirements specify that member states shall ensure that official controls are carried out regularly, on a risk basis and with appropriate frequency, and shall be carried without prior warning (including on an ad hoc basis) at any of the stages of production, processing and distribution of feed or food and of animals and animal products. The regulation specifies that methods of sampling and analysis shall be established, and if no such rules exist, member states should use internationally recognized rules or protocols (e.g. standards published by the European Committee for Standardisation ([CEN](#))).

The regulation also requires member states to designate official (national) reference laboratories, and identifies a list of current EU reference laboratories for specified chemical contaminants. The reference laboratories have a legal role in co-ordinating comparative trials between laboratories across Europe and providing scientific and technical assistance to the Commission. In turn the Commission has a legal responsibility, which it carries out through its 'Health and Food Audits and Analysis Directorate' ([Europa EU](#)), to inspect the application of food safety legislation in individual member states and in non-EU countries exporting to the EU.

To this end legal provisions for the sampling and analysis for the official control of the maximum levels for chemical contaminants have been laid down in several associated regulations. For example, [Regulation \(EC\) No 401/2006](#) sets out sampling and analysis requirements for mycotoxins, whilst Commission [Regulation \(EC\) No 333/2007](#) covers lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene, and [Regulation \(EU\) No 2017/644](#) covers dioxins, dioxin-like PCBs and non-dioxin-like PCBs. Further guidance, in particular to assist the competent authorities in application of the sampling and analysis requirements, are available via the Commission's website ([EC Europa EU](#)).

Many of the EU's regulations and directives on chemical contaminants specify that the analytical data which is obtained as part of their monitoring programmes are submitted direct to the European Food Safety Authority (EFSA) for use in subsequent risk assessments.

The Commission has also published several 'Commission Recommendations' regarding monitoring of certain foods and certain contaminants. These are principally aimed at member states (although third parties can also submit relevant data) and are intended to gather occurrence data to support future risk assessment or risk management options. Whilst such recommendations are not legally binding, they do suggest target foodstuffs and contaminants to be monitored, and in some cases the conditions under which they are to be sampled and analysed.

Lastly the European Commission has established legislation for monitoring and control of foods imported into Europe. For example, [Regulation \(EC\) No 669/2009](#) (as amended), sets an increased level of official monitoring controls on imports, at point



of entry into the EU, of certain feed and food of non-animal origin from specific third countries on the basis of ongoing or emerging food safety concerns.

Where analysed foods fail to meet the current EU maximum levels for a specified chemical contaminant, the competent authority or authorities in the relevant member state(s) may request the withdrawal or recall of affected products from the market, or in the case of products entering the EU, may refuse entry at the border. Actions taken are reported via the European Union Rapid Alert System for Food and Feed (RASFF), an online communication tool principally for use by competent authorities to share information on unsafe products which may have crossed EU borders.

## EU Risk Assessment for Chemical Contaminants in Food

Where maximum levels or other legal interventions have been established these are normally based upon EU risk assessments delivered by the EFSA (in a limited number of cases by the EFSA's predecessor, the Scientific Committee on Food ([EU Scientific Committee, 1997](#))).

The EFSA was established by [Regulation \(EC\) No 178/2002](#) as an independent risk assessment body. Its role is to assess the available evidence on a specific food safety related issue, and to provide risk assessments for use by the relevant EU risk management and legislative bodies (i.e. the European Commission, European Council and European Parliament). Whilst the EFSA is able to assign work to itself, most if its workload stems from existing legislative requirements, with priorities agreed with the European Commission and other partners, taking into account available resources.

The Commission, member states or the European Parliament may request the EFSA to deliver a food safety risk assessment (typically in the form of a 'scientific opinion') based upon the available evidence. The EFSA will in turn confirm or reject the request, and as appropriate agree a deadline for delivery. Once a request is accepted it becomes a formal mandate and the EFSA maintains a public register of all questions and mandates on its website.

For chemical food contaminants, risk assessments are provided by the EFSA Panel on Contaminants in the Food Chain. The panel is composed of independent scientific experts with a three-year mandate, and external experts may be invited to contribute on a regular or ad hoc basis. The EFSA staff may also produce some scientific outputs, such as responses to urgent requests for advice, and monitoring and analysis information, and data on chemical contaminants, food consumption and emerging risks.

The EFSA scientific opinions on chemical contaminants take into account relevant scientific colloquia and conferences, data collected and submitted by member states or received from open calls, studies which are shared with the EFSA by stakeholders, peer reviewed literature research, own-initiative studies, and where required expert elicitation to address data gaps ([EU-ANSA](#)). In some cases, a draft scientific opinion is issued for a period of public consultation prior to formal adoption. Adoption is via consensus within relevant panel and possible minority opinions are recorded. The text is published within the EFSA's Official Journal as an open access document and a copy is sent to the originator of the request.

The EFSA scientific opinions follow a standard risk assessment template, and include hazard identification and characterisation, exposure assessment and finally risk characterisation, from which probability of an adverse effect to sub-sets of the population are calculated. It should also be noted that several members states maintain separate food risk assessment agencies. On occasion these agencies may arrive at different risk assessment conclusions from the EFSA, and whilst they will typically defer to the EFSA scientific opinion there are rare occasions when this does not occur, and individual member states may then choose to set their own risk management measures, independent of the Commission, and on the basis of protecting their national population.

## Risk Management Measures

With the publication of an EFSA scientific opinion there is often sufficient evidence for the Commission and member states to start discussions on appropriate risk management measures or else to agree that no further action is required.

The Commission may propose draft legal measures based upon discussions within the relevant EU member state expert group, and taking into consideration any additional evidence that is of relevance, for example the practicalities associated with setting a specific maximum level for a specific commodity and reported occurrence levels in specific foodstuffs.

In some cases, such as ergot ([Recommendation, 2012/154/EU, 2012](#)), the EFSA scientific opinion may indicate that the available data do not indicate a concern, but that the occurrence data (and as a result the calculated dietary exposure) on which this opinion is based is limited. A risk management decision may be taken to collect further data or seek further external scientific advice and reconsider the issue at a later stage.

Regulatory texts can take some time to be agreed amongst all 28 EU member states, they may require consultation with relevant stakeholders and may be subject to a considerable number of revisions before they are finalised. Under the current Commission's 'better regulation' agenda, a draft measure such as new or amended maximum levels for chemical contaminants will also be subject to a four-week long public consultation prior to vote within the appropriate EU Standing Committee.

For chemical contaminants in food, the relevant committee is the Standing Committee on Plants, Animals, Food and Feed. The Standing Committee is composed of representatives from all member states and is presided over by a Commission representative. Records of all agenda's and minutes of the Standing Committees are maintained on the Commission's website under the 'commitology register'.

Voted text are submitted to both the European Parliament and the Council for a final 3-month scrutiny period before publication in the Official Journal of the European Union, and the measure may specify an additional transitional period before it applies.

### **Challenges for Food Businesses Posed by New and Emerging Contaminants**

One of the biggest challenges for any food business is how to ensure information on new and emerging contaminant issues are tracked, and to understand how these issues might potentially impact upon the ingredients and processes that the business employs, and ultimately on their finished products.

Due to resource, it can be difficult for a food business to identify relevant scientific research on all potential chemicals of concern. There are a vast number of papers and journals of varying quality to be tracked, and an infinite combination of ingredients and potential hazards. Whilst monitoring of research does take place, food business may find their priorities are determined more by monitoring the legislation process, and through discussions within legislative for a and professional associations.

In terms of legislation tracking there is already, thankfully, a large amount of transparency embedded within the decision-making process of the Commission's Directorate-General Health and Food Safety. As mentioned previously, the EFSA maintains a register of questions on contaminants, and materials such as Standing Committee agendas, minutes and voted regulatory texts are generally available from the Commission's website. The Commission does communicate on most of the work that it is proposing to carry out, via presentations at conferences and symposiums and through communications such as work programmes on its website. Similarly, authorities in individual EU members states will often communicate to stakeholder on proposed work.

However, there are also occasions when critical regulatory decisions on specific contaminants are made rapidly or else at a very early stage, and the materials and data related to those decisions may not always be made publicly available, or publicly consulted in a timely manner e.g. agendas, minutes and early regulatory drafts from member state expert group meetings are often unavailable to stakeholders without submission of specific requests.

Furthermore, whilst a decision to initiate a risk assessment activity on a potential food contaminant should be based upon the best available scientific research on hazard identification and hazard characterisation, and on accurate occurrence and consumption data, regulators may feel themselves under political pressure from external sources (e.g. MEPs, individual governments or lobby groups) to act quickly, particularly when new or potentially acute hazards are identified.

In such circumstances there may be limited knowledge of the full range of factors which are relevant to the finished foodstuffs as it is placed on the market e.g. differing production methods, processing factors, recipe design, supply chains, size of market and consumption patterns.

Failure of food businesses, authorities and the scientific community to communicate to one another at an early stage when any potential hazard is being reviewed can lead to emphasis being incorrectly placed on one aspect of a contaminant and requiring corrections to made to legislation later down the line. For example [Regulation \(EU\) No 594/2012](#), amending Regulation (EC) 1881/2006, set maximum levels for ochratoxin A in spices and mixtures of spices containing one or more of several named spices at 15 µg/kg. For paprika (*Capsicum* spp.) specifically this level would have been applicable from January 2015, but at a late stage industry was able to demonstrate that even with application of good agricultural and manufacturing practice this level was not technically achievable. As a result, the lower level did not come into force and a higher maximum level of 30 µg/kg was retained. However, the uncertainty that the legislation introduced did cause major problems for suppliers and for food businesses who wished to purchase compliant materials. Manufacturers would have had to make contingency plans to accommodate the potential shortage of materials and the spike in prices which would have occurred had the lower level been introduced.

Regulation (EC) 1881/2006 also poses another problem for manufacturers in that in some instances it sets maximum levels for a raw material, and then a separate maximum level for a finished product. Again, using ochratoxin A as an example, raw cereals have a maximum level of 5.0 µg/kg, whereas all products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption have a maximum level of 3.0 µg/kg. In practical terms this means that manufacturers need to ensure that, via processing, they are able to further reduce levels. Whilst this might pose only a limited issue for products with lower cereal content, for some minimally processed cereal products it considerably limits the raw materials that can be used, and may mean that farmers are contracted to provide raw materials that meet the lower processed maximum level.

Lastly in some instances, decisions of relevance for food businesses may also be taken in other non-food for a, for example regulatory discussions on standards for fertilisers which are managed under-European Commission's Directorate-General for Internal Markets, Industry and Entrepreneurship and SMEs, and the EU Water Framework Directive which is managed under the European Commission's Directorate-General for the Environment.

### **Quality and Comparability of Monitoring Data**

All EFSA risk assessments include an estimation of exposure for various subpopulations, which is based upon national food consumption surveys (compiled as the EFSA Comprehensive European Food Consumption Database ([EFSA](#))) and occurrence levels reported within individual foodstuffs. As the exposure assessment is a key determinant in determining appropriate risk measures, it is critically important that the national food consumption surveys are updated on a regular basis, that they cover all parts of the

population, and that they correctly categorise foodstuffs. This is an area that is largely out of control of food businesses, but it is a genuine concern in instances where countries do not have accurate consumption data as this may result in risk assessors overestimating or underestimating exposure for a particular market, based on non-representative data from other member states.

It is also important that analytical methods that are being used to determine levels in product are as accurate as possible, that descriptions of the products that are sampled are accurate (including where possible the method of production), and that the product is sampled in the most appropriate location.

It can sometimes be difficult to establish accurate analytical methods, especially at an early stage in the research, and this may lead to an overestimate or underestimate of levels in foodstuffs, and issue compounded by differences in limits of quantification, measurement uncertainty and other intra-laboratory differences. An example is furan, which is a volatile chemical and possible carcinogen that is formed in some heat processed foods (EFSA J). Şenyuva and Gökmen (2007), reported that during sample preparation for headspace GC–MS analysis, furan was formed during headspace equilibration leading to an overestimation of reported levels in foodstuffs. This was correctly picked upon in Commission Recommendation, 2007/196/EC, where it states that sample preparation before analysis should be carried out with the necessary care to ensure that the furan content of the sample is not altered. Nevertheless, there are currently no official standard methods available for the analysis of furan and neither are there any certified reference materials available.

In the case of Commission Recommendation (EU) 2017/84 of 16 January 2017 on the monitoring of mineral oil hydrocarbons in food and in materials and articles intended to come into contact with food, there is also no agreement amongst researchers and member states as to a suitable test method and at the time of writing (almost a year after the coming into force of the recommendation) guidance from the JRC is still awaited. It remains to be seen how the EFSA and the Commission will assess the results of the monitoring given the lack of standard method. In the case of both furan and mineral oil hydrocarbons should maximum levels be set in the future then for enforcement it would be imperative that a standardised analytical method was agreed.

A number of other factors also have the potential to indirectly bias data sets which then go to inform EU-wide risk assessments, for example, in 2012 the Commission published a recommendation on the monitoring of the presence of ergot alkaloids in feed and food (Recommendation (EU) 2017/84, 2017). This recommendation suggests that member states should perform monitoring on the presence of six ergot alkaloids and corresponding epimers in unground cereals and in cereal products and compound feed and food. However, ergot alkaloids are psychotropic substances, and therefore in some EU member states a laboratory wishing to purchase the standards would be required to apply for specific controlled substance licences, thus making laboratory testing for ergot alkaloids relatively expensive and thereby indirectly influencing the data collection activity by limiting the number of analytical results in some countries on the basis of cost.

In other instances, a data set may be subject to bias because a member state goes significantly beyond the voluntary monitoring recommendation. In its recent scientific report on furan (Recommendation, 2012/154/EU, 2012) the EFSA noted that ~80% of samples submitted came from only five member states (and ~60% originating from one single Member State). The EFSA highlighted the uncertainty on whether possible country-based differences in the levels of furan in diverse food commodities are well represented.

Lastly there may even be issues with the quality of the laboratories that are being used to undertake monitoring. In the EFSA furan report ~30% of the governmental data lacked information on the analytical method that was used to analyse some food samples. In its report on acrylamide in 2012 (EFSA, 2012), the EFSA reported that approximately 5% of the analytical results for the period 2007–09 did not include an indication of the analytical method used, and that data on the percentage of accredited laboratories responsible for the submitted data was often missing, for example in 2007 only 2% of the data reported by member states included information on the laboratories accreditation status (this increased to 6% in 2008, 83% in 2009 and 94% in 2010).

## Summary

On paper the EU legislative process for chemical contaminants in food appears to be relatively simple and the basis for setting controls is reasonably transparent. However, as this chapter hopefully highlighted, it can also be incredibly challenging for food businesses to keep a track of all current requirements and proposals, and all emerging hazards or risks.

Food safety is a major responsibility for a food business of any size, and it requires not only awareness of products and processes, but also awareness regarding the interplay between existing legislation, and an understanding of that way in which risk measures are developed.

There are numerous factors at play which can indirectly influence the direction of the data gathering activities, the risk assessment and ultimately risk management measures, and food businesses need to recognise that scientific knowledge is continually evolving, that new emerging hazards and risks are constantly being identified and characterised, and that they need to be in a position to react to accordingly.

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# Biocides: A Critical Review of Current and Proposed EU Legislation

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## Introduction

The Biocidal Products Regulation EU/528/2012 (BPR) is applicable since 1 September 2013 and has already been revised since its entry into force. Prior to the BPR system, disinfectants had to be registered with the competent authorities in the respective Member States. By contrast, the biocides legislation provides for a two-stage procedure for the authorisation of biocidal products: First, the active substance must be included in Annex I; next, the products formulated with this active substance must be approved separately in each Member State.

The status of the BPR as a regulation means that it applies equally in all Member States and should make a significant contribution to the harmonisation of the European internal market. However, in most respects the Biocidal Products Regulation is only a rough framework; almost all detailed rules and implementing provisions are not yet clearly defined and put into practice in the Member States. Work is currently underway at European level on implementing acts to bring the regulation in a more precise form. This is intended to clarify most of the open issues.

The complexity and “rigidity” of the rules and the cost-driving detailed provisions are mainly caused by the competent authorities of the Member States within the European coordination process. Industry representatives have gained the impression that the national competent authorities cannot agree on a common denominator. As a result, each authority can bring in its own priority which then emerges in the implementation regime.

This can be illustrated by the example of the mixture toxicity issue where, quite often, mixture toxicity is brought forward by one Member State but assessed to be less relevant by the other Member States. Germany's priorities are another example: They increasingly focus on environmentally sound uses. Instead of developing a common position, it is preferred to integrate both special topics into the regulation. However, this approach does not take into account the benefits of disinfectants for increasing the level of hygiene. The emphasis of the rules is clearly on occupational health and safety and environmental protection, based on the experiences with pesticides. But it is worth noting that disinfectants are drastically different from pesticides because of their chemistry and application. Disinfectants are intended to act quickly (almost immediately) and do not need to be chemically very stable and long-lasting. The output is diametrically different too. Disinfectants are almost always used in controlled conditions in objects with disposal via the waste water systems. Unlike in other biocide and pesticide applications, there is no uncontrolled release into the environment.

But it is not only the regulation as such that is proving problematic in terms of individual national interpretations. Moreover, within the approval procedures under the BPR it is increasingly found that the assessment authorities in the Member States have different interpretations of the law and of the guidelines so that they do not make uniform requirements regarding the data to be submitted and the resulting approval conditions. Also in the application of the BPR in other legal areas and concerning national recommendations on the selection and use of disinfectants, the competent authorities and technical societies are extremely persistent in adhering to older, partly outdated scientific principles.

## International Evaluation Methods and Proof of Efficacy

The biocides legislation integrates not only the chemical and physical characterisations of products, their environmental behaviour and various risk assessments but also efficacy tests to ensure adequate disinfection. At the end of 2017, a legal opinion was published on the legal significance of national assessment methods and listing systems for the microbial efficacy of disinfectants in the scope of the Biocidal Products Regulation. This opinion notes that the BPR clearly gives priority to international methods. National methods are only accepted if no international method is available for special applications (e.g. in case of epidemics). Harmonised European standards are the first choice for such internationally recognised methods. This is viewed similarly by scientists and even by federal law courts. The state of the art in science and technology explicitly requires that methods be internationally recognised. Conversely, this means that national methods can never represent the state of the art in science and technology.

Additionally to questions of standardisation and methods, the BPR also regulates the application of any products. Thus, the concentration or exposure times specified in the approval conditions of the assessing authorities must be observed by the users. Furthermore, manufacturers or other third parties (for example, professional societies or associations) may not recommend any other conditions. This means that existing recommendations, e.g. from manufacturers and industry associations or other national associations under private law, may no longer deviate from the authorisation conditions for authorised biocidal products. The consequence: Specialist tests of laboratory results and any risk premiums in listing processes would be rendered obsolete.



The legal basis suggests that the current situation regarding the proof of efficacy is facing a fundamental change in the EU Member States. National methods for testing the efficacy of disinfectants and disinfection measures, which have been dominant for several decades, will increasingly lose in importance and slowly disappear. The focus of possible listing systems will shift significantly, up to checking the reliability of expert opinions and their results. In addition, the high priority given at EU level to the free movement of goods means that restrictions and exemptions are considered near-impossible. In the future, recommendations by experts, professional associations or national authorities will have to comply with the methods and application instructions as described in the approval processes.

### **Costs of the Authorisation Process**

The authorisation of disinfectants according to the Biocidal Products Regulation is a bureaucratic, complex and time and cost-intensive procedure, similar to that for pharmaceuticals and pesticides. This increases the costs per product from a few 10,000 euros to up to 500,000 euros, with this cost estimate referring only to external regulatory costs. Approximately 50 percent each of the costs fall to the share of additional investigations and fees charged by the authorities for the procedure, respectively. Internal costs, e.g. for development, quality assurance and staff, are additionally incurred by the companies.

The enormous increases in fees by the European Chemicals Agency (ECHA) and the national authorities are deemed particularly critical for small and medium-sized enterprises (SMEs). For a standard biocidal product authorisation in several countries, initial experience shows that the amounts in fees to be expected are comparable to the amounts that had to be invested previously in the preparation of the required dossiers. In more detail, within the approval process of a disinfectant, thus roughly 200,000 euros (simple product) to 500,000 euros (complex product) of basic costs arise (for example, for dossier preparation) plus costs for the Letter of Access (for the use of the raw material), approval costs per country and any annual fees.

Within the framework of a study, the EU Commission had the financial burden on disinfectant manufacturers due to compliance with legal requirements in Europe investigated (cf.: <http://bit.ly/2gQNArY>). The results of the study give cause for concern: Due to the impending loss of raw materials and the existing cost burden, negative effects on product development and hygiene levels must be feared. Moreover, these regulatory restrictions and the resulting burdens are a “development brake” for new active ingredients. In Germany alone, the regulatory and administrative expenses doubled between 2015 and 2017 by more than one percentage point (in relation to total sales). In the same period, the industry’s research and development spending in Germany fell by around 0.8 percentage points (research ratio in relation to total sales). This shows that the registration and approval fees will have an enormous impact on the availability of products: The product range might be reduced dramatically.

For the authorisation of biocidal products in the health care sector, manufacturers of disinfectants in Germany invest roughly between 40 and 60 million euros with a total market volume of around 250 million euros. In view of the financial dimensions, a company must consider very carefully whether special products can continue to be offered in the portfolio. The same applies to new product developments: After the administration process, their formulations cannot be changed in the light of customer feedback or practical experience without further horrendous costs. Thus, it is questionable whether the development of application-specific, specialized and individualized solutions will remain possible in the current scope in the future. For facilities that depend on precisely this, there is the threat of special products being no longer available and of price increases.

### **Summary**

The requirements for authorisation are increasing enormously. The Biocidal Products Regulation contributes to this, especially for disinfectant products. The manufacturers fully support the objective of making the handling and use of such products safer and of minimizing risks. However, the new legislation goes far beyond the goal in terms of effort and costs for product authorisation. These are disproportionate to the sales that can be achieved and put pressure on an industry which is characterized by medium-sized enterprises.

This in two respects: firstly, with regard to the enormous costs and, secondly, regarding staff capacities. Preparing the necessary dossiers requires qualified, mostly technically experienced personnel who must be withdrawn from research and development and application technology in order to carry out this work. This leads to bottlenecks and thus to reduced innovation and, in some cases, less customer service. It is expected that large, multinational corporations, raw material manufacturers or large contract manufacturers will be able to implement this requirement more easily. By contrast, most medium-sized enterprises can no longer afford own products in the described situation. Especially in Germany, there are large numbers of medium-sized enterprises that are also technology leaders in Europe. A reduction of available raw materials and disinfectants is foreseeable. To date, around two-thirds of all active ingredients have been taken from the market as a result of the high costs connected with European legislation.

The problem is that the use of disinfectants is also essential as prophylaxis to prevent the spread of dangerous germs such as EHEC or multi-resistant hospital germs (MRSA). However, this is not taken into account, for example, in the European Biocidal Products Regulation. Rather, the regulation gives more focus on environmental impacts or other potential risks. But in the near future, an end to production is threatening even for products for extreme conditions, such as epidemics in the veterinary sector.

This triggers a process where there are only losers in the end: Very special but highly important active ingredients could disappear from the product range, smaller manufacturers are giving up business fields, and competition is increasingly concentrating on a few



products with the same active ingredients. In addition, a significant decline in innovation activities in the industry is driven forward and a reduction in personnel and services for customer support is provoked. There is reason to fear a drop in the level of hygiene and an increase in the risk of the spread of germs.

The changes will become apparent to users by 2020: In the event of an epidemic, important products could no longer be made available in a timely manner, with the outlined risks for prevention and hygiene. The one-sided criteria laid down in legislation for the assessment of active disinfectant substances urgently need to be rethought and restructured, with a much higher priority to health protection aspects. A further reduction or restriction of disinfectants cannot be justified.

What alternative solutions are conceivable? One proposal from the industry is a different basis for the assessment of disinfectants. It could be well-targeted and meaningful to carry out the risk assessment for occupational health and safety and environmental protection according to the REACH criteria without demanding a second risk assessment under the biocides legislation – in order to contribute to a genuine reduction of the costs for the market availability of disinfectants and products. The authorisation of biocidal products within the framework of European legislation should always focus on the efficacy of disinfectants. This approach wants to enable users to fulfil their hygiene tasks, thus ensuring the protection of patients, personnel and health as well as comprehensive food safety.

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Volume 2

# Encyclopedia of Food Chemistry

Editors

Peter Varelis, Laurence Melton and Fereidoon Shahidi



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# ENCYCLOPEDIA OF FOOD CHEMISTRY

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## PREFACE

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This is an exciting time for all who wish to understand the nature of food and how it changes during storage, processing, cooking and digestion. This is because science has turned its focus onto soft matter and food is very largely soft matter. Earlier scientific effort was preoccupied with simple molecules in solution and solids (preferably pure crystalline proteins). When you think about it, knowing the total protein, carbohydrate, lipid and vitamin contents of a food doesn't tell you much about the nature of the food and how it will provide nutrients for us. Now we are seeing a huge effort to understand soft matter, which is directly applicable to food. Meaning we are increasing our knowledge of how food components (both macromolecules such as proteins and polysaccharides as well as smaller molecules) interact chemically with each other; leading ultimately to understanding the complex chemical structure of foods (everything from apples and ice cream to vegetable protein meat-substitutes and food for a visit to Mars).

A golden age for food research has begun, with so many new techniques available such as CRISPR, isothermal calorimetry (ITC), analytical ultracentrifugation (AUC), small angle neutron and X-ray scattering (SANS, SAXS), surface plasmon resonance, electronic tweezers, and computer modelling (for example molecular dynamics simulations for understanding the interactions of proteins and polysaccharides, enzymes and substrates, bioactives and encapsulating agents). This is in addition to the tried and tested techniques of nuclear magnetic resonance (NMR), mass spectroscopy, electron and confocal microscopy, high performance liquid chromatography (HPLC), and gas chromatography. Biosensors publications are huge and nanotechnology is a hot topic for research.

The *Encyclopedia of Food Chemistry* is for people who have a basic knowledge of food chemistry and wish to expand their understanding of a topic based on information from a reliable source. No fake news here! We welcome you to enjoy the excitement captured here by experts from across the planet.

Laurence D. Melton

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Figure 4. Formation of Selected Heterocyclic Flavor Chemicals in Beverages.

Figure 1. Interactions between polyphenols and macromolecules: effect of tannin structure.

Figure 2. Interactions between polyphenols and macromolecules: effect of tannin structure.

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Table 1. Next-generation sequencing.

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# Antioxidant and Flavor in Spices Used in the Preparation of Chinese Dishes

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## Introduction

China has a long history of using spices to prepare dishes. Due to their strong and distinctive flavor and aroma in the fresh or dried state, they are used to mask or minimize the undesirable flavor, such as the bloody or raw flavor in the chicken, beef or other meats in the traditional Chinese cuisine (Lu et al., 2011). Some spices are native to China, such as the Sichuan Green pricklyash (Yang, 2008), while others are originally grown in the southern Asian countries such as India, Indonesia, Thailand, etc., but are now cultivated in China, such as the black pepper and cinnamon (Chomchalow, 2001).

Lipid is a major constituent in many commodities, such as in the meat of cattle, sea foods, and bakery products. Lipid oxidation has always been a major concern in the long-term preservation in the food industry. Oxidation of lipids will result in the production of undesirable volatile compounds. Such compounds may induce cancer or other diseases (Niki, 2016), and affect consumers' preference towards a food (Font-i-Furnols and Guerrero, 2014). Antioxidants are substances, when are present in the food at very low concentrations, could delay, control or prevent oxidative processes leading to the deterioration in food quality (Barden and Decker, 2016). Many researchers have reported the anti-oxidative property in spices. In fact, some spices have been used in the food industry as natural additives to maintain the food quality, and to provide stable sensory profiles (Radha krishnan et al., 2014). In this manuscript, we summarized some spices with focuses on their anti-oxidative property, their flavor descriptors and their applications in the food industry.

## Anti-oxidative Properties of Spices

### Mechanism of the Anti-oxidative Property in Spices

Antioxidants are broadly classified as primary and secondary ones. The primary antioxidants act as hydrogen donors or free radical acceptors, and generate more stable radicals (Nanditha and Prabhasankar, 2008). Spices as primary antioxidants (AH) could scavenge the peroxy and oxy radicals ( $RO_2\cdot$  and  $RO\cdot$ ) formed in the lipid propagation step to produce less active radicals ( $A\cdot$ ). These radicals ( $A\cdot$ ) will further react with each other or other radicals until they form stable molecules (ROOA and RA). In contrast to the peroxy and oxy radicals ( $RO_2\cdot$  and  $RO\cdot$ ), the less active radicals ( $A\cdot$ ) are not able to attract an H-atom from the unsaturated fatty acids (ROOH), thus, cannot initiate lipid peroxidation (Nanditha and Prabhasankar, 2008). Secondary antioxidants prevent or retard oxidation by suppressing the oxidation promoters, including metal ions, singlet oxygen, pro-oxidative enzymes and other oxidants (Barden and Decker, 2016). As secondary antioxidants, many spices act as chelating agents, binding to metal ions which act as catalysts for lipid oxidation.

### Chemical Assays for the Antioxidant Activity in Spices

The most commonly used assays to determine antioxidant activity in the food industry are chemical tests. Antioxidant activity can be monitored by a variety of assays which are grouped into four different methodologies as follows: 1) radical/ROS scavenging methods, such as ABTS/TEAC (Trolox equivalent antioxidant capacity) assay, and DPPH (2,2-diphenyl-1-picrylhydrazyl) assay; 2) non-radical redox potential-based methods, such as FRAP (ferric reducing antioxidant power) assay; 3) metal chelation capacity; and 4) total phenolic content (TPC) (Shahidi and Zhong, 2015). However, each type of assays has their limitations. For example, the results of ABTS/TEAC could be affected by their incubation times (Roginsky and Lissi, 2005), and the DPPH assay is only suitable for certain foods (Plank et al., 2012). Both TEAC and DPPH assays have been challenged for their lack in the biological relevance due to the use of artificial radical cation which is not found in food nor biological systems (Shahidi and Zhong, 2015). FRAP results might vary depending on the analysis time observed for the reaction between antioxidants and  $Fe^{3+}$  (Pulido et al., 2000). The total phenolic content assay is sensitive to changes in pH, temperature and reaction time, and the results could be overestimated by the non-phenolic reducing agents in the reaction system (Blasco et al., 2005). In Table 1, the anti-oxidative strengths of 19 commonly consumed spices in China were compared using ABTS, DPPH, FRAP, and total phenolic content assays. The results were similar to that of Risch and Ho's (1997) report in which spices are generally rich sources of antioxidants.

In Table 1, the anti-oxidant abilities determined by ABTS and FRAP ranged from 12.42 to 593.90  $\mu\text{mol TE g}^{-1}\text{ DW}$  and 37.80 to 771.00  $\mu\text{mol TE g}^{-1}\text{ DW}$ , respectively. The total phenolic content ranged from 3.53 to 58.25 mg GAE  $\text{g}^{-1}\text{ DW}$  among the spices (Table 1). The total phenolic content evaluates the anti-oxidant ability of phenolic content only, while ABTS and FRAP detect the ability of phenolic compounds in addition to other compounds such as flavonoids (Shahidi and Zhong, 2015). As a result, the anti-oxidant abilities of spices are different when different chemical assays are used, e.g. galangal and dried ginger using ABTS, DPPH, FRAP, and total phenolic content assays. Based on the results of ABTS, FRAP and total phenolic content assays, galangal had the highest strength among the 19 spices been tested (Table 1). However, its DPPH value was only 88.30%, much lower than

**Table 1** Comparison of anti-oxidant abilities among the 19 commonly consumed spices in China<sup>A, B</sup>

Common and scientific name	Botanical family	Edible parts tested	ABTS ( $\mu\text{mol TE g}^{-1} \text{ DW}$ ) <sup>C</sup>	DPPH Inhibition %	FRAP ( $\mu\text{mol TE g}^{-1} \text{ DW}$ ) <sup>C</sup>	Total phenolic content (mg GAE $\text{g}^{-1} \text{ DW}$ ) <sup>D</sup>
Galangal ( <i>Alpinia galanga</i> )	Zingiberaceae	Rhizome	593.90 $\pm$ 15.5 <sup>a</sup>	88.30 $\pm$ 0.39 <sup>bc</sup>	771.00 $\pm$ 8.72 <sup>a</sup>	58.25 $\pm$ 1.89 <sup>a</sup>
Dried ginger ( <i>Zingiber officinale</i> )	Zingiberaceae	Rhizome	75.66 $\pm$ 1.15 <sup>hi</sup>	32.38 $\pm$ 1.42 <sup>g</sup>	157.95 $\pm$ 2.2 <sup>h</sup>	9.20 $\pm$ 0.50 <sup>ghi</sup>
Villous amomum fruit ( <i>Fructus amomi</i> )	Zingiberaceae	Fruit	83.47 $\pm$ 2.08 <sup>gh</sup>	35.06 $\pm$ 1.14 <sup>f</sup>	102.30 $\pm$ 1.09 <sup>i</sup>	8.99 $\pm$ 0.09 <sup>ghi</sup>
Tsaoko amomum fruit ( <i>Fructus tsaolo</i> )	Zingiberaceae	Fruit	187.83 $\pm$ 5.16 <sup>f</sup>	78.08 $\pm$ 0.44 <sup>d</sup>	107.50 $\pm$ 0.66 <sup>ij</sup>	22.09 $\pm$ 0.26 <sup>d</sup>
Green prickleyash ( <i>Zanthoxylum schinifolium</i> Sieb. et Zucc)	Rutaceae	Fruit	405.01 $\pm$ 12.32 <sup>c</sup>	94.12 $\pm$ 0.24 <sup>a</sup>	585.25 $\pm$ 22.54 <sup>c</sup>	46.37 $\pm$ 1.08 <sup>b</sup>
Sichuan pepper ( <i>Zanthoxylum piperitum</i> )	Rutaceae	Fruit	368.47 $\pm$ 8.92 <sup>d</sup>	93.41 $\pm$ 0.30 <sup>a</sup>	461.50 $\pm$ 7.94 <sup>e</sup>	38.86 $\pm$ 2.34 <sup>c</sup>
Dried tangerine peel ( <i>Citrus reticulata</i> )	Rutaceae	Peel	43.00 $\pm$ 1.98 <sup>klm</sup>	16.83 $\pm$ 0.86 <sup>kl</sup>	124.60 $\pm$ 4.68 <sup>i</sup>	10.04 $\pm$ 0.55 <sup>gh</sup>
Bay leaf ( <i>Laurus nobilis</i> )	Lauraceae	Leaves	412.24 $\pm$ 12.59 <sup>c</sup>	89.20 $\pm$ 0.39 <sup>b</sup>	504.25 $\pm$ 26.74 <sup>d</sup>	46.79 $\pm$ 3.22 <sup>b</sup>
Cinnamon ( <i>Cinnamomum zeylanicum</i> )	Lauraceae	Bark	525.85 $\pm$ 27.65 <sup>b</sup>	87.45 $\pm$ 0.33 <sup>c</sup>	637.00 $\pm$ 46.78 <sup>b</sup>	45.24 $\pm$ 2.41 <sup>b</sup>
Fennel ( <i>Foeniculum vulgare</i> )	Umbelliferae	Fruit	55.77 $\pm$ 1.61 <sup>kl</sup>	25.03 $\pm$ 0.55 <sup>h</sup>	72.40 $\pm$ 1.24 <sup>k</sup>	6.76 $\pm$ 0.11 <sup>j</sup>
Cumin ( <i>Cuminum cyminum</i> )	Umbelliferae	Seeds	57.41 $\pm$ 2.73 <sup>jk</sup>	18.12 $\pm$ 0.35 <sup>j</sup>	68.70 $\pm$ 0.70 <sup>k</sup>	9.00 $\pm$ 0.15 <sup>ghi</sup>
Angelica dahurica root ( <i>Angelicae dahurica</i> )	Umbelliferae	Root	41.44 $\pm$ 2.73 <sup>jk</sup>	10.19 $\pm$ 0.78 <sup>m</sup>	35.20 $\pm$ 1.48 <sup>l</sup>	7.86 $\pm$ 0.21 <sup>ij</sup>
Nutmeg ( <i>Myristica fragrans</i> )	Myristicaceae	Fruit	213.91 $\pm$ 17.65 <sup>e</sup>	88.70 $\pm$ 0.48 <sup>b</sup>	369.50 $\pm$ 2.98 <sup>f</sup>	18.23 $\pm$ 0.55 <sup>e</sup>
Star anise ( <i>Illicium verum</i> )	Illicium	Fruit	188.13 $\pm$ 6.28 <sup>f</sup>	76.83 $\pm$ 0.90 <sup>e</sup>	255.40 $\pm$ 4.48 <sup>g</sup>	14.94 $\pm$ 0.08 <sup>f</sup>
Dried chili pepper ( <i>Capsicum annuum</i> )	Solanaceae	Fruit	41.92 $\pm$ 1.86 <sup>lm</sup>	8.31 $\pm$ 0.98 <sup>n</sup>	38.70 $\pm$ 1.31 <sup>l</sup>	9.30 $\pm$ 0.42 <sup>ghi</sup>
Mustard ( <i>Brassica nigra</i> )	Brassicaceae	Seed	39.21 $\pm$ 1.28 <sup>m</sup>	17.89 $\pm$ 0.71 <sup>jk</sup>	93.95 $\pm$ 1.37 <sup>j</sup>	7.62 $\pm$ 0.35 <sup>ij</sup>
White pepper ( <i>Piper nigrum</i> L.)	Piperaceae	Fruit	12.42 $\pm$ 0.32 <sup>n</sup>	5.35 $\pm$ 0.22 <sup>o</sup>	37.80 $\pm$ 1.55 <sup>j</sup>	3.53 $\pm$ 0.02 <sup>k</sup>
13 Scented spices powder	Mixed spices	NA	91.14 $\pm$ 0.79 <sup>g</sup>	22.48 $\pm$ 1.74 <sup>i</sup>	111.30 $\pm$ 2.07 <sup>ij</sup>	10.36 $\pm$ 0.13 <sup>g</sup>
Curry powder	Mixed spices	NA	63.68 $\pm$ 0.99 <sup>ij</sup>	16.39 $\pm$ 0.08 <sup>l</sup>	62.35 $\pm$ 2.09 <sup>k</sup>	8.28 $\pm$ 0.04 <sup>hij</sup>

A, Values were expressed as means  $\pm$  standard deviation.

B, Values marked by the same low-case superscript letters (from 'a' to 'o') within a column denote statistically significant differences ( $P < 0.05$ ).

C, Data were expressed as  $\mu\text{mol}$  Trolox equivalent (TE) per 1 g dry weight (DW).

D, Data were expressed as mg gallic acid equivalent (GAE) per 1 g dry weight (DW).

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that of Green prickleyash and Sichuan pepper which were 94.12% and 93.41%, respectively (Table 1). In Table 2, selected spices with their antioxidants commonly consumed in China were listed. Phenolic compounds and flavonoids are important antioxidants in spices, such as green prickleyash, Sichuan pepper, bay leaf, cinnamon, etc (Table 2). Most of the functional compounds could be found in the essential oils, such as in the clove and the cinnamon, which also contribute to the unique odor of the spices (Wei and Shibamoto, 2010).

The antioxidant abilities of different spices are affected by the differences in the chemical structures of the active components. For example, chemical structures of the pure ginger components (6-gingerol (6GE), 8-gingerol (8GE), 10-gingerol (10GE), and 6-shogaol (6SGE)) contain one hydroxyl group in their aromatic ring that contributes to their antioxidant properties (Fig. 1) (Pietta, 2000). The long hydrocarbon side chains present in the four compounds make them very soluble in oil, which in turn increases their ability to act as hydrophobic antioxidants (Fig. 1). However, their anti-oxidation abilities are not the same, which may be due to their differences in the hydrocarbon chain length (Fig. 2). In Figure 1, 6SGE contains a C=C double bond which is able to donate electrons easily and be oxidized. The side chain of 6GE contains a more polar hydroxyl group (–OH) which reduces its hydrophobicity as antioxidant in oil. Owing to the decrease in hydrophobicity, 6SGE has a higher anti-oxidant ability at 60 °C than that of 6GE in low fat foods (Fig. 2). The hydrophobicity increases with increasing length of the carbon side chain (Fig. 1), making 10GE more soluble in oil, and thus also a reasonably better hydrophobic antioxidant than that of 6GE (Fig. 2) (Si et al., 2018).

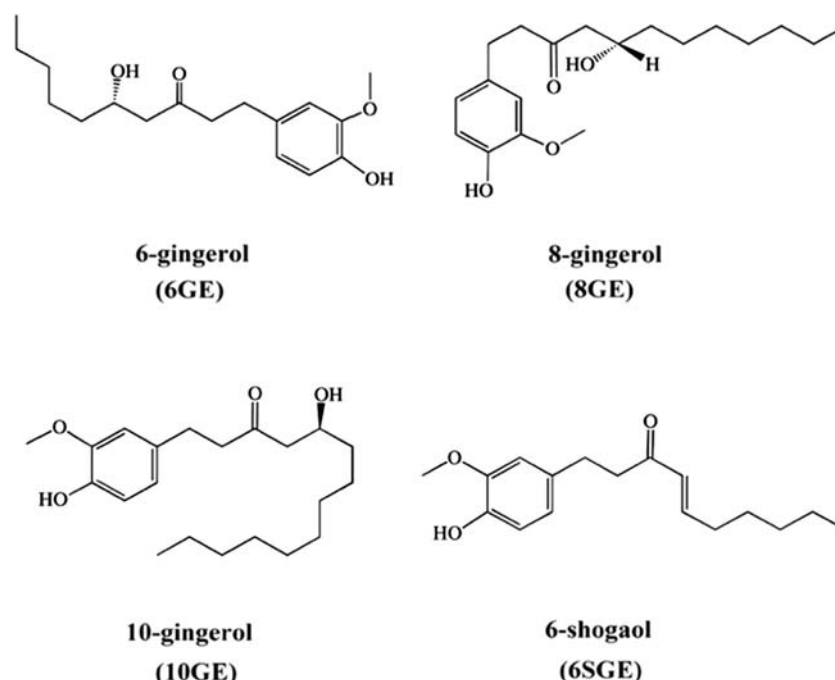
## Sensory Descriptors of Selected Spices Consumed in China

Sensory evaluation has been defined as a scientific method used to evoke, measure, analyze, and interpret those responses to products as perceived through the senses of sight, smell, touch, taste, and hearing (Stone and Sidel, 2004). Descriptors are a lexical language used in a sensory analysis to present sensation verbally by a subject when consuming food. When spices are served to panelists, different descriptors could be generated to profile them. Table 3 is an example of the sensory descriptors generated and used for the evaluation of the Chinese-style marinated chicken. To perform this evaluation, 25 consumers (ages between 20 and 30) were asked to rate the sensory properties of the products using six descriptors on color, texture, and flavor (Table 3) on the 10-cm line scales with increasing strength from left to right (Yusop et al., 2010).

In the Chinese cuisine, spices are often added to foods to mask the bloody or fishy flavor when preparing meat or fish dishes. Table 4 lists the odor descriptors in selected spices consumed in China. Ginger is a popular spice to increase the warm, spicy, and

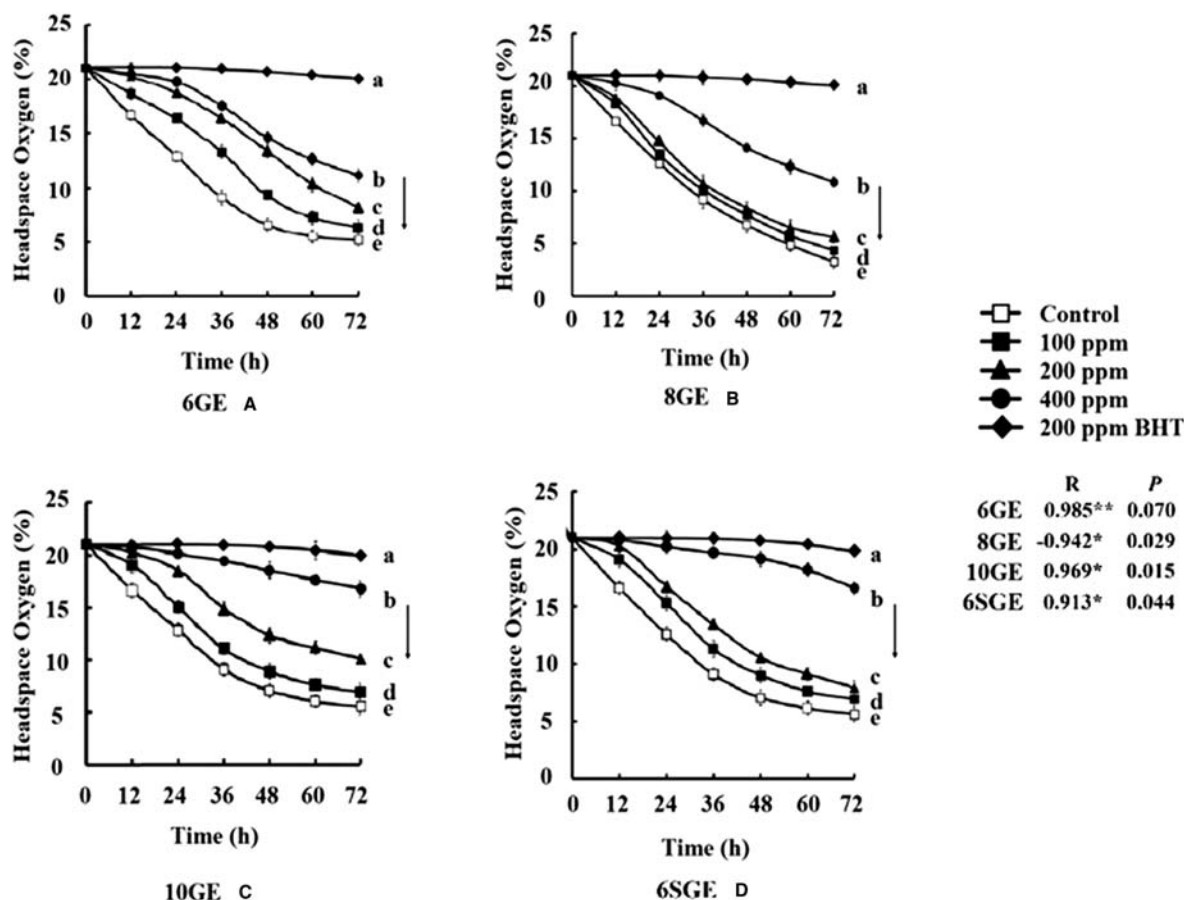
**Table 2** Antioxidant compounds in selected spices consumed in China

No.	Names	Anti-oxidant compounds
1	Galangal	Galangal (Lu et al., 2011)
2	Dried ginger	Gingerols, shogaols, zingerone (Embuscado, 2015)
3	Villous amomum fruit	Catechin and other flavonoids (Lu et al., 2011)
4	Tsaoko amomum fruit	Protocatechuic acid, and other phenolic acids; flavonoids (Lu et al., 2011)
5	Green prickleyash	Phenolic volatile oils (estragole, xanthoxylin), chlorogenic acid and other phenolic acids; quercetin, rutin and other flavonoids (Lu et al., 2011; Shan et al., 2005)
6	Sichuan pepper	Chlorogenic acid and other phenolic acids; rutin and other flavonoids (Lu et al., 2011)
7	Dried tangerine peel	Chlorogenic acid and other phenolic acids; rutin, naringin and other flavonoids (Lu et al., 2011)
8	Bay leaf	Phenolic acids; rutin, naringin and other flavonoids (Lu et al., 2011)
9	Cinnamon	Phenolic acids, phenolic volatile oil (2-hydroxycinnamaldehyde, cinnamyl aldehyde derivatives), flavan-3-ols (Shan et al., 2005)
10	Fennel	<i>trans</i> -Anethole, fenchone, estragole, limonene, camphene, $\alpha$ -pinene, fenchyl alcohol, anisaldehyde, myristicin, dillapiol (Lu et al., 2011; Hossain et al., 2008; Parthasarathy et al., 2008)
11	Cumin	Chlorogenic acid, ferulic acid and other phenolic acids; apigenin, luteolin, and other flavonoids (Lu et al., 2011)
12	Angelica dahurica root	Chlorogenic acid, caffeic acid, ferulic acid and other phenolic acids (Lu et al., 2011)
13	Nutmeg	Phenolic volatile oils, phenolic acid (caffeic acid, protocatechuic acid), rutin and other flavonoids, flavanols (Lu et al., 2011; Shan et al., 2005)
14	Star anise	Phenolic acids (protocatechuic acid), phenolic volatile oils (anethole), flavonoids (Shan et al., 2005)
15	Dried red chili pepper	Capsaicin, capsaicinol (Embuscado, 2015)
16	Mustard	Phenolic acids (Lu et al., 2011)
17	Black pepper	Volatile oils, phenolic amides, kaempferol, rhamnetin, quercetin (Embuscado, 2015; Shan et al., 2005)
18	White pepper	Volatile oils, phenolic amides (Shan et al., 2005)
19	Cloves	Phenolic acids (gallic acid), flavonol glucosides, phenolic volatile oils (eugenol, acetyl eugenol, isoeugenol), tannins (Embuscado, 2015; Shan et al., 2005)



**Figure 1** Structures of four constituents isolated from ginger: 6-gingerol (6GE), 8-gingerol (8GE), 10-gingerol (10GE), and 6-shogaol (6SGE). Reproduced from Si, W., Chen, Y.P., Zhang, J., Chen, Z.-Y., Chung, H.Y., 2018. Antioxidant activities of ginger extract and its constituents toward lipids. *Food Chem.* 239, 1117–1125, with the permission from Elsevier.

sweet flavors to food (Table 4), contributed by components such as  $\beta$ -sesquiphellandrene and (-)- $\alpha$ -curcumene,  $\alpha$ -Terpineol, citral  $\alpha$  and citral  $\beta$  and nerolidol  $\beta$  (Bednarczyk and Kramer, 1975). Huajiao is a very important ingredient in the preparation of the Chinese Sichuan cuisine. The most consumed species are the red huajiao (*bungeanum*) and the green huajiao (*schinifolium*) (Yang, 2008). Consumers could perceive the fresh, spicy, floral, cooling and green aroma in huajiao which contains a high content of essential oil (11%) in their raw state. In addition, most of them contribute distinctive tingling taste sensation (Table 4) (Yang,



**Figure 2** Percentage of headspace oxygen depletion determined for samples of canola oil containing 0, 100, 200, and 400 ppm of (A) 6-geringol (6GE), (B) 8-geringol (8GE), (C) 10-geringol (10GE), and (D) 6-shagaol (6SGE) and heated at 60 °C. Modified from Si, W., Chen, Y.P., Zhang, J., Chen, Z.Y., Chung, H.Y., 2018. Antioxidant activities of ginger extract and its constituents toward lipids. *Food Chem.* 239, 1117–1125, with the permission from Elsevier. Only Anti-oxidative ability of 6GE, 8GE, 10GE, and 6SGE heated at 60 °C were presented here.

**Table 3** Sensory terms used in the consumer evaluation of Chinese-style marinated chicken

Attribute	Description	
Color	Dark pink	0 = Light pink, 10 = dark pink
	Penetration	0 = No penetration, 10 = extreme penetration
Texture	Toughness	0 = Extremely tender, 10 = extremely tough
	Juiciness	0 = Extremely dry, 10 = extremely juicy
Flavor	Sourness	0 = Not sour, 10 = extremely sour
	Sweetness	0 = Not sweet, 10 = extremely sweet

Modified from Yusop, S.M., O'Sullivan, M.G., Kerry, J.F., Kerry, J.P., 2010. Effect of marinating time and low pH on marinade performance and sensory acceptability of poultry meat. *Meat Sci.* 85, 657–663, with the permission from Elsevier.

2008). Red chili pepper is another important ingredient in the Chinese Sichuan cuisine. Capsaicin (*trans*-8-methyl-N-vanillyl-6-nonenamide) is a major pungent ingredient in the chili pepper (Govindarajan and Salzer, 1986). The other sensorial feeling of chili pepper is contributed by flavor components such as 2,3-butanedione (caramel), 1-penten-3-one (pungent, spicy) and hexanal (grassy, herbal).

## Application in Food Industry

“Off-flavor” note in the rancid foods is perceived at very low threshold value which is contributed by the secondary oxidation products from lipid, including alcohols, aldehydes, ketones, hydrocarbons, volatile organic acids and epoxy compounds (Frankel, 1996; Kolakowska, 2003). Since concerns over the safety of the food additives have arisen in recent years, consumer demand for the use of

**Table 4** Odor descriptors in selected spices consumed in China

No.	Name	Odor descriptor
1	Galangal	Resembling ginger but with a distinct soapy overtone (Gautschi et al., 1999)
2	Dried ginger	Warm, spicy bite, a little bit sweet, not as strongly flavored as fresh ginger <sup>a</sup>
3	Villous amomum fruit	Pungent, warm <sup>b</sup>
4	Tsaoko amomum fruit	Specially fragrant, pungent, slight bitter <sup>c</sup>
5	Sichuan Huajiao	fresh, spicy, floral, cooling, and green aroma notes, distinctive tingling taste (Yang, 2008)
6	Dried tangerine peel	Sweet, fruity, incense, mellow
7	Bay leaf	Fresh green, eucalyptus, flowery, clove, black pepper (Kilic et al., 2004)
8	Cinnamon	Bark oil: A delicate aroma along with a sweet, pungent taste (Barceloux, 2009) Leaf oil: a warm, spicy and somewhat harsh odor that lacks the smooth consistency of bark oil (Barceloux, 2009)
9	Fennel	Metallic, anise-like, eucalyptus-like, almond-like, fir tree-like, moldy, geranium-like, citrus-like, vanilla-like, seasoning-like (Zeller and Rychlik, 2006)
10	Cumin	Seed oil: cumin, fatty, sweaty, pepper <sup>d</sup>
11	Angelica dahurica root	Acrid, warm, and with ascending and dispersing aroma in nature <sup>e</sup>
12	Nutmeg	Oil: sweet, woody, nutmeg, spicy with a slight terpy nuance <sup>d</sup>
13	Dried Red Chili Pepper	Caramel, pungent, spicy, grassy, herbal, fruity, sweet, almond (Junior et al., 2012)
14	Black pepper	Pine-like, warm-pepper (woody), pine-like(woody), warm-resinous, camphoraceous, then citrusy, floral-woody(citrusy), lilac-like (floral), spicy (clove-like), floral, woody-spicy, resinous, woody, floral, woody-floral (Pino et al., 1990)
15	White pepper	Fecal, swine-manure, horse-like, phenolic, cheese-like (Jagella and Grosch, 1999)
16	Cloves	Bud oil: sweet, woody, eugenol, spicy, medicinal, fresh and slightly balsamic with a nice bite <sup>d</sup>
17	Sesame seeds	Oil: popcorn-like, pungent, sweet, oily, solvent, peanut-like, burnt, aldehydic, spicy, anise-like, green (Shimoda et al., 1996)

<sup>a</sup>Data from Website: <https://www.thekitchn.com/inside-the-spice-cabinet-ground-ginger-211363>.

<sup>b</sup>Data from Website: <http://herbal.tcm.su.polyu.edu.hk/herbal/villous-amomum-fruit>.

<sup>c</sup>Data from Website: <https://www.myhealthwisdom.com/products/cao-guo-fructus-tsaoko-tsaoko-amomum-fruit>.

<sup>d</sup>Data from Website: <http://www.thegoodscentscompany.com/odor/clove.html>.

<sup>e</sup>Data from Website: <http://www.chineseherbshealing.com/bai-zhi-angelica-dahurica-angelica-root/>.

natural products as alternative preservatives in particularly fatty foods has increased (Govarís et al., 2010). Spices have been reported to contribute significantly to the stabilization of food color, aroma, and texture (Jinap et al., 2015; Yusop et al., 2010). In the presence of spices, oxidation of fats/oils and lipid-containing foods is prevented by excluding the initiator and promoter elements which promoted the lipid oxidation during processing and refrigerated storage (Barden and Decker, 2016). The application of spices in the meat industry could be very desirable. In fact, one of the common practices is to marinate meat with spices to extend its shelf life (Radha krishnan et al., 2014). Meat marinating with spices is not only popular in Asian countries, such as Malaysia and Singapore (Jinap et al., 2015), but also in Europe (Leatherhead Food International, 2007). China was the world's largest producer of meat in 2009 (28% of the world's total production), its meat products including both Chinese style (45%) and western style (55%) ones, but the value-added and enhanced products had a low market share (Zhou et al., 2012). Marination is a traditional Chinese meat-processing method with a long history (Li et al., 2016). In order to offer healthier and more flavored products, the combination of modern meat industrial technologies and spices is needed (Li et al., 2016).

Owing to the large variety and the amount of components in the essential oil of spices, components such as phenolic acids can not only inhibit lipid oxidation but control foodborne pathogens at the same time (Risch and Ho, 1997). Spices could slow down rancidity and provide flavor (Dwivedi et al., 2006), leading to the retardation of spoilage and the extension of shelf-life, thus, maintaining the desirable quality and safety of food (Devatkal and Naveena, 2010). For example, Chinese 5-Spices often used in the preparation of ground beef can be used to extend the shelf-life of meat-based products for both shopkeepers and consumers (Dwivedi et al., 2006). The rancid odor and flavor from the spices-treated cooked ground beef were found to be significantly lower after 15-day storage at 2 °C when compared to that of the control (Dwivedi et al., 2006). The addition of spice extracts (*Syzygium aromaticum*, *Cinnamomum cassia*, *Origanum vulgare*, *Brassica nigra*, and their mixtures) effectively maintained or improved sensory attributes and extended the shelf-life of raw chicken meat during refrigerated storage (Table 5) (Radha krishnan et al., 2014). Sensory evaluation from experienced judges concluded that no significant differences in the sensory scores on the attributes between the chicken meats treated with and without spice extracts in the first 3 days of storage (Table 5). But the sensory scores of the control samples (positive control with 0.02% BHT; and negative control with no extract) started to decline after 3 days of storage, but not for the spice extract-treated samples (Table 5). The poor sensory scores in the control samples were due to the off-odor generated from lipid oxidation and protein degradation (Radha krishnan et al., 2014).

Both marination time and pH affect sensory properties of spices marinated meat (Yusop et al., 2010). In Table 6, Chinese-style marinated chicken was subjected to different marination times (30, 60, 120 and 180 min) and acidity (pH 3.0, 3.2, 3.4, 3.6, 3.8, 4.0 and 4.2), the sensory attributes (dark pink, color penetration, toughness, sourness, sweetness and juiciness) of the cooked products were evaluated. Results showed that all attributes were significantly ( $P < 0.05$ ) affected by marination time (Table 6). Dark pink color increased when marination time was extended. Samples marinated for 120 min received a consistently higher score in every



**Table 5** Effect of spice extracts on sensory properties (odor and taste) of raw chicken meat stored at 4 °C

Sample	Days					
	0	3	6	9	12	15
<b>Odor</b>						
NC	9.0 ± 0.4	7.7 ± 0.2	6.3 ± 0.3	4.9 ± 0.4	2.9 ± 0.3	1.8 ± 0.2
PC	9.0 ± 0.4	7.6 ± 0.4	6.1 ± 0.3	5.2 ± 0.3	4.2 ± 0.3	2.9 ± 0.3
T-W-SA	9.0 ± 0.4	8.6 ± 0.3	7.8 ± 0.2	6.7 ± 0.4	6.3 ± 0.2	4.5 ± 0.1
T-W-CC	9.0 ± 0.4	8.5 ± 0.3	7.5 ± 0.4	6.4 ± 0.1	6.2 ± 0.4	4.3 ± 0.3
T-W-OV	9.0 ± 0.4	8.6 ± 0.1	7.2 ± 0.3	6.3 ± 0.3	5.7 ± 0.3	4.1 ± 0.2
T-W-BN	9.0 ± 0.4	7.9 ± 0.4	6.8 ± 0.1	5.9 ± 0.3	5.6 ± 0.4	4.2 ± 0.3
T-W-SA + T-W-CC	9.0 ± 0.4	8.6 ± 0.3	7.9 ± 0.4	6.8 ± 0.3	6.5 ± 0.3	4.8 ± 0.4
T-W-SA + T-W-OV	9.0 ± 0.4	8.6 ± 0.2	7.8 ± 0.3	6.7 ± 0.4	6.4 ± 0.2	4.6 ± 0.2
T-W-CC + T-W-OV	9.0 ± 0.4	8.5 ± 0.3	7.3 ± 0.3	6.9 ± 0.1	6.2 ± 0.3	4.7 ± 0.3
T-W-SA + T-W-CC + T-W-OV	9.0 ± 0.4	8.6 ± 0.4	8.1 ± 0.4	7.4 ± 0.4	6.8 ±	6.1 ± 0.2
<b>Taste</b>						
NC	9.0 ± 0.4	7.5 ± 0.4	5.2 ± 0.2	3.6 ± 0.3	NT	NT
PC	9.0 ± 0.4	7.8 ± 0.3	5.7 ± 0.3	4.1 ± 0.4	NT	NT
T-W-SA	9.0 ± 0.4	8.6 ± 0.3	7.7 ± 0.4	7.2 ± 0.2	6.1 ± 0.2	4.7 ± 0.2
T-W-CC	9.0 ± 0.4	8.6 ± 0.4	7.6 ± 0.3	7.2 ± 0.4	6.1 ± 0.2	4.6 ± 0.4
T-W-OV	9.0 ± 0.4	8.6 ± 0.3	7.3 ± 0.2	7.1 ± 0.3	6.3 ± 0.3	4.5 ± 0.3
T-W-BN	9.0 ± 0.4	8.2 ± 0.4	7.1 ± 0.3	6.9 ± 0.4	5.4 ± 0.4	4.3 ± 0.4
T-W-SA + T-W-CC	9.0 ± 0.4	8.7 ± 0.3	7.8 ± 0.4	7.5 ± 0.3	6.4 ± 0.3	4.9 ± 0.3
T-W-SA + T-W-OV	9.0 ± 0.4	8.7 ± 0.2	7.8 ± 0.2	7.6 ± 0.4	6.3 ± 0.3	4.8 ± 0.2
T-W-CC + T-W-OV	9.0 ± 0.4	8.6 ± 0.3	8.1 ± 0.4	7.3 ± 0.3	6.3 ± 0.3	4.8 ± 0.2
T-W-SA + T-W-CC + T-W-OV	9.0 ± 0.4	8.7 ± 0.2	8.3 ± 0.3	7.8 ± 0.2	6.5 ± 0.3	5.1 ± 0.4

NT: not tasted.

NC: negative control; no extract.

PC: positive control with 0.02% BHT.

T-W-SA: Treatment with *Syzygium aromaticum* (1% v w<sup>-1</sup>).T-W-CC: Treatment with *Cinnamomum cassia* (1% v w<sup>-1</sup>).T-W-OV: Treatment with *Origanum vulgare* extract (1% v w<sup>-1</sup>).T-W-BN: Treatment with *Brassica nigra* (1% v w<sup>-1</sup>).T-W-SA + T-W-CC: Treatment with *Syzygium aromaticum* (0.5% v w<sup>-1</sup>) + *Cinnamomum cassia* (0.5% v w<sup>-1</sup>).T-W-SA + T-W-OV: Treatment with *Syzygium aromaticum* (0.5% v w<sup>-1</sup>) + *Origanum vulgare* (0.5% v w<sup>-1</sup>).T-W-CC + T-W-OV: Treatment with *Cinnamomum cassia* (1% v w<sup>-1</sup>) + *Origanum vulgare* (0.5% v w<sup>-1</sup>).T-W-SA + T-W-CC + T-W-OV: Treatment with *Syzygium aromaticum* (0.33% v w<sup>-1</sup>) + *Cinnamomum cassia* (0.33% v w<sup>-1</sup>) + *Origanum vulgare* (0.33% v w<sup>-1</sup>).Reproduced from Babuskin, S., Babu, P.A.S., Sasikala, M., Sabina, K., Archana, G., Sivarajan, M., Sukumar, M., 2014. Antimicrobial and antioxidant effects of spice extracts on the shelf life extension of raw chicken meat. *Int. J. Food Microbiol.* 171, 32–40, with the permission from Elsevier.**Table 6** Effect of marinating time and pH on sensory attributes of cooked marinated chicken

	Attributes					
	Dark pink	Color penetration	Toughness	Sourness	Sweetness	Juiciness
<b>Marinating time (min)</b>						
30	4.99 ± 2.18 <sup>b</sup>	3.05 ± 2.14 <sup>a</sup>	3.68 ± 2.22 <sup>a</sup>	3.14 ± 2.37 <sup>b</sup>	4.06 ± 2.11 <sup>c</sup>	5.54 ± 2.00 <sup>a</sup>
69	5.01 ± 2.48 <sup>b</sup>	2.75 ± 2.08 <sup>ab</sup>	4.01 ± 2.53 <sup>a</sup>	3.32 ± 2.48 <sup>ab</sup>	3.72 ± 2.32 <sup>bc</sup>	5.84 ± 2.00 <sup>a</sup>
120	5.05 ± 2.28 <sup>b</sup>	2.60 ± 1.92 <sup>bc</sup>	3.51 ± 2.39 <sup>a</sup>	3.51 ± 2.56 <sup>a</sup>	4.37 ± 2.56 <sup>a</sup>	5.69 ± 2.14 <sup>a</sup>
180	5.55 ± 2.33 <sup>a</sup>	2.42 ± 1.85 <sup>c</sup>	3.70 ± 2.61 <sup>a</sup>	3.59 ± 2.68 <sup>a</sup>	4.02 ± 2.71 <sup>ab</sup>	5.51 ± 2.30 <sup>a</sup>
<b>Marinade pH</b>						
3.0	4.64 ± 2.09 <sup>cd</sup>	2.36 ± 1.74 <sup>b</sup>	3.21 ± 2.55 <sup>c</sup>	3.36 ± 2.48 <sup>a</sup>	3.66 ± 2.31 <sup>a</sup>	6.04 ± 2.23 <sup>a</sup>
3.2	4.59 ± 2.26 <sup>d</sup>	2.54 ± 1.83 <sup>ab</sup>	3.10 ± 2.19 <sup>c</sup>	3.20 ± 2.45 <sup>a</sup>	3.68 ± 2.38 <sup>a</sup>	5.72 ± 2.05 <sup>ab</sup>
3.4	5.38 ± 2.23 <sup>b</sup>	2.91 ± 2.16 <sup>a</sup>	3.86 ± 2.46 <sup>ab</sup>	3.16 ± 2.47 <sup>a</sup>	3.89 ± 2.49 <sup>a</sup>	5.64 ± 2.24 <sup>ab</sup>
3.6	4.95 ± 2.40 <sup>bcd</sup>	2.60 ± 1.86 <sup>ab</sup>	3.89 ± 2.18 <sup>ab</sup>	3.52 ± 2.57 <sup>a</sup>	3.90 ± 2.33 <sup>a</sup>	5.46 ± 2.21 <sup>bc</sup>
3.8	5.27 ± 2.33 <sup>b</sup>	2.95 ± 2.14 <sup>a</sup>	3.47 ± 2.32 <sup>bc</sup>	3.34 ± 2.52 <sup>a</sup>	4.11 ± 2.48 <sup>a</sup>	5.71 ± 2.15 <sup>ab</sup>
4.0	5.15 ± 2.37 <sup>bc</sup>	2.76 ± 2.06 <sup>ab</sup>	4.16 ± 2.54 <sup>a</sup>	3.59 ± 2.64 <sup>a</sup>	4.24 ± 2.53 <sup>a</sup>	5.06 ± 2.19 <sup>c</sup>
4.2	6.07 ± 2.33 <sup>a</sup>	2.82 ± 2.21 <sup>a</sup>	4.39 ± 2.61 <sup>a</sup>	3.57 ± 2.57 <sup>a</sup>	5.18 ± 2.61 <sup>a</sup>	2.00 ± 1.95 <sup>bc</sup>

Values correspond to mean data ± corresponds to standard deviation.

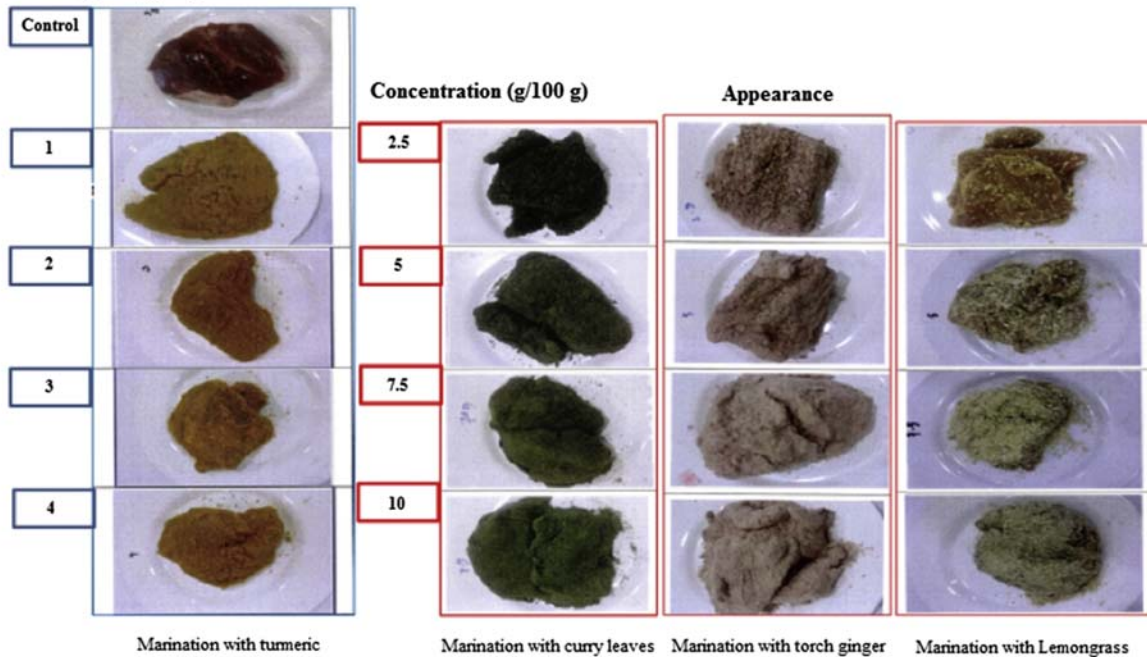
a-d With different letters in columns are significantly different ( $P < 0.05$ ).Modified from Yusop, S.M., O'Sullivan, M.G., Kerry, J.F., Kerry, J.P., 2010. Effect of marinating time and low pH on marinade performance and sensory acceptability of poultry meat. *Meat Sci.* 85, 657–663, with the permission from Elsevier.



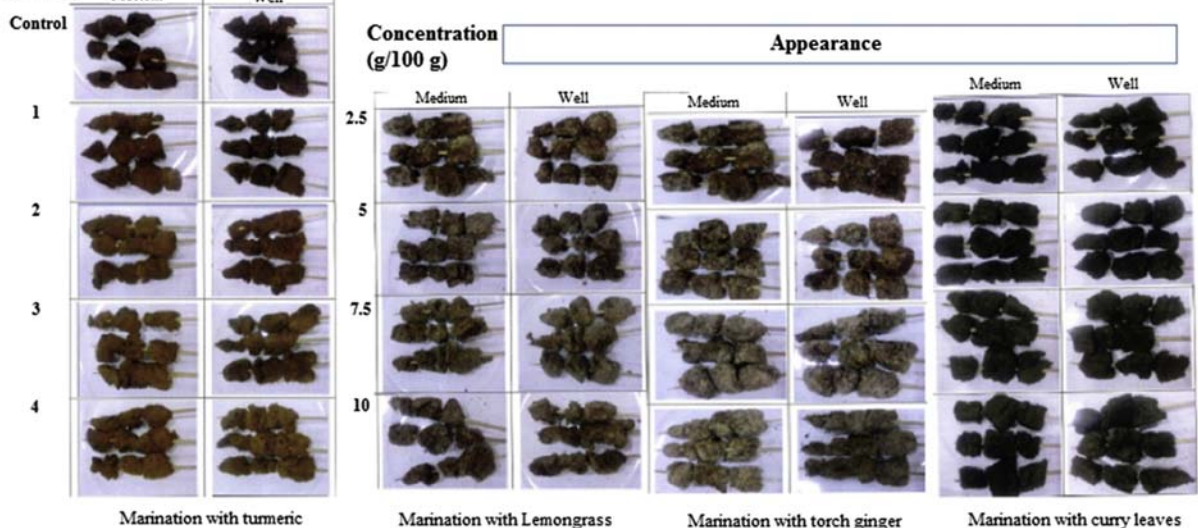
sensory attribute measured than other samples (Table 6). Sourness and sweetness were not found to be significantly affected by the marinade pHs. But the intensity of juiciness decreased significantly when marinade pH increased from 3.0 to 4.2 (Table 6).

Heterocyclic aromatic amines (HAs) are important mutagens/carcinogens generated during the cooking of meat at high temperature (Jägerstad and Skog, 2005), which also increase the risk of cancer (Zheng and Lee, 2009). However, they could be reduced by marinating the meat with spices (Jinap et al., 2015; Viegas et al., 2012). For example, Viegas et al. (2012) reported the inhibitory effect of antioxidant-rich meat flavoring (garlic, ginger, thyme, rosemary, and red chili pepper) reduced 90% HAs formed in the pan-fried beef. After 4-h marination, all beef samples had good overall quality even though only the surface was treated (Viegas et al., 2012). Fig. 3 shows the observed results on the color intensity of grilled meat previously marinated with selected spices (turmeric) at the concentrations of 1, 2, 3, and 4 g/100 g and curry leaves, torch ginger, and Lemongrass at the concentrations of 2.5, 5, 7.5, and 10 g/100 g. Browning of cooked meat suggested the formation of chemical complexes from the Maillard reaction (Jinap et al.,

#### A Concentration (g/100 g) Appearance



#### B Concentration (g/100 g) Appearance



**Figure 3** (A): The effect of different concentrations of selected spices i.e. turmeric, curry leaves, torch ginger and lemon grass on meat. (B): The effect of different concentrations of selected spices i.e. turmeric, lemon grass, torch ginger and curry leaves on satay. Reproduced from Jinap, S., Iqbal, S.Z., Selvam, R.M., 2015. Effect of selected local spices marinades on the reduction of heterocyclic amines in grilled beef (satay). LWT Food Sci. Technol. 63, 919–926 with the permission from Elsevier.

2015). All medium-heat cooked meats (controls), as well as the lemongrass- and torch ginger-treated ones showed less browning than that of the well-cooked meats (Fig. 3). Apparently, marinating meats with spices reduced their browning while samples without marination had more browning (Smith et al., 2008).

## Conclusions

Spices used in the preparation of Chinese dishes are rich sources of antioxidant and flavor making them good alternative source of food preservative and flavoring. Chemical assays are often used to determine the strength of antioxidants in spices. The antioxidant abilities of different spices are affected by the content of their phenolic, flavonoids and other compounds, as well as by the different chemical structures of the active components. Since spices possess both antioxidant and unique flavor, their application, particularly in the meat products, is more desirable.

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## Anthocyanins in Food

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### Chemical Structure of Anthocyanins

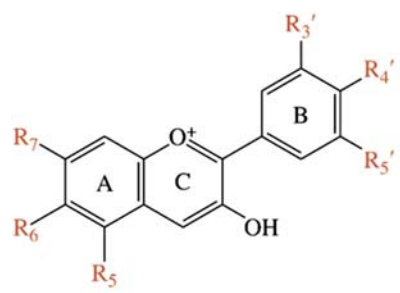
Being known for centuries as “coloured cell sap”, anthocyanins were named for the first time in 1835 by German botanist Ludwig Marquart from the Greek words of *anthos* (means flower) and *kyanos* (means blue) (Boldt et al., 2014). Anthocyanins are among the most important part of substances known as flavonoids, which are bioactive water-soluble plant pigments. They are widely extracted from flowers (e.g. peony, morning glory and geranium), vegetables (e.g. radish, curly kale, eggplant), and fruits (e.g. grapes, blueberries, cherries) where they are responsible for bright colours including red, blue, purple, and many more (Chung et al., 2015; Fernandes et al., 2013; Wiczowski et al., 2014).

Anthocyanins exist in plants in the form of glycosides predominantly, and the basic structure of anthocyanins contains an anthocyanidin core that is attached with sundry sugars and organic acids in the case of acylated anthocyanins. Anthocyanin core, also called anthocyanidin, consists of two benzoyl rings (A ring and B ring) and a heterocyclic ring (C ring), presenting a C6—C3—C6 basic skeleton with a total of 15 carbons (Fig. 1). To date, over seven-hundred anthocyanins have been identified from various plants; however, there are about twenty-three anthocyanidins (Andersen and Jordheim, 2014). According to the different numbers and positions of hydroxyl and methoxyl groups in the A and B rings, there are some selected anthocyanidins that are distinguished and valued by specialists (Fig. 1) (Cavalcanti et al., 2011; Chung et al., 2015; Tsuda, 2012).

An anthocyanidin can yield different types of anthocyanins through glycosylating or acylating different sugar moieties and phenolic or aliphatic acids (Bueno et al., 2012b). The most common glycoside derivatives found in nature are 3-monosides, 3-bisides, 3,5-, and 3,7-diglucosides (Kong et al., 2003). Cyanidin glycoside is the most predominant anthocyanin discovered in many fruits and vegetables (Boldt et al., 2014). The distribution of anthocyanins in some fruits is shown in Table 1 (Patras et al., 2010). Some common types of glucosides with different molecular weight (MW) for anthocyanins are pentoside moiety (MW: 132 Da), rhamnoside moiety (146 Da), glucoside moiety (162 Da), rutinoid moiety (rhamnose + glucose, 308 Da), neohesperidoside moiety (308 Da), sophoroside moiety (324 Da), glucosylrutinoside moiety (rhamnose + glucose + glucose, 470 Da), acetylglucoside moiety (204 Da), acetylrutinoside moiety (350 Da), and coumaroylglucoside moiety (308 Da).

### Function of Anthocyanins

Anthocyanins are known as natural antioxidants due to their ability to donate protons to highly reactive free radicals (Sadilova et al., 2007). In plants, anthocyanins are considered to act as photoprotectants preventing plants from the free radicals that are formed during photosynthesis (Hiemori et al., 2009). The antioxidant activity of anthocyanins is conferred by their chemical structure: the number and organisation of phenyl groups, the availability of electron-donating and electron-withdrawing groups in the ring structure, the degree of structural conjugation and the positive charge of anthocyanins (Castañeda-Ovando et al., 2009). Anthocyanins are reported to prevent further radical generation and protect cells from oxidative damage which is often associated with aging and various diseases caused by oxidative stress (Bueno et al., 2012a). Recently, anthocyanins were found to possess anti-inflammatory ability through modulating the NF-κB signaling pathways, thereby inhibiting the expression of proinflammatory cytokines (such as TNF-α, IL-6, and IL-10), the production of the inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), and the utterance of adhesion molecules (ICAMs and VCAM-1) (Vendrame and Klimis-Zacas, 2015).



Anthocyanidin	R <sub>5</sub>	R <sub>6</sub>	R <sub>7</sub>	R <sub>3</sub> '	R <sub>4</sub> '	R <sub>5</sub> '
Aurantidin	OH	OH	OH	H	OH	H
Capensinidin	OCH <sub>3</sub>	H	OH	OCH <sub>3</sub>	OH	OCH <sub>3</sub>
Cyanidin	OH	H	OH	OH	OH	H
Delphinidin	OH	H	OH	OH	OH	OH
Europinidin	OCH <sub>3</sub>	H	OH	OCH <sub>3</sub>	OH	OH
Hirsutidin	OH	H	OCH <sub>3</sub>	OCH <sub>3</sub>	OH	OCH <sub>3</sub>
Malvidin	OH	H	OH	OCH <sub>3</sub>	OH	OCH <sub>3</sub>
Pelargonidin	OH	H	OH	H	OH	H
Peonidin	OH	H	OH	OCH <sub>3</sub>	OH	H
Petunidin	OH	H	OH	OH	OH	OCH <sub>3</sub>
Pulchellidin	OCH <sub>3</sub>	H	OH	OH	OH	OH
Rosinidin	OH	H	OCH <sub>3</sub>	OCH <sub>3</sub>	OH	H

Figure 1 Chemical structure of selected anthocyanidins.



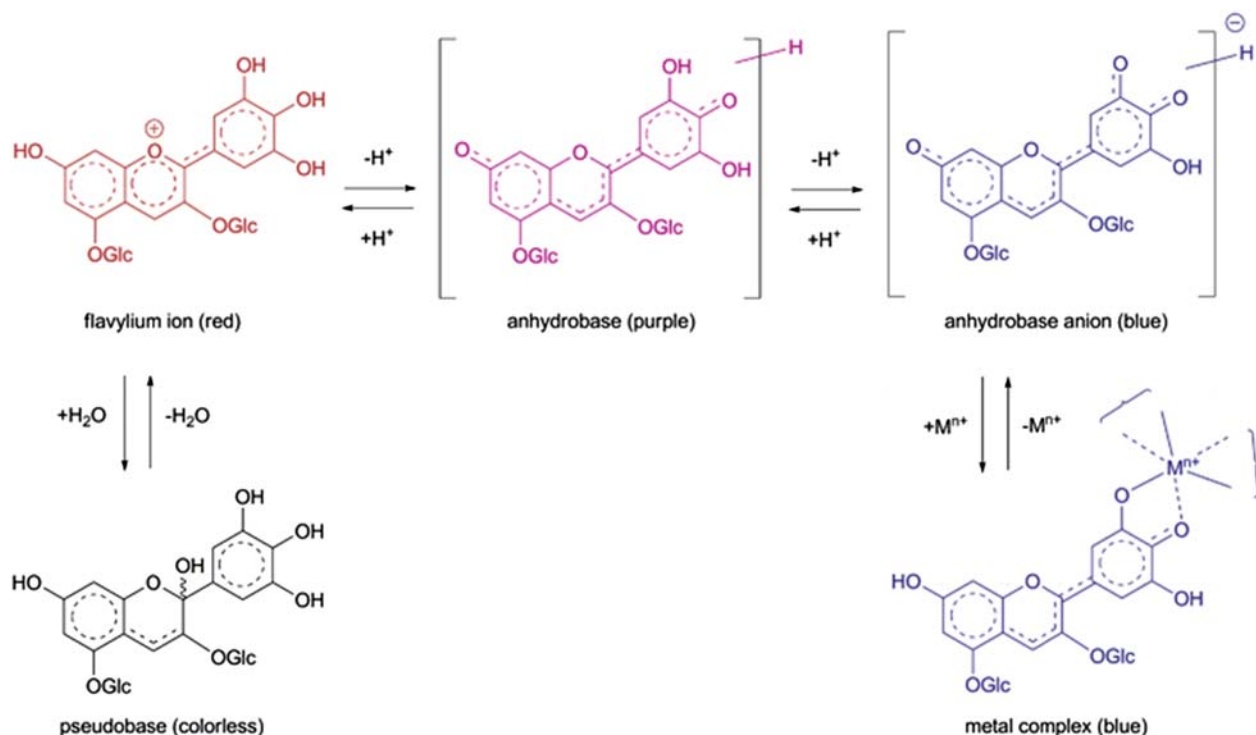
**Table 1** Occurrence of anthocyanins in some fruits

Sources	Major anthocyanin	Minor anthocyanins
Strawberry	cyanidin-3-glucoside	cyanidin-3-glucoside, pelargonidin-3-rutinoside
Blackberry	cyanidin-3-glucoside	cyanidin-3-rutinoside, malvidin-3-glucoside
Raspberry	cyanidin-3-glucoside	pelargonidin-3-glucoside, pelargonidin-3-rutinoside
Sweet cherry	cyanidin-3-rutinoside	cyanidin-3-glucoside, peonidin-3-rutinoside
Blackcurrant	cyanidin-3-rutinoside	cyanidin-3-glucoside, delphinidin-3-glucoside
Bilberry	delphinidin-3-galactoside	pelargonidin-3-glucoside, pelargonidin-3-galactoside

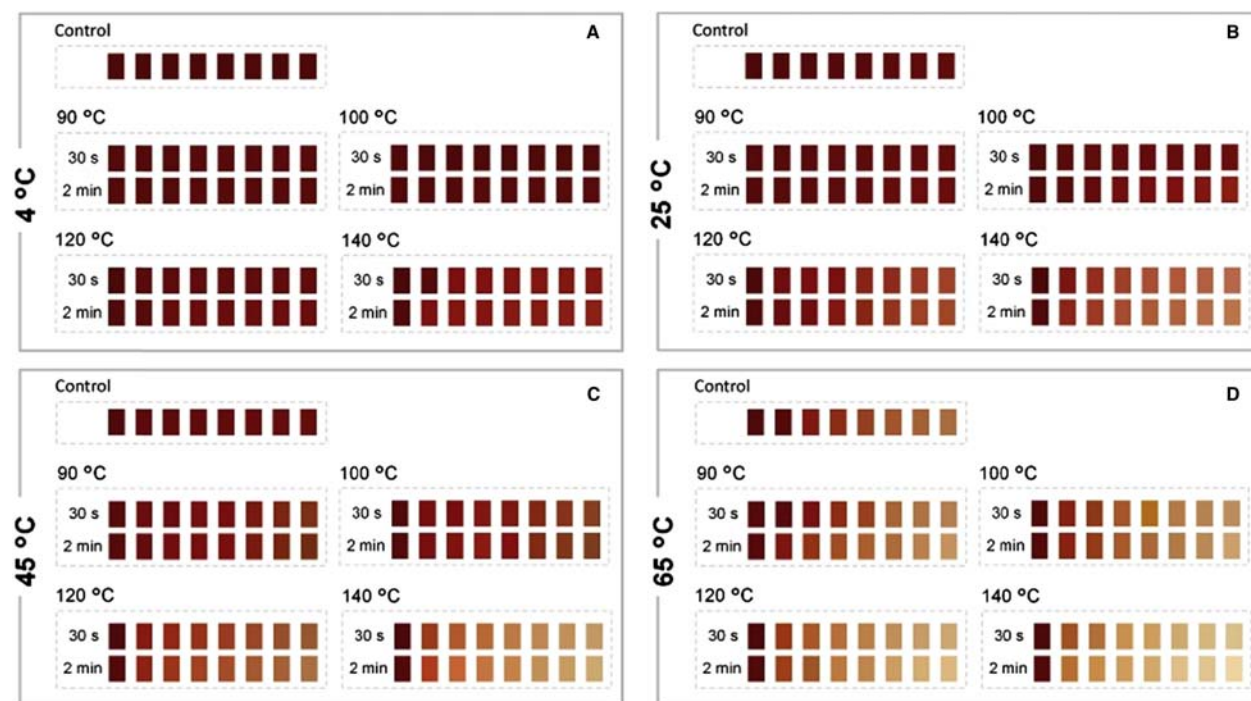
The intake of anthocyanins in the US diet was previously estimated to be between 180 and 215 mg per day, whereas the intake of other dietary flavonoids, such as genistein, quercetin and apigenin, was only between 20 and 25 mg per day (Hertog et al., 1993). However, the National Health and Nutrition Examination Survey (NHANES) 2001–2 highlighted the inaccurate anthocyanins intake data in the previous study, and pointed out that the average intake of anthocyanins in the US was much lower and at about 12.5 mg per day. Of the different aglycones, cyanidin, delphinidin, and malvidin were estimated to contribute 45, 21, and 15%, respectively, to the total intake of anthocyanins per day. Besides, nonacylated anthocyanins predominantly contributed 77% to the total intake of anthocyanins per day (Wu et al., 2006).

### Colour of Anthocyanins

Anthocyanins give plants their colours, which is important for their survival as they attract pollinators (Glover and Martin, 2012). Their range of brilliant colours has led to industrial use of anthocyanins as natural colourants in foods as substitutes to synthetic food colourants, and they have been recognised as one of the nine European Union selected natural colour classes. The resonance structure of anthocyanidin, comprising the flavylium cation (also known as 2-phenylbenzopyrylium) enables anthocyanins to act as colourants (Castañeda-Ovando et al., 2009). Within the same class of anthocyanins, there is a variation in colour, e.g. the increase of hydroxylation in the B-ring results in a bathochromatic shift from red to violet (pelargonidin → cyanidin → delphinidin) (Mateus and de Freitas, 2009). It has been found that the colour of anthocyanins varies significantly with pH (Fernandes et al., 2014). The pH dependent structural changes of anthocyanidin and resulting colour are illustrated in Fig. 2 (Yoshida et al.,



**Figure 2** pH dependent structural changes in the anthocyanidin chromophore and the resulting colour changes in aqueous solution. From Yoshida, K., Mori, M., Kondo, T., 2009. Blue flower color development by anthocyanins: from chemical structure to cell physiology. Nat. Product. Rep. 26 (7), 884–915, with permission.



**Figure 3** Colour swatches for anthocyanin in aqueous solution during storage. From Sui, X., Bary, S., Zhou, W., 2016. Changes in the color, chemical stability and antioxidant capacity of thermally treated anthocyanin aqueous solution over storage. *Food Chem.* 192, 516–524, with permission.

2009). Furthermore, the differences among various colours contributed by anthocyanins are found to be determined, to a great degree, by two groups of factor: internal factors and external ones. The internal factors are the structure of anthocyanins; with an increase of hydroxylation and the presence of methoxyl groups in the B ring, the hue of anthocyanins becomes lighter. The external factors, such as temperature, pH, and the type of solvent, also have a profound impact on the colour of anthocyanins (Mazza, 2007; Tsuda, 2012). For example, our previous work reported that anthocyanin aqueous solutions with or without a heat treatment showed excellent colour at the storage temperature of 4 °C; however, the anthocyanin aqueous solutions thermally treated at higher temperatures lost colour more easily than those heated at lower temperatures throughout storage (Fig. 3) (Sui et al., 2016a).

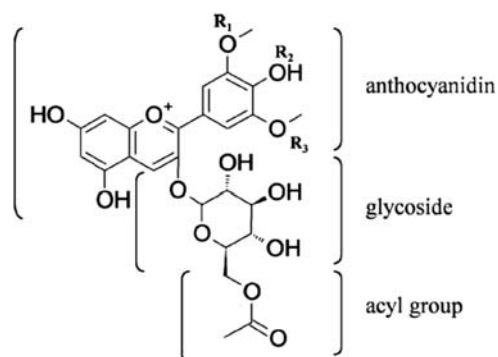
### Stability of Anthocyanins

The stability of anthocyanin compounds in a food matrix is dependent on several factors, such as their chemical structure, temperature, pH, light intensity, oxygen availability, and solid content of the matrix (He et al., 2016). In particular, temperature is reported as one of the major factors that impact the stability of anthocyanins. Moreover, anthocyanins show a greater stability in acidic media than in alkaline media (Cavalcanti et al., 2011). Meanwhile, isolated anthocyanins are unstable and highly susceptible to degradation (Giusti and Wrolstad, 2003).

### Chemical Structure Influence

The stability of anthocyanins is influenced by their chemical structure, i.e. substitutes in the B ring and the presence of additional hydroxyl (–OH) or methoxyl (–OCH<sub>3</sub>) groups (Fleschhut et al., 2006). Anthocyanins with complex structures of glycosylation and acylation exhibit remarkable stability to pH changes, thermal treatment, light exposure, etc (Dangles et al., 1993). Acylated anthocyanins are found to be more stable than their corresponding non-acylated forms. An example of an acylated anthocyanin is shown in Fig. 4. The acylation reaction is believed to enhance the stability of anthocyanins through protecting them from hydration (Brouillard, 1981; Goto, 1987). Acylated anthocyanins appear to prolong their half-life and slow down their decaying process under mild thermal treatment (Sadilova et al., 2006). However, high heat can easily break the chemical structure of acylated anthocyanins. The degradation of acylated anthocyanins starts from the degradation of their glycosylated and acylated groups, followed by hydrolyzing the sugar, leaving an anthocyanidin structure solely (Prasain et al., 2004).





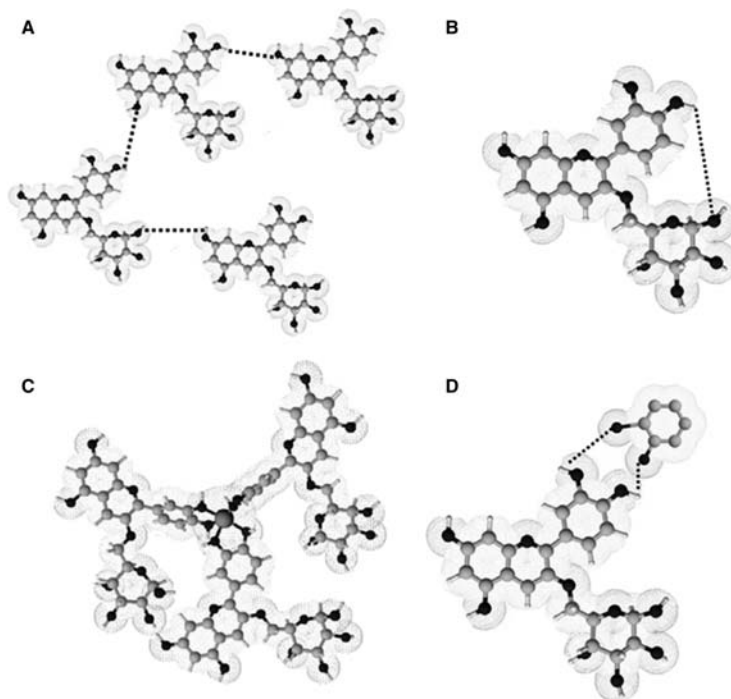
**Figure 4** Chemical structure of an acylated anthocyanin, malvidin-3-(6''-acetyl)glucoside (monoisotopic mass = 535.14517 amu). From Barnes, J.S., Nguyen, H.P., Shen, S., Schug, K.A., 2009. General method for extraction of blueberry anthocyanins and identification using high performance liquid chromatography-electrospray ionization-ion trap-time of flight-mass spectrometry. *J. Chromatogr. A*. 1216 (23), 4728–4735, with permission.

### Co-pigmentation Influence

Co-pigmentation is a phenomenon during which the pigments and other colourless organic compounds or metallic ions form molecular or complex associations (Boulton, 2001). The stabilization of anthocyanins could be improved through intramolecular and intermolecular co-pigmentations, self-association, and metal complexing (Fig. 5) (Malien-Aubert et al., 2001). Thus, co-pigmentation of anthocyanins is the major mechanism for the stabilization of anthocyanins and their colour in plants (Davies and Mazza, 1993). Dyrby et al. (2001) reported that the co-pigmentation of anthocyanins in red cabbage offered the anthocyanins with greater stability than the isolated anthocyanins from blackcurrant, grape skin and elderberry in a soft drink model system.

### Temperature Influence

Temperature has been reported as one of the major factors in determining the stability of anthocyanins. The stability of anthocyanins was fast decreased as temperature rises (Maccarone et al., 1985). Total monomeric anthocyanins extracted from Bordo grape skin showed a loss of 95.1% at 55 °C and 90% R.H. for 35 days (Kuck et al., 2017). Castagnini et al. (2015) observed that increasing



**Figure 5** Anthocyanins co-pigmentation (A) self-association (B) intramolecular co-pigmentation (C) metal complexation, and (D) intermolecular co-pigmentation. From Castañeda-Ovando, A., Pacheco-Hernández, M.d.L., Páez-Hernández, M.E., Rodríguez, J.A., Galán-Vidal, C.A., 2009. Chemical studies of anthocyanins: a review. *Food Chem.* 113 (4), 859–871, with permission.

the temperature from 30 to 50 °C caused the loss of total anthocyanins in impregnated apple increased from 70% to 75%. Similarly, a decrease of 68% of total anthocyanins in mortino fruits was found during the drying at 60 °C for 10 h (López-Vidaña *et al.*, 2016). Even at lower temperatures, the degradation of anthocyanins is still a concern. Hellström *et al.* (2013) reported that the half-life ( $t_{1/2}$ ) of anthocyanins in both laboratory-prepared and commercial berry juices was much shorter at room temperature than at 4 °C and 9 °C. In our laboratory, we observed that in an aqueous solution the content of anthocyanins extracted from black rice was relatively constant at low temperatures (4 and 25 °C); however it significantly decreased at 120 and 140 °C (Sui *et al.*, 2016a).

### pH Influence

Anthocyanins are found in different chemical forms depending on the pH of the environment (Costa *et al.*, 1998; Fleschhut *et al.*, 2006). Typically, anthocyanins are more stable in acidic media of low pH than alkaline ones of high pH (Castañeda-Ovando *et al.*, 2009). At the pH of 1, flavylium cation is a dominant species with purple and red colours. At the pH between 2 and 4, quinoidal form is predominant with blue colour. At the pH between 5 and 6, two colourless species can be observed, which are carbinol pseudobase and chalcone. When the pH is greater than 7, the degradation of anthocyanins occurs, which depends on their substituents (Castañeda-Ovando *et al.*, 2009).

### Oxygen Influence

The presence of oxygen accelerates the degradation process of anthocyanins (Cavalcanti *et al.*, 2011). Specifically, this influence of oxygen can take place through a direct oxidative mechanism or through the action of oxidising enzymes, such as polyphenol oxidase (PPO). PPO catalyses the oxidation of anthocyanins to form brown condensation products (Patras *et al.*, 2010). It was reported that oxygen-restricted atmospheres retained the initial antioxidant capacity and anthocyanin content of fresh-cut strawberries over a storage period of 25 days (Odrizola-Serrano *et al.*, 2009). Under modified atmosphere storage conditions, the oxygen content of 60%–100% was found to increase the content of anthocyanins of freshly harvested blueberries in the initial period (0–7 days) of cold storage, however the content of anthocyanins was decreased with prolonging the storage (Zheng *et al.*, 2003).

### Solid Content and Water Activity Influence

Anthocyanins showed faster degradation in more concentrated liquid systems. Garzón and Wrolstad (2001) reported that the degradation of anthocyanins was greater in concentrates compared to juices in their study evaluating the stability of anthocyanins in strawberry juices and concentrates. Similar trends have been reported for anthocyanins in sour cherry juice (Cemeroglu *et al.*, 1994). This could be due to the closeness of reacting molecules in juices of higher soluble solid content (Patras *et al.*, 2010).

In solid/semi-solid systems, water activity is another factor influencing the stability of anthocyanins. Anthocyanins exhibited remarkable stability when stored in dry crystalline form (Markaris *et al.*, 1957). The degradation of anthocyanins in freeze-dried strawberry puree increased when being stored at increasing relative humidity conditions among 11%, 32%, 57%, 75% and 100% (Erlandson and Wrolstad, 1972). It was also reported that reducing water activity from 1.0 to 0.44 enhanced the stability of anthocyanins in model systems that were prepared using glycerol and pH 3.4 citrate buffer (Garzón and Wrolstad, 2001).

### Degradation of Anthocyanins

Knowledge about degradation mechanisms of anthocyanins is relatively little (Patras *et al.*, 2010; Sadilova *et al.*, 2006). It was believed that anthocyanins were decomposed via two possible pathways to end up with either chalcone and coumaric acid glycosides or aldehyde and benzoic acid derivatives (Patras *et al.*, 2010). Anthocyanins would decompose upon heating into a chalcone structure, the latter being further transformed into a coumarin glucoside derivative with the loss of the B-ring (Hrazdina, 1971). Sadilova *et al.* (2007) reported successive deglycosylation reactions in the initial steps of anthocyanin degradation at the pH of 1, yielding the respective aglycones. The sugar-free anthocyanidin aglycones latter were cleaved into a phenolic acid and a phenolic aldehyde. The aglycone-sugar bond is found to be more labile than other glycoside bonds at the pH of 2 to 4. However, at the pH of 1 all glycosidic bonds are accessible to hydrolysis; e.g. heating cyanidin-3-rutinoside at the pH of 1 results in the formation of rhamnose and glucose, but only traces of rutinose (Adams, 1973). In the case of acylated anthocyanins, the degradation of the anthocyanins was slowed down because the intermediate compounds formed during degradation were coloured flavylium cation before further degradation proceeds the same way as for their glycoside derivatives (Sadilova *et al.*, 2006). Through kinetic and Monte Carlo modeling, the degradation of cyanidin-3-glucoside and cyanidin-3-rutinoside can be modeled as first-order reactions in both aqueous solutions and semi-solid foods such as bread (Sui and Zhou, 2014; Sui *et al.*, 2014, 2015).

### Interactions of Anthocyanins With Proteins

The interactions between phenolic compounds and proteins are known to affect the structure of proteins, content of free polyphenols, antioxidant capacity and bioavailability of phenolic compounds in foods (Ozdal *et al.*, 2013). Specifically, both covalent and

non-covalent interactions between proteins and phenolic compounds frequently occur in food systems (Jakobek, 2015), which were reported to change the structural, functional, and nutritional properties of proteins (Ozdal et al., 2013). The non-covalent interactions between proteins and polyphenols are via hydrogen bonding and hydrophobic bonding and are reversible, i.e. van der Waals forces are involved (Arroyo-Maya et al., 2016; Ghorbani Gorji et al., 2015; Wang and Wang, 2015). In the case of covalent interactions, polyphenols are oxidized to quinones under alkaline conditions and incorporated into the side chains of proteins (Ozdal et al., 2013). They can irreversibly interact with the sulfhydryl and amino groups of proteins to form covalent C–N or C–S bonds with the phenolic rings. It was shown that anthocyanins were more likely to form covalent interactions with soy protein isolates (SPI) instead of non-covalent interactions (Sui et al., 2018). The addition of anthocyanins in SPI sodium phosphate buffer at the concentration of 0.05, 0.1%, and 0.2% (w/v) changed the secondary structure of SPI with a decreased amount of  $\beta$ -sheets and an increased amount of  $\beta$ -turns and random coils. The emulsifying and foaming properties of SPI were improved after its complexation with anthocyanins (Sui et al., 2018). Moreover, the complexation of SPI with anthocyanins was found to improve the digestibility of both native SPI and thermally denaturized SPI (Zhang et al., 2018). This observation suggested that anthocyanins may have an ability to alter the thermally-induced negative influence on the digestibility of SPI.

## Fortification of Anthocyanins in Foods

Potential use of natural “green” plant-extracted anthocyanins could offer the food processing industry an alternative solution to synthetic chemical antioxidants. Therefore, considering also the beneficial effect of these compounds on health, their incorporation in food products is of important value (Cevallos-Casals and Cisneros-Zevallos, 2004). Kazemi et al. (2017) fortified salted and yellow alkaline noodles with an anthocyanin extract from pomegranate peel, at 0.75% and 1.50% of the total weight of prepared noodles, to improve their antioxidant capacity. For bread fortified with anthocyanin-rich black rice extract powder at a relatively low level of 0.2% (i.e. 0.04% anthocyanins, flour based), the antioxidant capacity of its crumb decreased while that of the crust showed an increase (Sui et al., 2015). Bread fortified with various levels of anthocyanin-rich black rice extract powder (up to 4%, flour based) was further studied for its digestibility, and the rate of digestibility was found to be retarded by the anthocyanins in a dose-dependent manner (Sui et al., 2016b). Fortification of anthocyanins in foods may provide new opportunities to produce functional food products by improving their existing functionalities as well as creating new functionalities, thereby providing extra health benefits to consumers.

## Summary

Like many other polyphenols, stability and functionality are the two major issues about anthocyanins. Due to the varieties in their chemical structure, anthocyanins possess different stability. The environment factors, such as pH, temperature, and food components, are known to affect the stability of anthocyanins to certain extent. The antioxidant capacity of anthocyanins has been intensively studied over the years. However, variations in the antioxidant capacity of anthocyanins in food matrices and during metabolism are still unclear and debatable. Since the attention of today's consumers has been drawn to choose healthier foods, the application of anthocyanins in foods is expected to increase in the near future. Although the studies of anthocyanins have reached some milestones, clearly more research is still needed to well understand and confidently apply anthocyanins in foods in order to utilise, as well as further extend, their beneficial functionalities.

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# Caramelization in Foods: A Food Quality and Safety Perspective

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## Introduction

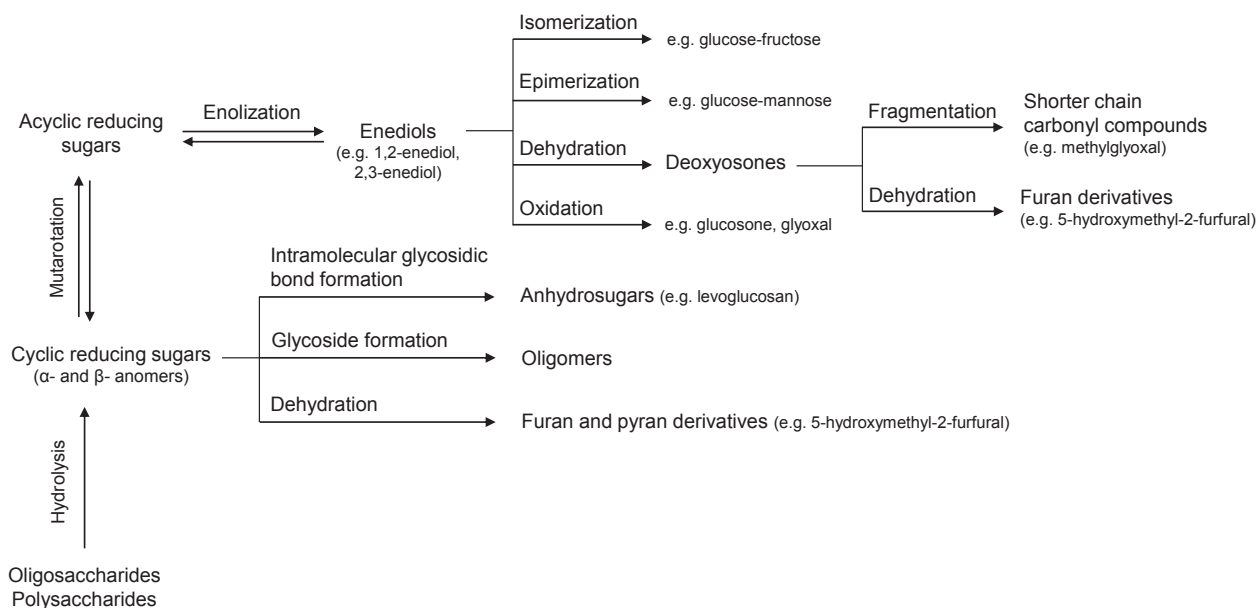
Caramelization is a nonenzymatic browning reaction of sugars providing a caramel-like flavor during high temperature treatment of foods. Degradation of sugars is catalyzed by amino acids during the Maillard reaction, which is characterized by nitrogen containing low and high molecular weight compounds. At elevated temperatures, both reactions proceed together in a way that one affects the other.

The Maillard reaction may take place under milder conditions, but sugars are caramelized at temperatures above 120°C. Food products reach these elevated temperatures during processes like roasting (180–240°C) and baking (160–240°C). After drying of the outer layers of bakery products during heating, the surface can reach temperatures above 100°C where the browning and flavor development begin (Mogol and Gökmen, 2014a,b). Caramelization reactions are also observed in jams, canned fruit products, fruit juices and concentrates, soft drinks, honey, and sugar syrups during thermal treatment or storage. At milder temperatures, pH below three and above nine are often required to observe reactions in moderately higher rates (Kroh, 1994).

Food caramels are commercially available as caramel color, burnt sugars, aromatic caramel, and caramelized sugar syrups. Burnt sugars, aromatic caramel, and caramelized sugar syrups are produced without using additives, while additives are used for caramel color production. Caramel color is used as a colorant in food and beverages, and it is not usually a flavoring.

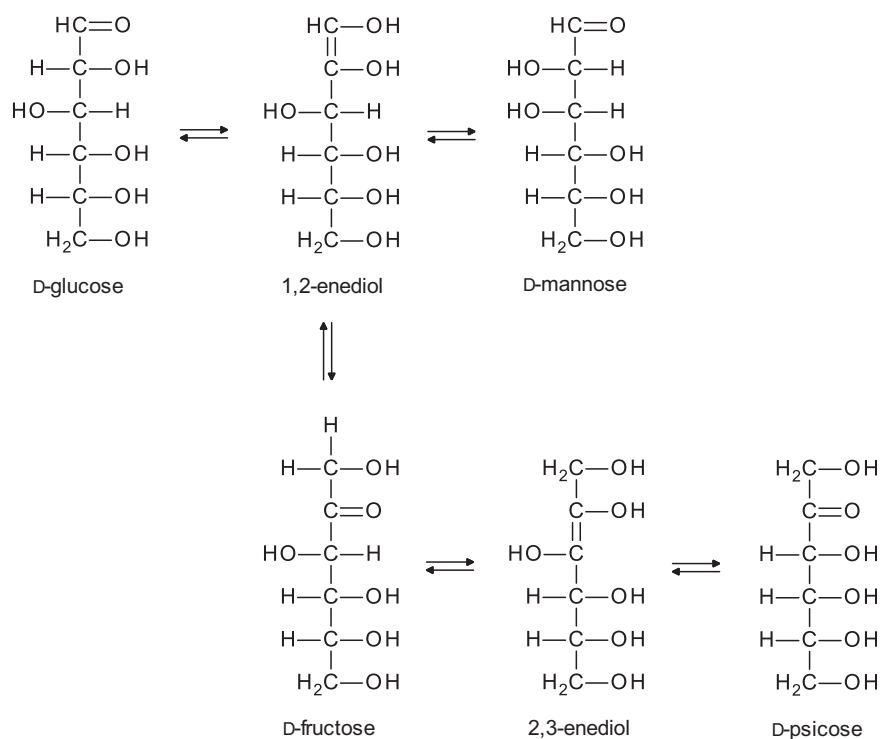
## Caramelization Reactions

The main reactions of sugar degradation are schematized in Fig. 1. The acyclic forms of sugars are very reactive, and more acyclic forms are found by increasing temperature. Isomerization, epimerization, dehydration, and oxidation reactions of the cyclic reducing sugar are initiated by ring opening followed by enolization (Speck, 1958). Aldose and ketose sugars isomerize via their common intermediate, 1,2-enediol. This isomerization also involves epimerization, that is, the change of the configuration of C-2 in aldoses and C-3 in ketoses. Glucose, fructose, and mannose are found in equilibrium in alkaline solutions (Fig. 2). Similarly, galactose, tagatose, and talose may interconvert each other. These aldose–ketose isomerization and epimerization reactions are known as the Lobry de Bruyn–Alberda van Ekenstein transformation (rearrangement) (LdB-AvE) (Angyal, 2001). Within these reactions, aldose–ketose interconversion predominates, and to a lesser extent epimerization is observed. Although the LdB-AvE rearrangement is observed faster in alkaline media, it can also occur limitedly in acidic media (Zhao et al., 2007). In addition to the alkaline conditions required for the isomerization, a considerable amount of glucose–fructose interconversion is observed during the heating of sugar-rich low-moisture foods and dry heating of sugars at elevated temperatures (Kocadağlı and Gökmen, 2016a,b).



**Figure 1** Main reactions of sugar degradation.





**Figure 2** Lobry de Bruyn-Alberda van Ekenstein transformation.

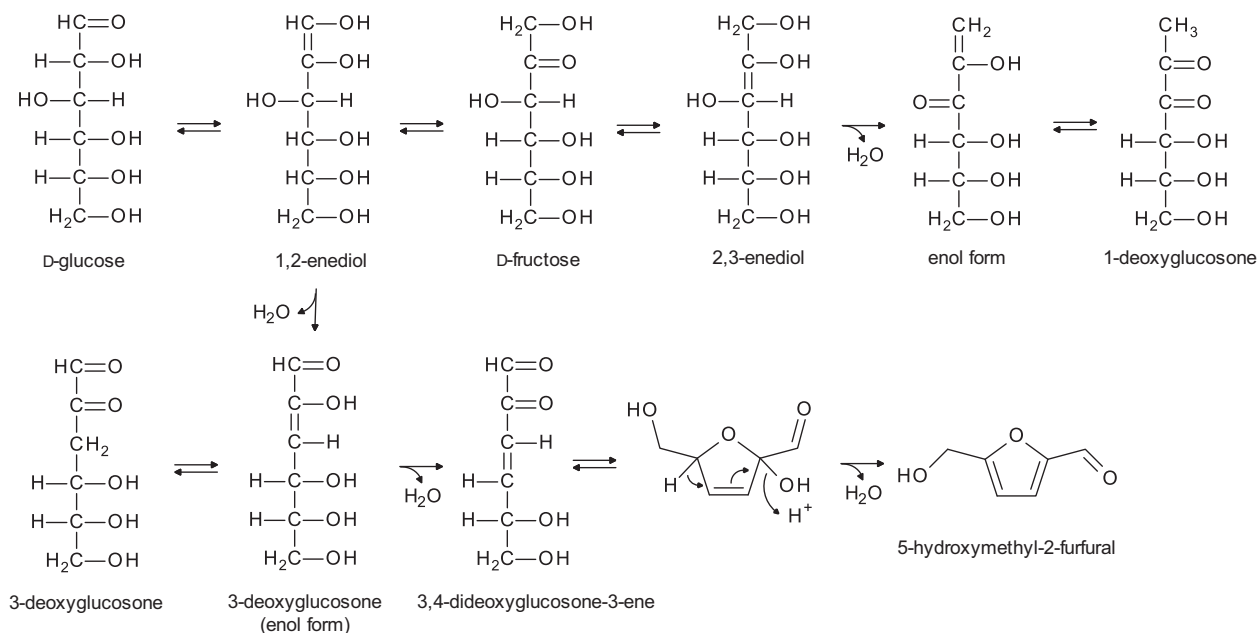
Aldose–ketose isomerization is substantially catalyzed in the presence of alkali and alkaline earth metal cations ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{+2}$ ) by increasing the rate of chain opening (Speck, 1958).

Aldose–ketose isomerization (LdB-AvE) is also observed in the reducing end of the oligosaccharides, such as maltose (4-*O*- $\alpha$ -D-glucopyranosyl-D-glucose) to maltulose (4-*O*- $\alpha$ -D-glucopyranosyl-D-fructose) and lactose (4-*O*- $\beta$ -D-galactopyranosyl-D-glucose) to lactulose (4-*O*- $\beta$ -D-galactopyranosyl-D-fructose). Maltose/maltulose ratio can be used as a marker of thermal input in certain cereal products such as cookies, crackers, breakfast cereals (García-Baños et al., 2004a), pasta (García-Baños et al., 2004b), and infant formula (Morales et al., 2004). Lactulose is an indigestible disaccharide with laxative and prebiotic properties. It can be formed during heating infant formula, milk, and milk products and can also be used as an ingredient. Isomerization reaction of lactose to lactulose during thermal treatment of milk is quantitatively more important than the Maillard reaction for the degradation of lactose (Berg, 1993).

In an acidic medium, enolization and subsequent  $\beta$ -elimination of 1–3 water molecules (dehydration) predominates. In an alkaline medium, enolization is followed by fragmentation reactions (including fragmentation after dehydrations and isomerizations) via splitting of carbon bonds, and subsequently recombination is observed via aldol addition. Enediols are oxidized via transition metal catalysis, such as  $\text{Cu}^{2+}$ , and also in the presence of oxygen.

During heating or drying of fruit products, heating of sugar with or without additives, and baking of sourdough, many dehydration products of sugars may form because of the acidic conditions of the products and high temperature. Enediols are also the key intermediates for the dehydration of sugars under acidic conditions. Dehydration refers to the removal of a water molecule by  $\beta$ -elimination mechanism from the carbon skeleton of a carbohydrate. Heating accelerates the dehydration especially under low moisture conditions and even in milder pH conditions because of an increment in acyclic forms. The dehydration may also occur under alkaline condition during heating, followed by extensive fragmentation. Dehydration of sugars produces several reactive deoxyosones, and more stable furan and pyran derivatives, which are important for the formation of caramel color and flavor.

3-Deoxyosone, having an  $\alpha$ -dicarbonyl structure, is formed by the removal of a water molecule from the third carbon of an aldohexose or a ketohexose (Fig. 3). When hexoses are glucose and fructose, 3-deoxyglucosone (3-deoxy-D-*erythro*-hexos-2-ulose) is formed. Similarly, galactose yields 3-deoxygalactosone (3-deoxy-D-*threo*-hexos-2-ulose) (Hellwig et al., 2010). Dehydration from C-4 of 3-deoxyglucosone produces 3,4-dideoxyglucosone-3-ene (3,4-dideoxy-D-*glycero*-hex-2-enos-2-ulose). Dehydration from a hemiacetal structure of 3,4-dideoxyglucosone-3-ene results in 5-hydroxymethyl-2-furfural (HMF, 5-hydroxymethylfuran-2-carbaldehyde). HMF is a rather stable product, and it is often used as an indicator of the thermal load applied to foods (Gökmen and Senyuva, 2006; Gökmen et al., 2007, 2008; Göncüoğlu and Gökmen, 2013). However, HMF might also behave as a reactive intermediate, especially at elevated temperatures, because of its carbonyl group (Nikolov and Yaylayan, 2011a,b; Gökmen et al., 2012).



**Figure 3** Dehydration of glucose and fructose.

Fructose gives rise to the formation of a wide variety of products because of its ability to be involved in both 1,2- and 2,3-enolization. Dehydration of fructose from C-1 through 2,3-enediol yields 1-deoxyglucosone (1-Deoxy-D-*erythro*-hexo-2,3-diulose) (Fig. 3). 1-Deoxyglucosone is the key reactive intermediate in the formation of many other reactive intermediates and important aroma compounds (Hirsch et al., 1995; Voigt and Glomb, 2009). Isomerization of 1-deoxyglucosone along the carbon chain is an important aspect in the formation of a wide range of products that are important for color and flavor development (Belitz et al., 2009).

Glucose and fructose oxidation produces glucosone (D-*arabino*-hexos-2-ulose) by removal of two protons, especially by transition metal catalysis and in the presence of oxygen. Nonoxidative degradation of glucosone yields C-5 intermediates, which are pentosone, 3-deoxypentosone, and 1-deoxypentosone (Gobert and Glomb, 2009).

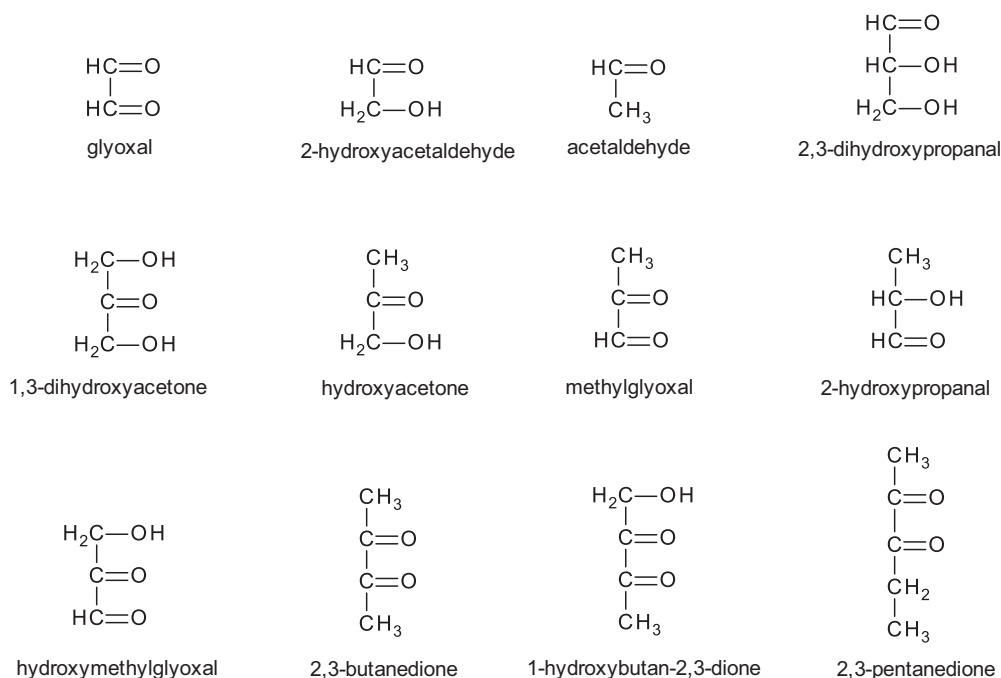
Shorter chain  $\alpha$ -dicarbonyl compounds,  $\alpha$ -hydroxyaldehydes, and  $\alpha$ -hydroxyketones are formed by the cleavage of the carbon skeleton, especially through deoxyosones. Alkaline conditions accelerate the formation of fragments. These shorter chain carbonyl compounds are of importance because they substantially contribute to browning, and involve in the formation of flavor compounds (Hollnagel and Kroh, 1998).

Typical products of the fragmentation with one carbon is formaldehyde; with two carbons are glyoxal, 2-hydroxyacetaldehyde (glycolaldehyde), and acetaldehyde; with three carbons are 2,3-dihydroxypropanal (glyceraldehyde), 1,3-dihydroxyacetone, hydroxyacetone (acetol), methylglyoxal (pyruvaldehyde), 2-hydroxypropanal (lactaldehyde), hydroxymethylglyoxal; with four carbons are 2,3-butanedione (diacetyl), 1-hydroxybutan-2,3-dione, and with five carbons is 2,3-pentanedione (Fig. 4) (Smuda and Glomb, 2013). These can be formed by direct degradation of the carbon chain (after isomerization and/or dehydration) and also by condensation of the fragments. The mechanisms largely depend on the reaction conditions, and the source of carbon may come from different regions of the carbon skeleton. Some of these  $\alpha$ -dicarbonyl compounds may also form as secondary lipid oxidation products and in ascorbic acid degradation (Fujioka and Shibamoto, 2004; Schulz et al., 2007).

In addition to the formation of short-chain carbonyl compounds, carboxylic acids are also formed. Formic acid and acetic acid are formed from 3-deoxyglucosone and 1-deoxyglucosone, respectively. The main mechanism is hydrolytic  $\beta$ -dicarbonyl cleavage (Davidek et al., 2005; Smuda and Glomb, 2013), and to a lesser extent oxidative cleavage (Davidek et al., 2006).

Glycosidic bonds of oligosaccharides are cleaved by acid catalysis. Hydrolysis of the glycosidic bond is affected especially by pH and temperature, and also depends on the structure. Reducing oligosaccharides, such as maltose and lactose, may participate in browning reactions by producing various reactive intermediates (Hollnagel and Kroh, 1998). Degradation of polysaccharides might also be important at very high temperatures as in the case of coffee roasting.

Hydrolysis and decomposition of sucrose in sweet bakery products are of importance as inversion products glucose and fructose participate in nonenzymatic browning reactions. Moreover, cleavage of a glycosidic bond of sucrose releases glucose and fructofuranosyl cation (Fig. 5) (Perez Locas and Yaylayan, 2008). At elevated temperatures and under dry heating conditions, the fructofuranosyl cation produces HMF via elimination of a proton and dehydration (Perez Locas and Yaylayan, 2008). HMF formation with this pathway is more efficient than dehydration of 3-deoxyglucosone to HMF through acyclic intermediates (Perez Locas and Yaylayan, 2008). Another possible pathway for HMF formation is dehydration of fructose over cyclic intermediates (Antal et al., 1990; Mayes et al., 2014; Kocadağı and Gökmen, 2016a,b).



**Figure 4** Fragments with carbonyl moieties formed in caramelization.

Reactions of sugars are not only limited to the decomposition reactions but inter- and intramolecular glycoside bond formations are also observed. Oligomers with up to six carbohydrate units may form during caramelization of glucose, fructose, and sucrose via glycosidic bonding (Golun and Kuhnert, 2012; Golun and Kuhnert, 2013). These oligomers are also found in their dehydrated (up to eight  $\text{H}_2\text{O}$  loss) and hydrated forms.

Anhydrosugars (sugar anhydrides) are formed from aldohexoses by intramolecular condensation of the hemiacetal and other hydroxyl groups. Levoglucosan (1,6-anhydro- $\beta$ -D-glucopyranose or  $\beta$ -glucosan) is abundantly formed during heating of glucose, starch, and cellulose at high temperatures (Fig. 6). To a limited extent, its furanose form (1,6-anhydro- $\beta$ -D-glucofuranose) may also form (Velisek, 2014).

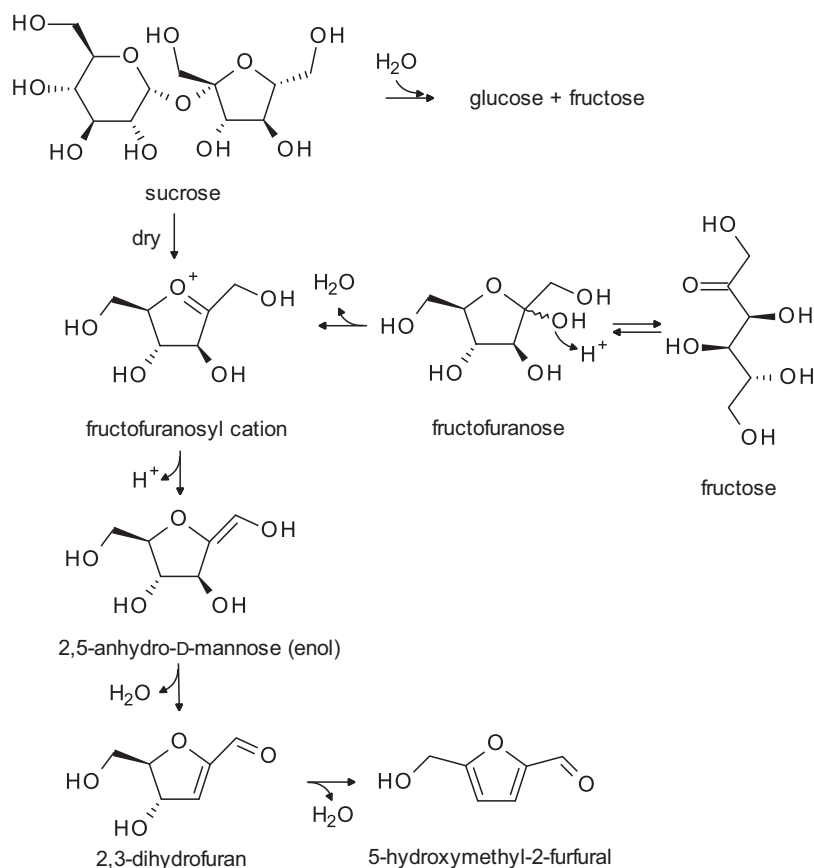
Intramolecular glycosidic bond formation is not observed in fructose, but difructose dianhydrides (Fig. 6) and their glycosylated derivatives are formed via fructofuranosyl cation (Audemar et al., 2017). Glycosidic bond formation between fructofuranosyl cation and fructose produces fructodisaccharides (fructobioses), which then form a tricyclic structure via intramolecular glycosidic bonding. Isomerization in the molecule gives various diastereomers and further glycosylation may give glycosylated derivatives. These difructose dianhydrides and their glycosylated derivatives are prebiotic compounds found in caramel (Mellet and Garcia Fernandez, 2010). Isomers of difructose dianhydrides are also important as they can be used for the authenticity of caramel and for the detection of addition of caramel in foods (Ratsimba et al., 1999).

## Flavor Development

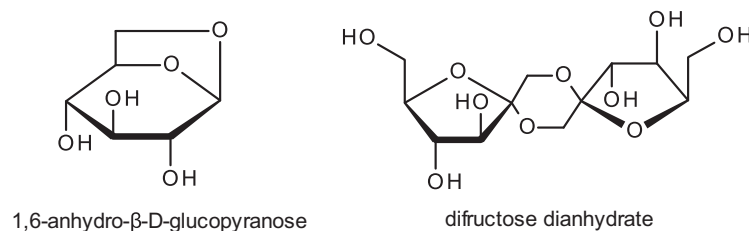
Major flavor compounds formed during caramelization of sugars are oxygen-containing heterocyclic compounds, furanones and pyranones, and also carbocyclic compounds, cyclopentenolones. In these groups of molecules, caramel aroma appears if a planar vicinal enol-oxo configuration is found (Fig. 7) (Belitz et al., 2009). A volatile fraction of caramel constitutes low molecular weight compounds derived from degradation of sugars. These compounds may also play a role in the aroma of baked and roasted foods via caramelization reactions; however, many characteristic aroma compounds with relatively low odor thresholds are formed via involvement of amino acids in the Maillard reaction. Increment in fragmentation reactions during the Maillard reaction may also provide short-chain carbonyl compounds for aldol reactions required for the formation of compounds with a caramel flavor.

One of the important compounds is furaneol (4-hydroxy-2,5-dimethyl-2H-furan-3-one) (Fig. 8) with an intensive caramel flavor and a relatively low odor threshold (Zabetakis et al., 1999). It is found in caramel, biscuits, coffee, and dark beer. It can be formed via aldol condensation of hydroxyacetone and methylglyoxal followed by cyclization and dehydration (Velisek, 2014). It is mainly formed from L-rhamnose (6-deoxy-L-mannose) (Haleva-Toledo et al., 1997; Illmann et al., 2009). Another furanone with a higher odor threshold, norfuraneol (4-hydroxy-5-methyl-2H-furan-3-one), can be formed from the furanose form of 1-deoxyglucosone upon dehydration and splitting of formaldehyde (Velisek, 2014).

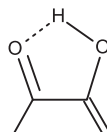
Sotolon (caramel furanone, 3-hydroxy-4,5-dimethyl-5H-furan-2-one) and abhexon (maple furanone, 5-ethyl-3-hydroxy-4-methyl-5H-furan-2-one) are powerful aroma compounds with relatively low odor thresholds (Maarse, 1991). Sotolon can be



**Figure 5** Degradation of sucrose and fructose to HMF. Adapted from Antal Jr., M.J., Mok, W.S., Richards, G.N., 1990. Mechanism of formation of 5-(hydroxymethyl)-2-furaldehyde from d-fructose and sucrose. *Carbohydr. Res.* 199, 91–109 and Locas, C.P., Yaylayan, V.A., 2008. Isotope labeling studies on the formation of 5-(hydroxymethyl)-2-furaldehyde (HMF) from sucrose by pyrolysis-GC/MS. *J. Agric. Food Chem.* 56, 6717–6723.



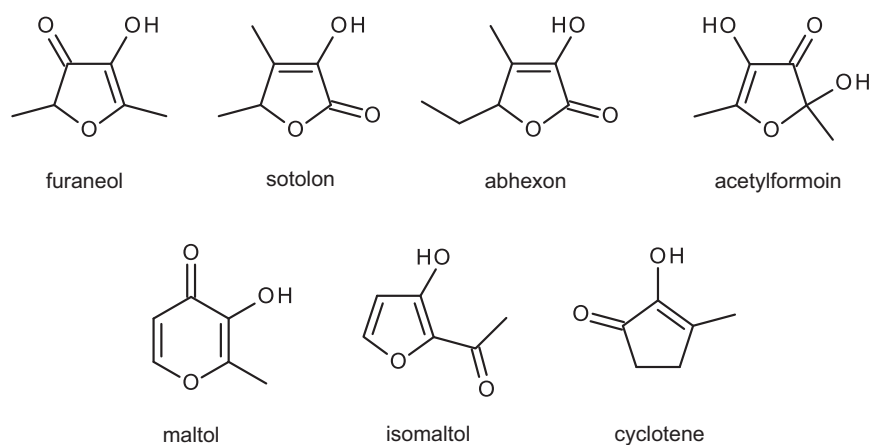
**Figure 6** Levoglucosan and difructose dianhydride



**Figure 7** Enol-oxo configuration found in compounds with caramel aroma.

produced from glycolaldehyde with 2,3-butanedione, and abhexon from glycolaldehyde and 2,3-pentanedione via aldol condensation (Belitz et al., 2009). Sotolon has caramel, maple syrup, and a burnt sugar odor at low concentrations, and fenugreek or curry odor at high concentrations (Tokitomo et al., 1980; Girardon et al., 1986). Abhexon has an intense maple syrup and typical protein hydrolyzate aroma. Both sotolon and abhexon can be formed from different precursors in different food products (Velisek, 2014).

Acetylformoin (2,4-dihydroxy-2,5-dimethyl-2H-furan-3-one) is another important furanone with a caramel flavor (Cutzach et al., 1999). It is formed from the dehydration of hexoses through 1-deoxyglucosone (Hofmann, 1998). Acetylformoin is found in many tautomeric forms in its acyclic structure (Goto et al., 1963). It is very reactive and unstable because of its reductone structure



**Figure 8** Compounds with distinct caramel flavor.

in the acyclic form. Acetylformoin is also an intermediate in furaneol formation (Hofmann and Schieberle, 2000). It can be found in aromatic caramel and burnt sugar (Paravisini et al., 2012).

Maltol is found in biscuits, roasted coffee, chocolate, caramel, malt, and beer. It has a high odor threshold and may not always contribute to the aroma of food (Belitz et al., 2009). It is one of the important odorants in burnt sugar caramels (Paravisini et al., 2012). Maltol is formed when glucose has a 4-*O* glycoside linkage, like in disaccharides maltose and lactose, and its formation from monosaccharides is negligible (Yaylayan and Mandeville, 1994). Isomaltol (2-acetyl-3-hydroxyfuran) also has a caramel-like flavor, and it is formed from hexoses, and especially from 4-*O*-substituted glucoses at higher concentrations.

Cyclopentenolones are alicyclic compounds formed from fragments of sugar degradation and have a distinct caramel flavor. Cyclotene (2-hydroxy-3-methyl-2-cyclopenten-1-one) has an intense burnt sugar and caramel flavor, and it can be formed from hydroxyacetone and 2-hydroxypropanal (Velisek, 2014).

The volatile fraction of sugar degradation contains many more compounds with different aroma properties and odor thresholds. Among others, diacetyl, 2,3-pentanedione, acetoin, furan, 2-methylfuran, 3-methylfuran, furfural, 2-acetylfuran, and 2-hydroxyacetylfuran are the constituents of the volatile fraction in thermally treated foods. Although they can be directly formed from sugar backbone during caramelization, carbon atoms from amino acid fragmentation may also be involved, and the quantitative distribution largely depends on the conditions and food product composition (Kerler et al., 2010).

## Browning Development

The nature of the pigment molecules responsible for the color development during heating sugars is largely unknown, similar to melanoidins formed in the Maillard reaction. During heating and storage of several food products, the Maillard reaction is responsible for nonenzymatic browning via catalysis of sugar dehydration and fragmentation by involvement of amino acids and incorporation of nitrogen to the polymers. At low pH values, amino compounds get protonated, making them less susceptible to participate in carbonyl-amine condensation, which is the first step of the Maillard reaction, because of the decrement in the nucleophilic strength of the amino group (Martins et al., 2000; Martins and Van Boekel, 2005). Browning in dessert wine, where these conditions are observed, is favored via caramelization reactions (Kroh, 1994). Removal of amino acids from citrus juices is known to decrease undesired nonenzymatic browning during thermal treatment and storage (Bharate and Bharate, 2014). However, browning still progresses to a limited extent via caramelization reactions and ascorbic acid degradation. Caramelization reactions of fructose might account for 10%–36% of the browning observed during heating fructose–lysine aqueous mixture (Ajandouz and Puigserver, 1999; Ajandouz et al., 2001). However, it is generally hard to make a conclusion to distinguish between caramelization and the Maillard reactions, especially at very high temperatures, for the contribution to browning.

During dry heating of solid sugars, reactivity is observed after melting or glass transition temperatures because transition from solid to liquid brings mobility to the molecules, which is required to observe any reaction at a considerable rate (Roos et al., 2013). Therefore the caramelization and browning in sugar solids requires higher temperatures. Melting temperatures of fructose, glucose, and sucrose are 127, 158, and 190°C, respectively (Roos and Drusch, 2016). Increasing temperature increases the kinetic energy of the molecules and thus caramelization reactions get faster. In parallel with that, more acyclic sugar molecules are found at higher temperatures, and thus fragmentation reactions provide an intensive dark color development. The increments in short-chain  $\alpha$ -dicarbonyl compounds,  $\alpha$ -hydroxycarbonyl compounds, and furan derivatives are associated with an increment in browning intensity (Hollnagel and Kroh, 1998; Kroh et al., 2008).

The presence of alkali metal cations (like NaCl, KCl, CaCl<sub>2</sub>) enhances the browning during caramelization of sugars. Color intensity increases with higher concentrations of NaCl and KCl in cereal model systems and breakfast cereals (Moreau et al., 2009b, 2011). Browning provided by NaCl is linked neither to the hygroscopic behavior of NaCl nor to the physical state of the cereal

**Table 1** Classification of caramel colors

Name	Plain	Caustic sulfite	Ammonia	Ammonium sulfite
Class	Class I, E150a	Class II, E150b	Class III, E150c	Class IV, E150d
Additive (promoter)	NaOH, KOH, Ca(OH) <sub>2</sub> , Na <sub>2</sub> CO <sub>3</sub> , K <sub>2</sub> CO <sub>3</sub> , H <sub>2</sub> SO <sub>4</sub> , acetic acid, citric acid	H <sub>2</sub> SO <sub>4</sub> , Na <sub>2</sub> SO <sub>3</sub> , K <sub>2</sub> SO <sub>3</sub> , NaOH, KOH, Ca(OH) <sub>2</sub>	NH <sub>4</sub> OH, (NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub> , NH <sub>4</sub> HCO <sub>3</sub> , ammonium phosphate, Na <sub>2</sub> CO <sub>3</sub> , NaOH, KOH, Ca(OH) <sub>2</sub>	NH <sub>4</sub> OH, (NH <sub>4</sub> ) <sub>2</sub> CO <sub>3</sub> , NH <sub>4</sub> HCO <sub>3</sub> , ammonium phosphate, (NH <sub>4</sub> ) <sub>2</sub> SO <sub>3</sub> , NH <sub>4</sub> HSO <sub>3</sub> , Na <sub>2</sub> CO <sub>3</sub> , NaOH, KOH, Ca(OH) <sub>2</sub> , H <sub>2</sub> SO <sub>4</sub> , Na <sub>2</sub> SO <sub>3</sub> , NaHCO <sub>3</sub> , K <sub>2</sub> SO <sub>3</sub> , KHCO <sub>3</sub>
Color	golden-yellow	reddish-brown	brown-yellow	dark brown-grey
Colloidal charge	weak	negative	positive	negative
Uses	alcoholic beverages, coffee extract, bakery products, confectionary	spirits, vermouth, brandies, bakery products, confectionary	beer, vinegar, acidic foods, sauces, bakery products, confectionary	soft drinks, bakery products, confectionary

model systems (Moreau et al., 2009a). In cookies, the presence of NaCl, KCl, and CaCl<sub>2</sub> increases 2-furfural and 5-hydroxymethyl-2-furfural in parallel with browning (Kocadağlı and Gökmen, 2016c). Contribution of fructose to browning development is generally higher than glucose. Heating glucose with alkali metal cations increases the rate of isomerization of glucose to fructose; therefore more furan derivatives are formed from fructose, which might be associated with the colored pigment formation (Hollnagel and Kroh, 1998; Mayes et al., 2015; Kocadağlı and Gökmen, 2016a).

Alkaline pH is also necessary to increase browning intensity during heating of sugars and sugar-rich foods. Higher pH values also increase the rate of browning by increasing enolization, as temperature does. Enhancement of browning at alkaline conditions is related to the increasing rate of fragmentation reactions.

Caramel colors are produced by controlled heating of sugars at temperatures above 120°C, and pressure can also be applied. Sucrose, glucose, fructose, sugar syrups, and starch are the main sources of raw material. Caramel colors are classified into four categories depending on the additives used (FAO, JECFA) (Table 1). The additives are used as catalysts to promote browning and to achieve desired properties (such as colloidal charge) for specific food products. Ammonia-sulfite caramel is the darkest colored caramel, and it is commonly used in soft drinks. Nitrogen-containing heterocyclic compounds pyrroles, pyridines, pyrazines, and imidazoles are formed in the case of using ammonium salts, which are characteristic to the Maillard reaction.

## Potential Toxicants Formed During Caramelization

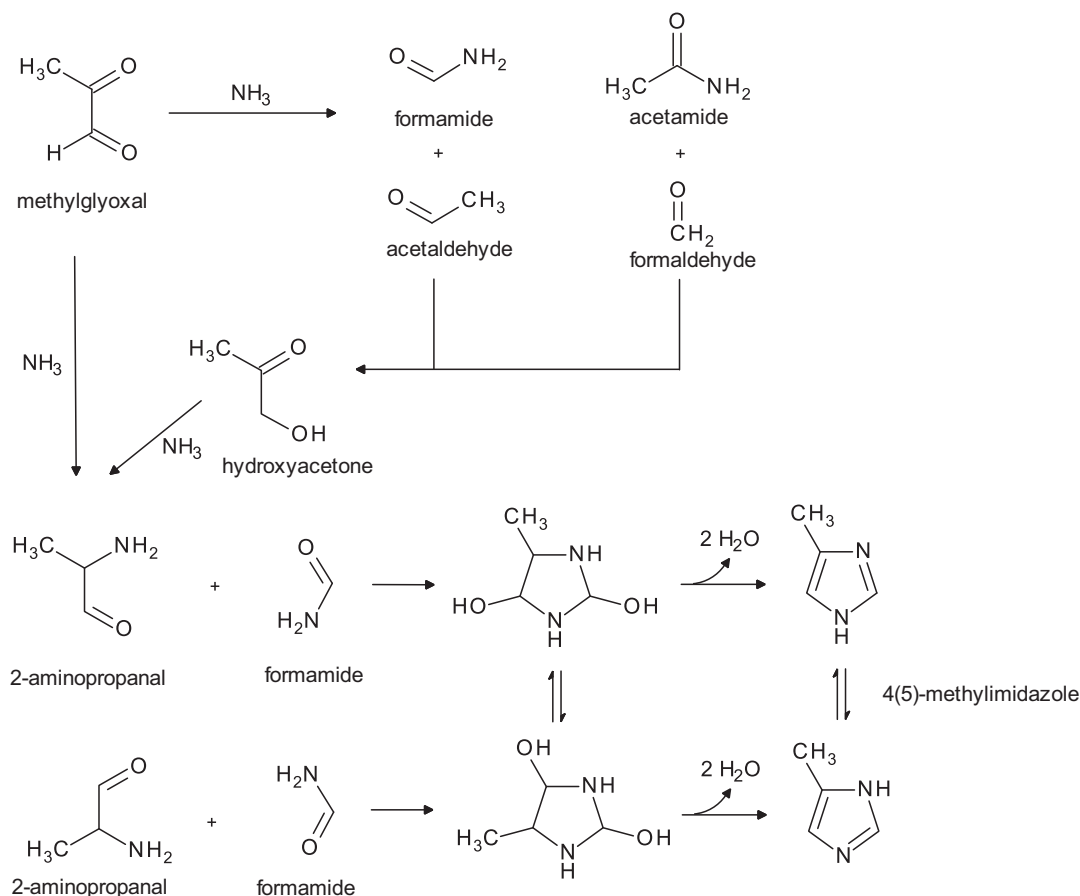
### Imidazoles

4(5)-Methylimidazole (4-MI) and 2-methylimidazole (2-MI) are found in class III and IV caramel colors, and 2-acetyl-4-tetrahydroxybutylimidazole (THI) is found only in class III caramel color (EFSA, 2011). 4-MI and 2-MI are classified as “possibly carcinogenic to humans (Group 2B)” by the International Agency for Research on Cancer (IARC, 2013). This classification is based on the sufficient evidence in experimental animals for the carcinogenicity of both compounds (IARC, 2013). THI is a potent immunosuppressant, which means it reduces the strength of the body’s immune system (EFSA, 2011). Maximum residue limits have been set for 4-MI as 200 mg kg<sup>-1</sup> in class III and 250 mg kg<sup>-1</sup> in class IV, and for THI as 10 mg kg<sup>-1</sup> on equivalent color basis by the European Union. 4-MI can be present in caramel colors up to 1000 mg kg<sup>-1</sup>, being higher in class IV, and varies largely (Henggel and Shibamoto, 2013). Concentration of THI can be up to 50 mg kg<sup>-1</sup> (Elsinghorst et al., 2013). 2-MI is generally found in lower amounts.

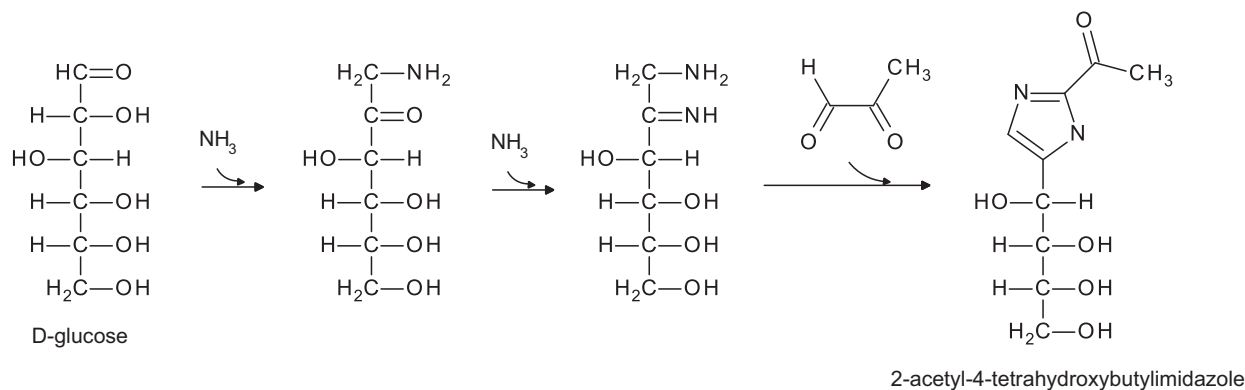
The imidazole ring is formed via reactions of reactive carbonyl compounds and ammonia that is found in class III and IV caramel colors. 4-MI has been proposed to be formed from methylglyoxal in the presence of ammonia (Fig. 9) (Moon and Shibamoto, 2011). THI is formed via iminofructosamine, which is formed in the reaction of hexoses with ammonia (Fig. 10) (Kroeplien et al., 1985). Although their presence is commonly of concern in caramel colors, they may also form in thermally processed foods during the Maillard reaction (Mottier et al., 2017). The source of nitrogen or ammonia in this case is the Strecker degradation of amino acids (Yaylayan and Haffenden, 2003).

Divalent cations Ca<sup>2+</sup> and Mg<sup>2+</sup> have been shown to decrease the formation of 4-MI (Wu et al., 2016). The levels of sulfide play an important role for the formation of 4-MI during class IV caramel color production, and addition of appropriate levels of sulfide decreases 4-MI formation (Lee et al., 2013). Due to the wide usage of caramel colors in several food products, controlling the exposure to imidazoles and other toxicants is of importance from the point of view of food safety.





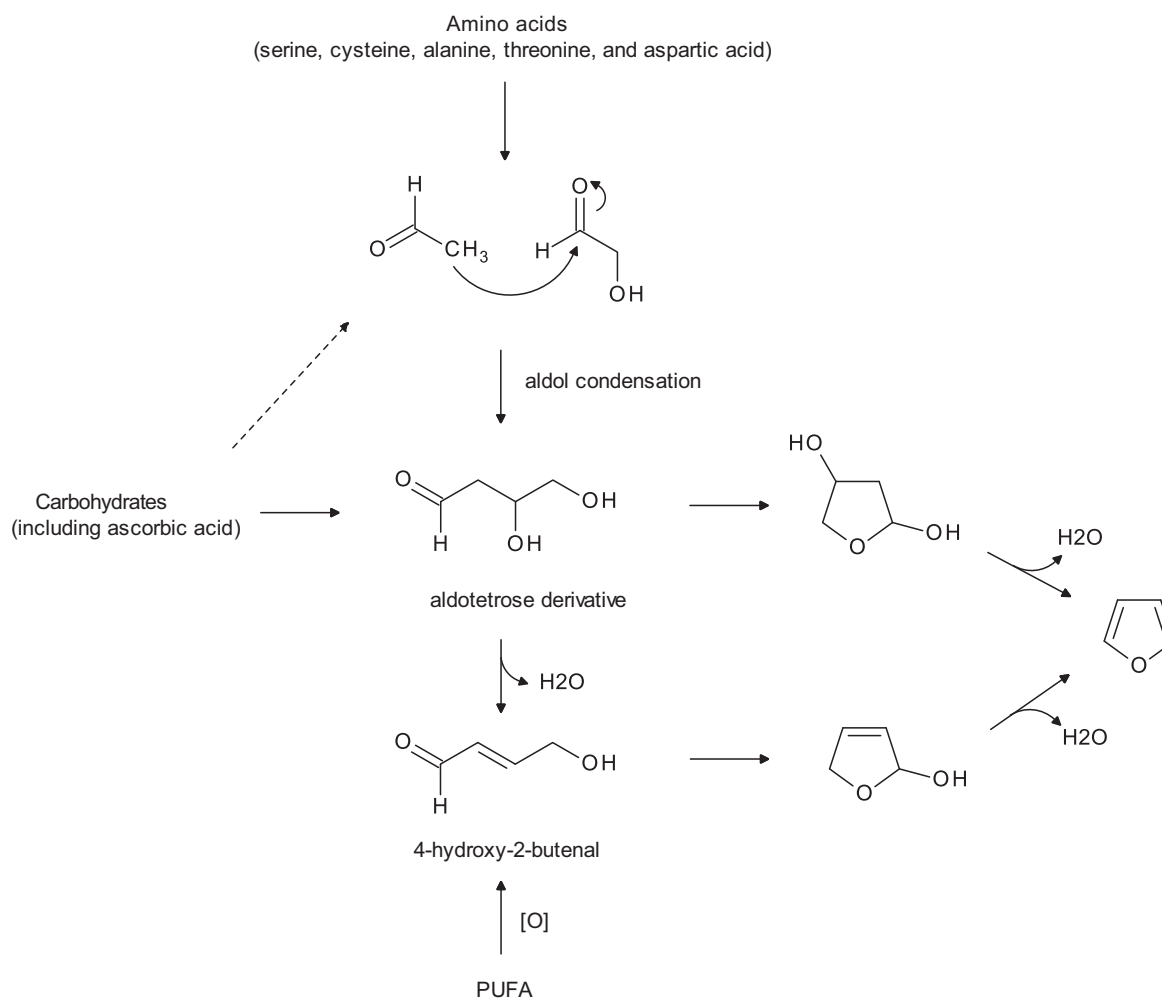
**Figure 9** Formation of 4(5)-methylimidazole. Adapted from Moon, J.K., Shibamoto, T., 2011. Formation of carcinogenic 4(5)-methylimidazole in maillard reaction systems. *J. Agric. Food Chem.* 59, 615–618.



**Figure 10** Formation of 2-acetyl-4-tetrahydroxybutylimidazole. Adapted from Kroeplien, U., Rosdorfer, J., Van Der Greef, J., Long, R.C., Goldstein, J.H., 1985. 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazole: detection in commercial caramel color III and preparation by a model browning reaction. *J. Org. Chem.* 50, 1131–1133.

### Furan and Derivatives

IARC has classified furan as “possibly carcinogenic to humans (Group 2B)” (IARC, 1995). The precursors of furan are sugars, amino acids, polyunsaturated fatty acids, carotenoids, and ascorbic acid in foods (Fig. 11) (Yaylayan, 2006). The reactivity of sugars for furan formation is in the order of erythrose > ribose > sucrose > glucose = fructose (Perez Locas and Yaylayan, 2004). It is a very volatile compound, and its presence in foods highly depends on the characteristics of foods. In caramel colors, its concentration can be ranged from 52 to 177  $\mu\text{g kg}^{-1}$  (EFSA, 2011).



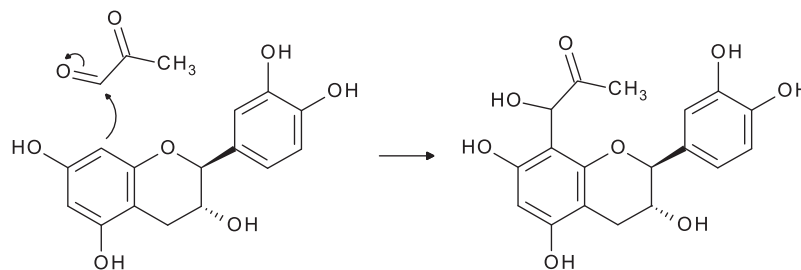
**Figure 11** Furan formation pathways. Adapted from Perez Locas, C., Yaylayan, V.A., 2004. Origin and mechanistic pathways of formation of the parent furan: a food toxicant. *J. Agric. Food Chem.* 52, 6830–6836.

The main furan compound formed during heating of hexoses is HMF, and various other furan compounds are also formed to a lesser extent as mentioned above. HMF can reach extremely high concentrations up to  $30 \text{ g kg}^{-1}$  in caramel products (EFSA, 2011), and its levels might exceed  $1 \text{ g kg}^{-1}$  in dried fruits, roasted coffee, malt, vinegar, and various thermally treated sugar-rich foods (Morales, 2008). Its metabolite 5-sulfoxymethyl-2-furfural is of serious concern because of its genotoxicity (Capuano and Fogliano, 2011). Formation of furan derivatives highly depends on the thermal load applied, and therefore limiting thermal input is one of the main strategies that could be applied. Formulation changes, such as choice of sugar, is also important to mitigate furan derivatives (Şenyuva and Gökmen, 2007; Gökmen et al., 2008; Kocadağlı et al., 2012; Van der Fels-Klerx et al., 2014; Taş and Gökmen, 2016).

### $\alpha$ -Dicarbonyl Compounds

$\alpha$ -Dicarbonyl compounds are highly reactive compounds, and therefore their presence might be a concern both in foods and in vivo. In addition to their cytotoxic effects, they may also react with free or bound lysine and arginine, yielding advanced glycation end products, which are related to various health consequences (Henle, 2007; Hellwig and Henle, 2014).  $\alpha$ -Dicarbonyl compounds found in foods may cause dicarbonyl stress mainly in the gastrointestinal lumen (Degen et al., 2013; Degen et al., 2014; Rabbani and Thornalley, 2015). It has been shown that dietary exposure to glyoxal has tumor growth-promoting properties in the small intestine in mice (Svendsen et al., 2016). Moreover,  $\alpha$ -dicarbonyl compounds play a role in the formation of other toxicogenic compounds during food processing, such as acrylamide, furan, heterocyclic aromatic amines, and 4(5)-methylimidazole.

The major  $\alpha$ -dicarbonyl compound found in foods is 3-deoxyglucosone, among others (Degen et al., 2012; Gensberger et al., 2012, 2013; Kocadağlı and Gökmen, 2014). The concentration of 3-deoxyglucosone is generally quite higher than HMF. Thermal input, sugar composition, and pH are critical factors for the formation of  $\alpha$ -dicarbonyl compounds. Phenolic compounds are known to trap  $\alpha$ -dicarbonyl compounds in foods and in physiological conditions in vitro (Totlani and Peterson, 2005; Lo et al.,



**Figure 12** Reaction of methylglyoxal with (–)-epicatechin via electrophilic aromatic substitution.

2006).  $\alpha$ -Dicarbonyl compounds react with certain positions on the phenol ring via electrophilic aromatic substitution as shown for (–)-epicatechin in Fig. 12.

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# Carbohydrate Active Enzymes Applied in the Production of Functional Oligosaccharides

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## Introduction

The seek for foods that possess physiological benefits apart from nutritional properties has increased in the last decades. These natural healthy products are called functional foods and are directly related to health promotion, reduction of disease risks and health care costs (Shahidi, 2009). Biologically-active food products comprehend a broad variety of molecules, such as polyunsaturated fatty acids, polyphenols and bioactive peptides, among others.

Prebiotics are non-digestible short-chain carbohydrates and have received extensive attention as functional foods because of their selective stimulation of the growth and/or activity of different benefic bacteria (Gibson and Roberfroid, 1995). These compounds are composed of sugars with different degree of polymerization (DP) and possess different molecular structures (Table 1).

Prebiotic oligosaccharides are naturally found in different vegetables such as chicory, onions, asparagus and garlic (Van Loo et al., 1995). However, the growing demand for industrial use requires more efficient processes. In this context, microbial enzymes can be applied to efficiently produce bioactive prebiotics. These biocatalysts are highly selective for the substrate allowing the obtainment of a large variety of functional oligosaccharides (Manas et al., 2017).

The most used enzymes are hydrolases that often catalyze transglycosylation reactions. Several glycoside hydrolases can form oligosaccharides with a great variety in nature. In addition, protein engineering strategies are a great alternative to modulate hydrolysis and transglycosylation activities, resulting in tailor-made oligosaccharides (Manas et al., 2017).

This review chapter focuses on the application of different enzymes in the production of biological oligosaccharides. Functional aspects of the prebiotics and enzyme characteristics are also reported.

## Oligosaccharides as Functional Foods

After a meeting organized by the Food and Agriculture Organization of the United Nations (FAO), prebiotic was defined as “a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota” (Pineiro et al., 2008). This broader concept was thought to encompass many prebiotics that had not been considered. However, three criteria must be fulfilled in order to a candidate be a prebiotic: i) non-digestible by hydrolytic enzymes of gut microbiota and resistant to gastrointestinal absorption; ii) fermented selectively by specific bacteria present at intestinal microflora; iii) to be able to stimulate the growth and/or the activity of bacteria related to benefits to the host (Gibson et al., 2004).

Until 2024, it is expected that the prebiotic market size of food and beverage exceeds USD 7 billion (Global Market Insights, 2017). Following this trend, pharmaceutical and food industries have made efforts to develop and explore functional oligosaccharides such as fructooligosaccharides (FOS) and inulin.

It has been reported for a long time that prebiotics boost the selective growth of mainly lactobacilli and bifidobacteria. These microorganisms produce short-chain fatty acids (SCFAs), including propionate, acetate and butyrate. These SCFAs turn the intestinal lumen alkaline and, therefore, inhibit the proliferation of bacterial pathogens, such as *Salmonella* and *Helicobacter* (Subin et al., 2017). Also, SCFAs induce the proliferation of enterocytes by the production of ATP. As a result, the cells produce more mucin and make the epithelial barrier less permeable to invasion of pathogens. As additional consequence of oligosaccharides fermentation, the immune system is strengthened by the production of cytokines modulators (Krumbeck et al., 2016).

**Table 1** Molecular structure of most known non-digestible oligosaccharides

Oligosaccharide	Molecular structure <sup>a</sup>	Glycosidic bond
Fructooligosaccharides	(Fr) <sub>n</sub> – Gu	β (2–1)
Galactooligosaccharides	(Ga) <sub>n</sub> – Gu	β (1–3) and β (1–4)
Xylooligosaccharides	(Xy) <sub>n</sub>	β (1–4)
Maltooligosaccharides	(Gu) <sub>n</sub>	α (1–4)
Isomaltooligosaccharides	(Gu) <sub>n</sub>	α (1–6)
Chitooligosaccharides	(GlcNAc) <sub>n</sub> or (GlcN) <sub>n</sub>	β (1–4)
Gentiooligosaccharides	(Gu) <sub>n</sub>	β (1–6)
Pectic oligosaccharides	(GalA) <sub>n</sub>	α (1–4)

<sup>a</sup>Ga: galactose; Gu: glucose; Fr: fructose; Xy: xylose; GlcNAc: N-acetyl-D-glucosamine; GlcN: glucosamine; Gal: galacturonic acid.

Modified from Singh et al. (2017).



Prebiotics are still associated with a vast range of beneficial effects, including improvement of the mineral absorption, prevention of cardiovascular diseases, diabetes, gastrointestinal infections and even some types of cancers (Rastall, 2010). Besides, due to their functional properties, prebiotics can be used as fat replacer and sugar in food industry. In addition, functional oligosaccharides are used in beverages, milk products, meat products, confectionary, breakfast cereals, bakery products, food for infants and other applications (Patel and Goyal, 2011).

## Carbohydrate Active Enzymes

Prebiotics can be produced by extraction from natural sources and by chemical, physical or biotechnological processes using enzymes. The enzymatic approach is normally the most reliable choice due to its high reproducibility and yield, focusing mainly on large scale oligosaccharide production (Moreno et al., 2017). In addition, enzymes normally act on mild reaction conditions. Besides the possibility of obtaining enzymes from different sources, their microbial production has shown to be more interesting for industrial production of prebiotics (Díez-Munício et al., 2014). The main advantages of using microbial enzymes include easier handling, higher production yield, economic feasibility and absence of seasonal fluctuation (Filice and Marciello, 2013).

The enzymes used in the production of different oligosaccharides are called carbohydrate-active enzymes (CAZymes) which are categorized in different classes in the CAZy database (<http://www.cazy.org>). The classes comprise glycoside hydrolases (GHs), glycosyltransferases (GTs), carbohydrate esterases (CEs), polysaccharide lyases (PLs) and auxiliary activities (AAs) (Lombard et al., 2014). These classes are subcategorized into families based on genomic, biochemical and structural information of the enzymes (André et al., 2014) (Fig. 1).

The glycoside hydrolase (GH) and glycosyltransferase (GT) are the most represented CAZymes. GHs normally act on O- and S-glycosyl compounds, hydrolyzing and/or rearranging glycosidic bonds. On the other hand, GTs are able to transfer glycosyl groups (donor to acceptor), catalyzing oligosaccharide synthesis (Díez-Munício et al., 2014). Several of these enzymes are used after cloning and expressing their respective genes in many hosts, including *Saccharomyces cerevisiae*, *Bacillus* sp., *Kluyveromyces* sp., *Aspergillus* sp., *Trichoderma* sp. and others (Patel and Goyal, 2011).

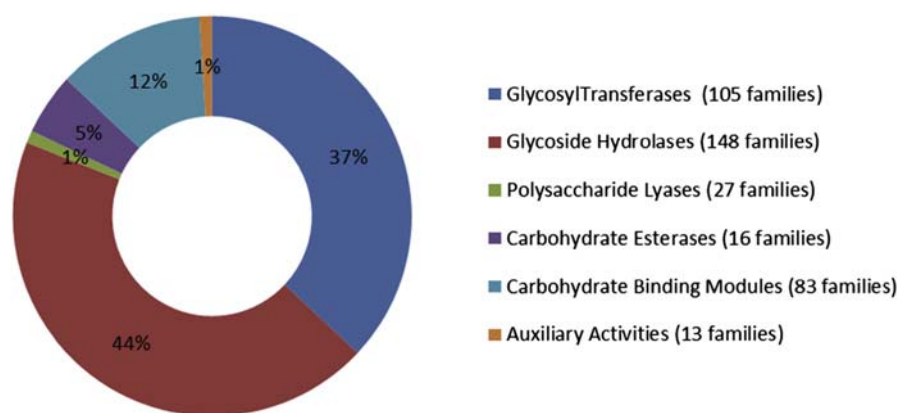
## Oligosaccharides: Enzymatic Production, Biological Activities and Applications

### Fructooligosaccharides

Fructooligosaccharides (FOSs) are oligosaccharides composed of 2–10 fructosyl residues linked by  $\beta$ -(2–1) glycosidic bonds (Rastall, 2010). FOSs are present in trace amounts in honey and several fruits and vegetables. These compounds show moderate sweetness (one-third of sucrose), low glycemic index and low calorimetric value. They can also be manufactured by three different approaches: (i) extraction from plant materials, (ii) enzymatic degradation of inulin, and (iii) enzymatic synthesis from sucrose (Dominguez et al., 2014). However, most of FOSs marketed have been produced either from sucrose or by degradation of inulin using enzymes (Singh et al., 2017).

Inulin is composed of fructosyl units linked by  $\beta$ -(2–1) bonds, with one terminal  $\alpha$ -(1–2) glucosyl residue. FOSs production from inulin is possible by controlled hydrolysis using endoinulinases (enzymes from family GH32) produced by filamentous fungi (*Aspergillus* sp. and *Penicillium* sp.), yeasts (*Kluyveromyces* sp.) and bacteria (*Bacillus* sp., *Pseudomonas* sp. and *Streptomyces* sp.) (Ganaie et al., 2014; Sarup et al., 2016). The action of endoinulinases on inulin randomly breaks down  $\beta$ -(2–1) glycosidic linkages resulting in a mixture of FOSs with DP from 2 to 7 molecules of fructose (Rastall, 2010; Sarup et al., 2016).

On the other hand, FOSs production from sucrose is carried out by enzymes with fructosyltransferase activity. These enzymes are found in family GH32 and possess both transfructosylation and hydrolytic activities, depending on the concentration of sucrose in the reaction medium. At low sucrose concentration, fructosyltransferases have mainly hydrolytic activity. However, the transfructosylating activity is more pronounced at high substrate concentration (Kovács et al., 2013). In the last case, the enzyme



**Figure 1** Diversity of CAZymes according to the CAZy database (<http://www.cazy.org>) (Accessed December 2017).

acts on sucrose by cleaving  $\beta$ -(1–2) linkages and transferring the fructosyl group to an acceptor molecule, such as FOSs and sucrose, with glucose release as a byproduct. The FOSs produced from sucrose contain 2–4 fructofuranosyl residues linked by  $\beta$ -(2–1) bonds, featuring a terminal glucose joined by  $\alpha$ -(1–2) linkage. These are mainly composed of 1-kestose (GuFr<sub>2</sub>), nystose (GuFr<sub>3</sub>) and 1- $\beta$ -fructofuranosyl nystose (GuFr<sub>4</sub>) (Rastall, 2010; Singh et al., 2017). Fucosyltransferase from *Aspergillus* sp. and *Aureobasidium pullulans* have been widely investigated to produce FOSs from sucrose (Ganaie et al., 2014). Sucrose-based FOSs can also be produced from sucrose present in different sources, such as agro-wastes and other low-cost byproducts (Dominguez et al., 2014).

FOS can inhibit the growth of harmful bacteria, improve mineral absorption in the gut, enhance immunity, reduce cholesterol level, prevent the development of colon cancer, promote the synthesis of complex B vitamins, control diabetes mellitus and obesity, and act on intestinal traffic control (Bali et al., 2015).

### Galactooligosaccharides

Galactooligosaccharides (GOSs) are oligosaccharides composed of different galactosyl residues (from 2 to 9 units) and a terminal glucose linked by  $\beta$ -glycosidic bonds, such as  $\beta$ -(1–2),  $\beta$ -(1–3),  $\beta$ -(1–4), and  $\beta$ -(1–6) (Illanes et al., 2016). They naturally occur at low concentrations in the milk of many animals, including humans and cows, but they also can be produced by chemical glycosylation or biocatalysis (Ackerman et al., 2017).

The enzymatic GOSs production can be performed by using galactosyltransferases as well as  $\beta$ -galactosidases. Galactosyltransferases exhibit high catalysis selectivity and produce higher GOSs yield, however they are not used commercially because of the requirement of specific sugar nucleotides as substrates and the extremely expensive costs (Contesini et al., 2013; Osman, 2015). On the other hand,  $\beta$ -galactosidases are robust GHs found in families GH1, GH2, GH35 and GH42 that can catalyze, when the acceptor is water, the hydrolysis of lactose into galactose and glucose. Alternatively, when the acceptor is a sugar such as lactose,  $\beta$ -galactosidases can catalyze transgalactosylation reactions giving rise to oligosaccharides (Hernández-Hernández et al., 2011). Thus, GOSs have been typically synthesized by the action of microbial  $\beta$ -galactosidases, produced by *Kluyveromyces lactis*, *Bacillus circulans*, *Bifidobacterium bifidum*, *Aspergillus oryzae* or *Streptococcus thermophiles*, by transgalactosylation of pure lactose solutions and low-value whey lactose from dairy (Kovács et al., 2013; Illanes et al., 2016; Ackerman et al., 2017).

Some conditions, such as elevated temperatures, high lactose concentrations and low water activity, are required in the reaction medium for GOSs production by  $\beta$ -galactosidases (Gosling et al., 2010; Illanes et al., 2016). Some studies have demonstrated that the concentration of GOSs, yields, and types of GOS formed during their enzymatic synthesis depend on the reaction conditions (e.g. time of reaction, pH, inhibitors) and on the source of enzyme (Gosling et al., 2010; Osman, 2015). For example, it was observed that the  $\beta$ -galactosidase from *B. subtilis* shows the highest GOSs yield (41% after 5 h using 49% lactose), which were mainly tri- and tetrasaccharide linked by  $\beta$ -(1–4) glycosidic linkages (Frenzel et al., 2015).

Currently, different possible human health benefits have been associated with GOSs consumption. Examples are mineral absorption, stool improvement, weight management, carcinogenesis, reduction of the cholesterol level and allergy alleviation (Illanes et al., 2016).

### Xylooligosaccharides

Xylooligosaccharides (XOSs) are sugar oligomers composed of xylose units linked by  $\beta$ -(1,4) bonds, with DP ranging from 2 to 10. They are named according to the number of xylose residues in their composition, i.e.: xylobiose, xylotriose, xylotetraose and so on. XOSs are known to be produced by *Aspergillus*, *Streptomyces*, *Trichoderma*, *Penicillium* and *Bacillus* from xylan rich materials (Belorkar and Gupta, 2016), although they are naturally found in fruits, vegetables, honey, bamboo and milk (Vázquez et al., 2000; Mandelli et al., 2014).

Production of XOSs usually includes chemical and enzymatic methods, as well as a combination of both approaches. The major drawback of chemical methods is the formation of toxic components that include acetic acid, furfural, protein-derived products and soluble inorganic compounds. This approach requires previous purification to obtain food grade XOSs. On the other hand, enzymatic process can be of great interest as it overcomes the limitations of chemical process. However, the direct enzymatic treatment is only suitable for susceptible materials such as citrus peels (Wang and Lu, 2013).

XOSs production from xylan through enzymatic methods is generally obtained after alkaline extraction (KOH or NaOH) from lignocellulosic materials, which are then converted to XOSs by xylanases from GH families 5, 7, 8, 10, 11 and 43. In XOSs production, xylanases having endoxylanase activity are preferred rather than exoxylanase activity since it can minimize xylose production and subsequently increase the XOSs production (Samanta et al., 2015).

Among the biological activities of XOSs it can be included antioxidant, immuno-modulatory, anti-inflammatory, anti-cancer and anti-microbial activities, antiallergy properties, active against diabetes, treatment of arteriosclerosis, among others (Belorkar and Gupta, 2016).

In addition to their biological properties, XOSs stand out for their potential to be used as ingredients in functional foods. They are competitively priced prebiotic ingredients which can be used at low doses comparing to other oligosaccharides. XOSs are safe and effective ingredients, presenting excellent stability over a wide range of pH (2.3–8) and temperatures (up to 120 °C). Moreover, they are suitable for use in a broad range of foods and formulations, are slightly sweet, being 40% as sweet as sucrose, but low in calories, what makes them suitable for low-calorie products. Finally, XOSs can be used with protein powders, overcoming constipation and other digestion problems associated with high protein diets (Kumar et al., 2013).

## Other Relevant Functional Oligosaccharides

Maltooligosaccharides (MOSs) are usually composed of 2 to 10 D-glucopyranosyl residues linked by  $\alpha$ -1,4 glycosidic linkages, while isomaltooligosaccharides (IMOSs) are generally linked by  $\alpha$  (1–6)-, and sometimes by  $\alpha$  (1–2),  $\alpha$  (1–3) or  $\alpha$  (1–4)-linkages (Singh et al., 2017). Both, MOS and IMOS, can be obtained from starch by the action of debranching enzymes. The difference between them is that MOSs are produced by the action of amylases and pullulanases, while IMOSs are obtained after starch liquefaction by amylases, demanding a second stage which involves transglucosylation by  $\alpha$ -transglucosidase (Mussatto and Mancilha, 2007). MOSs and IMOSs are also associated with a lower risk of diarrhea and infections, increase in short-chain fatty acid production, antidiabetic effects, obesity management and improvement of lipid metabolism (Meyer et al., 2015).

Gentiooligosaccharides (GeOSs) consist of glucose units linked by  $\beta$ -(1–6) glycosidic bonds, and can be obtained from pustulan (lichen polysaccharide) hydrolysis. The enzymatic production of GeOS is performed in two steps, starting with the conversion of gentiobiose in gentotriose by the action of  $\beta$ -glucosidase, followed by the conversion of gentotriose into gentiooligosaccharides by the action of  $\beta$ -(1–6)-glucanase (Jung and Park, 2014). In addition to the prebiotic activity, GeOSs can be used as a low-glycemic sweetener and as a taste improver in certain beverages due the bitter taste (Côté, 2009; Singh et al., 2017). Moreover, GeOSs were shown to be an anti-cancer agent against human colon carcinoma (Kothari and Goyal, 2015).

Pectic oligosaccharides (POSs) are a class of emerging prebiotics resulting from partial hydrolysis of the complex heteropolysaccharide, pectin. POSs are composed of D-galacturonic acid units, both in its acetylated or methylated forms, linked by  $\alpha$  (1–4) bonds. POSs production from purified pectins or agro-industrial byproducts can be achieved by hydrothermal processing, physical degradation, and by acid or enzymatic hydrolysis (Gullón et al., 2013). Because of the structural complexity of pectin, different enzymes, such as esterases, pectin lyases and hydrolases are necessary to achieve its degradation (Bonnin et al., 2014). Apart from promoting the bifidiogenic flora, some studies showed others benefits from POSs, such as lowering serum levels of total cholesterol, inhibition in the accumulation of body fat, protection against cardiovascular diseases (Li et al., 2010), reduction of damage by heavy metals, stimulation of apoptosis in human colonic adenocarcinoma cells, antioxidant, antiinfection, and antibacterial properties (Míguez et al., 2016).

## Conclusions

As discussed in this chapter, several oligosaccharides present remarkable functional properties, based mainly on the stimulation of benefic bacteria growth in human gut. Thus, several preventive effects for health can be achieved, including anticarcinogenic properties. Enzymatic synthesis of oligosaccharides is considered a promising alternative to the industrial production of this functional products, since it does not have the common drawbacks found in chemical routes or in the case of extraction from natural products. Many studies have focused on the optimization of CAZymes production and oligosaccharide production through enzymatic routes. However, efforts still must be made in protein engineering to obtain tailor-made functional oligosaccharides. Furthermore, genetic engineering of industrial strains is another bottleneck that must be focused. Finally, the precise identification of oligosaccharides, including branched ones, is still an obstacle to be overcome in order to maximize oligosaccharide market.

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# Chemically Reducing Properties of Maillard Reaction Intermediates

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## Introduction

The designation Maillard reaction is in fact somewhat of a misnomer in that the so-called “reaction” represents a large number of discrete, but closely related separate reactions. The MR is initiated by the fundamental interaction of carbonyl and amine-containing reactants. In foods and biological systems the MR begins with a reducing sugar (a carbonyl derivative) and amino acids and/or proteins representing the amine component. Early studies on the MR dealt mainly with reaction end products consisting of colored polymeric materials today commonly known as melanoidins or browning products. Historically, the MR was first investigated by the French/Algerian scientist **L. C. Maillard (1912)** during his researches on peptide syntheses. Many intermediates in the MR have since proven to have potent chemically reducing properties some of which comprise the subject of this chapter.

MR intermediates are typically highly reactive, often present in low concentration and were therefore seldom isolated in early studies. In the decades following the work of Maillard MR chemistry has been thoroughly investigated internationally and results are represented by numerous comprehensive books (**Pigman et al., 1980; Nursten, 2005; Ikan, 1996**) and reviews (**Ledl and Schleicher, 1990**). In the USA pioneering research by J. H. Hodge et al. in the 1950s led to the first mechanistic understanding of the MR and to a sequence of reactions still valid today (**Hodge, 1953**) [vide infra].

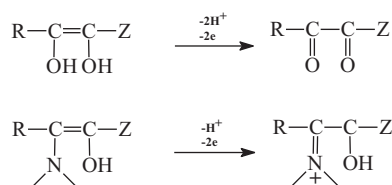
## Origin of MR Reducing Properties

Reduction in the chemical sense takes place when a species adds one or more electrons to its structure. Thus substances which can provide these electrons are referred to as reducing agents. In the MR key intermediates with reducing properties are structures containing acyloin ( $\alpha$ -hydroxycarbonyl) or related tautomeric 1,2-enediol elements. Structures containing the predominantly 1,2-enediol tautomeric forms are known collectively as REDUCTONES (**Schank, 1972**). 1,2-Enediols act as reducing agents by their conversion to 1,2-diketones via elimination of two electrons and two hydrogen ions. The equilibrium between acyloins and 1,2-enediols normally favors the acyloin form which is lower in energy. Acyloins such as reducing sugars and Amadori compounds (discussed below) exhibit moderate reducing power and will be described in a later paragraph, however the similarity in their oxidative mechanism is shown in **Fig. 1**.

The presence of strongly acidifying groups e.g. like a carbonyl group on one or both oxygen-bearing acyloin carbon atoms tends to stabilize the enediol form as an  $\alpha$ -oxo-enediol as for example in ascorbic acid. The enhanced acidity of the  $\alpha$ -oxo-enediol hydroxyls readily leads to relatively stable anionic enediolates. And, once formed, these anionic species can act as relatively more powerful reducing agents than simple acyloins by more facile donation of one or two electrons to accepting species. Compounds containing the  $\alpha$ -oxo-enediol moiety, including ascorbic acid, are known collectively as aci-reductones. In addition, compounds in which one or both hydroxyl groups have been replaced by SH, NH<sub>2</sub> or –NHR also belong to the reductone class. Nitrogen-containing reductones were first recognized in disaccharide/amino acid reactions (**Ledl, 1984**) and an aminoreductone, 1-alkylamino-1,2-dehydro-4-deoxy-3-hexulose has been prepared in a reaction of lactose and n-butylamine (**Pischetsrieder et al., 1998**).

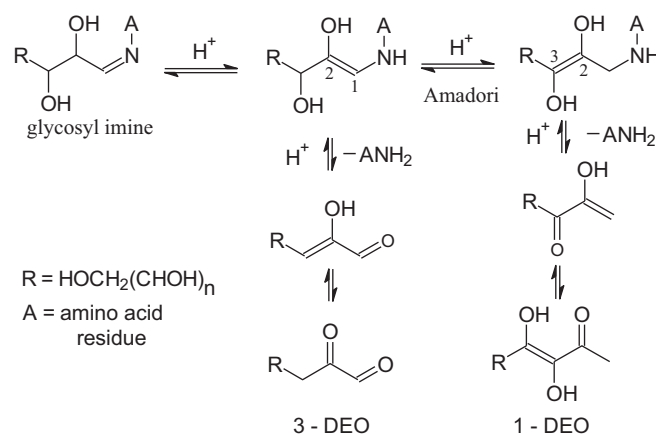
## Formation of Reducing Compounds in the Maillard Reaction

The early stages of a typical MR are illustrated in **Fig. 2**. Amino acids and/or proteins react with reducing sugars (aldoses or ketoses) with loss of water to form glycosyl imines (shown in open chain form). In **Fig. 2** an aldose-derived imine undergoes prototropic rearrangement to form a relatively stable isomeric aminoketone known as an Amadori compound (**Yaylayan and Huyghues-Despointes, 1994**). Under similar conditions ketoses yield aminocarbonyls known as Heyns compounds. In the MR thermal or acid catalyzed fragmentation of relatively stable Amadori or Heyns intermediates leads directly to more highly reactive 1,2-dicarbonyl structures most commonly called 1-deoxyosones (1-DEOs) and 3-deoxyosones (3-DEOs). Elimination of the amine function takes place from the two enolic forms of the Amadori compound. The 1,2-enol yields 3-DEOs whereas the 2,3-enol affords 1-DEOs. Both types of DEOs are highly reactive in subsequent reactions by virtue of their 1,2-dicarbonyl functionality. But, 1-DEOs



**Fig. 1** Oxidative mechanisms for tautomers of acyloins and Amadori compounds.





**Fig. 2** Early stage of Maillard reaction.

being  $\alpha$ -oxo-enediols as well are therefore by definition aci-reductones and show characteristic enhanced reducing capability. To date 1-DEOs have escaped detection in the MR although their presence has been proven by chemical derivatization. 1-DEOs share highly reducing properties with similar substances like ascorbic acid and reductic acid.

### Unique Reducing Chemistry of 1-Deoxyosones

The special reactivity of acyclic  $\alpha$ -oxo-enediols (i.e. 1-DEOs) is explained by their ability to form a double 5-membered ring protonic chelate (Fig. 3) which has a central hydroxyl group. The chelate structure provides enhanced protic activity thus allowing enhanced ionization and subsequent ease of electron abstraction. As also shown in Fig. 2 the resulting two electron oxidation products of 1-DEOs are initially 1,2,3-tricarbonyl compounds commonly called dehydroreductones. Carbohydrate derived dehydroreductones are highly reactive and probably undergo facile redox disproportionation with concomitant loss of carbon dioxide (Schank, 1972), a putative scheme for this is shown in Fig. 3. Typical products predicted from such degradation of a 5-carbon tricarbonyl compound are, besides  $\text{CO}_2$ , a 4-carbon diketone and a regenerated 5-carbon dihydroxyoneone.

In practice carbohydrate derived triketoones have not been observed, but their presence has been rationalized in terms of isolated fragmentation products (Voight et al., 2010). Interestingly, the well known bicyclic tricarbonyl ninhydrin is completely stable in the form of its monohydrated central carbonyl carbon. Whereas the reducing power of reductones is well known and can be demonstrated readily in terms of analytical reagents (vide infra) the question of what substances are actually being reduced during the MR remain unanswered. Molecular oxygen has been suggested as a possible electron recipient leading eventually to fragmentation of 1-DEOs without the formation of a dehydroreductone intermediate with formation of acetic acid (Robert et al., 2005), but MR conducted under anoxic conditions suggest alternate possibilities. For example, ample evidence exists for the presence of free radical species during the MR and it's possible that reductones may contribute to their formation in situ (Hayashi and Namiki, 1981). The single electron donating capability of 1-DEOs is also evident in the molecule's antioxidant behavior (Kanzler et al., 2014). Using EPR (electron paramagnetic resonance) measurements, for example special radical scavenging activity was reported for 1-deoxy-D-erythro-hexo-2,3-diulose, a typical 1-DEO.

In contrast to the highly reactive 1-DEOs the 3-DEOs are not reductones and do not exhibit the reductive reactivity associated with enediols. However, 3-DEOs do participate in important subsequent reactions like the Strecker degradation, a well documented source of volatile flavor compounds (Rizzi, 2008).

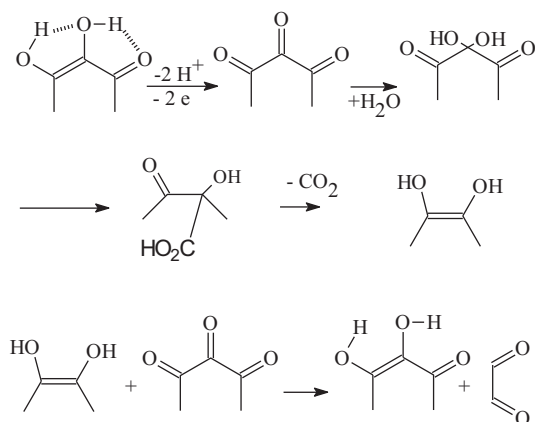
### Detection and Measurement of MR Reductones

#### Chemical Reagents for Reductone Analysis

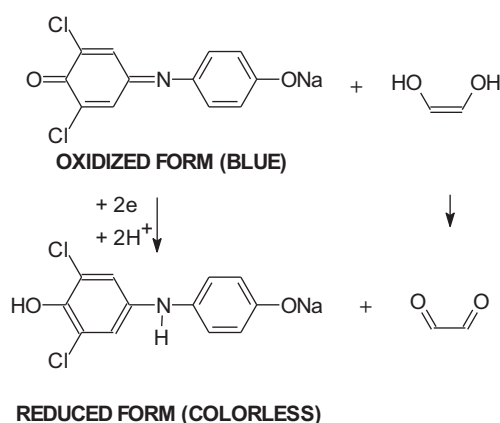
Early studies on the MR indicated intermediate compounds were present capable of reducing common reagents such as: methylene blue, ferricyanide, dichlorophenolindophenol, Fehling solution ( $\text{Cu}^{+2}$ ) and Tollens reagent ( $\text{Ag}^{+1}$ ) (Hodge, 1953). Further detailed studies characterized reducing bodies as Amadori compounds and recognized the formation of what were then simply called reductones and dehydroreductones.

Reductones decolorize weakly acidic solutions of iodine by forming carbonyl oxidation products. The oxidation products can be determined gravimetrically using 2,4-dinitrophenylhydrazine (Schank, 1972). A more recent and convenient colorimetric method uses the commercially available reagent 3'-[1-(phenylamino)carbonyl-3,4-tetrazolium]bis (4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT). Tetrazolium reagents like XTT are readily reduced by reductones to form highly colored formazan derivatives with a UV/VIS max at 470 nm easily quantified spectrophotometrically. The method is practical and has been used to quantify the reductones present in UHT-treated milk and in a model MR of lactose (Shimamura et al., 2000). Using post-column detection





**Fig. 3** Chelate structure and related oxidation and decomposition of 1-deoxyosones.



**Fig. 4** Redox behavior of 2,6-dichloroindophenol.

with alkaline triphenyltetrazolium chloride 16 Amadori compounds were quantified in dried vegetable products (Reutter and Eichner, 1989).

A popular laboratory method for reductone analysis is the use of Tillman's reagent based on the commercially available indophenol dye, 2,6-dichloroindophenol (DCIP). The dye in its normal oxidized state is deep blue, but when used to titrate reductones in solution a sharp colorless endpoint is obtained. The redox chemistry of DCIP with a generalized enediol substrate is shown in Fig. 4.

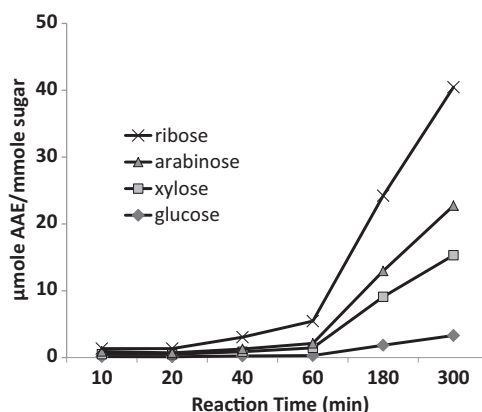
Results showing DCIP analysis of reductone formation during the phosphate ion catalyzed degradation of reducing sugars (Rizzi et al., 2010) are shown in Fig. 5.

The ordinate (AAE) ascorbic acid equivalents represents the amount of ascorbic acid standardized Tillman's reagent used in the titrations. Under standardized conditions DCIP will respond to aci-reductones like 1-DEOs and Amadori compounds, but is unreactive to less reactive acyloins like reducing sugars.

## Electrical Properties of MR Intermediates

The inherent redox nature of reductones implies electrochemical behavior with both theoretical and practical value (Rizzi, 2003a). In some cases Galvanic activity has been observed in which redox pairs constitute a source of EMF (electromotive force) whose presence can serve to detect the presence of reductones in MR systems and to follow their rates of formation and decay. The magnitude of Galvanic EMF can also serve to estimate the relative propensity of reducing sugars for producing reductones in the MR.

The reversible character of redox pairs also permits the use of electrolytic action in which application of external potentials are used to alter redox equilibria. Typical applications of electrolysis in MR systems are: electrochemical detectors for GC or HPLC analysis; polarography; cyclic voltammetry; amperometry and more recently pulsed amperometry (Blank et al., 2002). With suitable voltage ranges many types of latent enediol systems e.g. acyloins like reducing sugars, Amadori compounds, polyphenolic compounds and late phase MR heterocyclic materials like pyranones may be detected and quantified.



**Fig. 5** Use of Tillman's reagent to titrate reductones in reducing sugar degradation reactions. Reprinted with permission from Ref. Rizzi et al. (2010), Copyright 2010 American Chemical Society.

### Galvanic Activity in the MR

Open circuit EMFs have been measured between platinum and Ag/AgCl electrodes in a model MR systems consisting of reducing sugars,  $\beta$ -alanine in bis/tris buffer at 100 °C (Rizzi, 2003a). Increasing negative voltages were observed with time indicative of reductone production. The existence of a measurable EMF is evidence for the spontaneous ability of a reducing substance (most probably an aci-reductone) present to convert silver ion into metallic silver. In these experiments independent chemical derivatization verified the presence of an aci-reductone and other reductones. Data obtained after 1.0 h (Table 1) indicated greatest reductone activity for pentoses, ribose, arabinose and xylose compared with less reactive glucose. Negative controls i.e. negligible EMF was observed by 2-deoxy-D-glucose, sorbitol and xylitol, compounds incapable of undergoing MR.

### Electrolytic Behavior in MR

Electrolytic aspects of the MR and Galvanic activity have been reviewed recently (Rizzi, 2013). Little published information is available on polarography of the MR. Hayashi and Namiki (1981) observed anodic waves in model MR systems indicative of oxidation and consistent with the presence of enediol moieties. Concurrent DCIP titration data was in agreement with their polarographic results. Cyclic voltammetry (CV) is a type of polarography combining both oxidation and reduction. A CV route was envisioned for electrical conversion of an Amadori compound (eneaminol tautomer) to an otherwise elusive dehydroreductone (Rizzi et al., 2010). A model Amadori compound, N-(1-deoxyfructos-1-yl)piperidine (Fig. 6) was CV analyzed at pH 7.14) Two oxidation waves were observed: + 0.7 V (oxidation of the eneaminol) and + 0.9 V (oxidation of the piperidinium osone, leading ultimately to a piperidinium reductone and presumably by hydrolysis to a triketonic dehydroreductone. However, total lack of

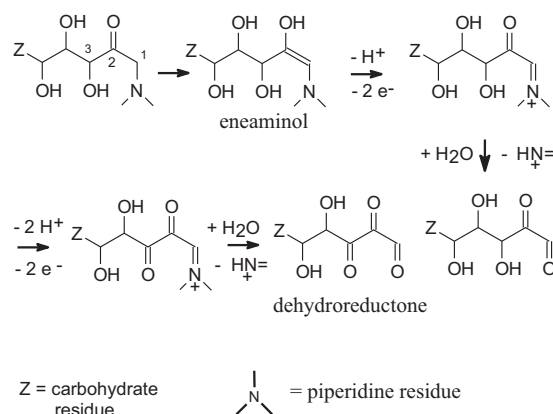
**Table 1** Changes in redox potential during  $\beta$ -alanine/carbohydrate Maillard reactions

Carbohydrate Compound	$E_f - E_i$			
	$\Delta$ in mV	$\pm \sigma$	Replicates	Buffer pH
D-glucose	-102	1	3	6.94-7.22
D-mannose	-100	...	1	6.94
L-rhamnose	-105	3	2	6.94
D-fructose	-83	...	1	6.94
2-deoxy-D-glucose	-22	8	2	6.85
D-sorbitol	-3.7	27	3	6.94-7.22
D-xylose	-155	27	3	7.22
D-arabinose	-157	7.5	2	6.94
D-ribose	-192	4.5	2	6.94
D-xylitol	-7.7	27	3	6.94-7.22
Lactose	-99	...	1	6.94

Initial concentration: carbohydrates, 0.20 M;  $\beta$ -alanine, 0.067 M refluxed 1.0 h in 0.10 M bis-tris buffer.

$E_f - E_i$  = potential difference before and after heating.

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**Fig. 6** Putative oxidation scheme for N-(1-deoxyfructos-1-yl)piperidine.

corresponding reduction waves suggested that initially formed oxidation products had rapidly decomposed to electroinactive products.

The observation of two oxidation (anodic) waves was indicative of a stepwise transfer of electrons possibly involving free radical intermediates in the reduction process. Free radical behavior is known in the chemical reduction reaction of Amadori compounds with nitroblue tetrazolium (NBT) to form the tetrazinoly radical (NBT<sup>•</sup>) (Baker et al., 1994) and during autooxidation induced by copper I ion (Kawakishi et al., 1991). The subject of free radicals in the MR has been recently reviewed (Rizzi, 2003b).

CV results reported by Chaung and Chou (2008) on the Amadori compound, N-(1-deoxy-β-D-fructopyranos-1-yl)-L-valine, revealed a single anodic wave at ca. + 1.0 V with no compounds detected in a cathodic scan. A practical application of voltammetry for determination of reducing substances in beer has been described (Sobiech et al., 1998).

## Electrochemical Detectors (ECD)

Whereas many acyloins (including reducing sugars), Amadori compounds and various reductones exist in the MR electrochemical methods have only occasionally been employed for their unique detection or measurement. Acyloins and Amadori compounds because of their greater stability are generally separated or isolated from MR mixtures by chromatography and detected by optical (diode array detection) or mass spectrometric techniques (Davidek et al., 2005; Yuan et al., 2017). In ECD the emerging analyte is oxidized in a fixed potential electrolytic cell [amperometric analysis (EC/AA)] and resulting current flow provides detection.

An early example of EC/AA was the detection and quantification of the MR product 2,3-dihydro-3,5-dihydroxy-6-methyl-4-H-pyran-4-one, a.k.a. 2,3-dihydro-3-hydroxymaltol (Schnee and Eichner, 1987). This late stage MR product is formed during cacao roasting and is isolated among other volatile flavor compounds during steam distillation. HPLC analysis coupled with UV detection verified its MR origin and complete absence in unroasted cocoa. The pyranone derivative was electroactive by virtue of its inherent enediol structure and was readily detected (oxidized) in an ECD cell maintained at + 0.8 V. Using ECD detection the pyranone was readily quantitated and showed a linear correlation with respect to roasting degree thus providing a practical guide to follow the extent of cocoa bean roasting.

A modification of (EC/AA) called pulsed amperometric detection (EC/PAD) optimizes electrode function and has proven useful for analysis of MR mixtures. Using EC/PAD Amadori compounds were detected and quantified in model MRs of glucose/morpholine and glucose/tryptophan at 100 °C during 3.5 h (Huyghues-Despointes and Yaylayan, 1994). Later, by using synthesized Amadori compounds glu/morpholine and glu/proline their decomposition was studied using EC/PAD in aqueous solution at 100 °C, several electroactive products were observed but none were identified. More recently, refinements in the AA/EC and EC/PAD methodology led to, for example, analysis of the labile pentose-derived compound xyl/gly i.e. N-(1-deoxy-D-xylulos-1-yl) glycine and the analysis of Amadori compounds xyl/ala and xyl/ile synthesized in a mixture MR containing alanine and isoleucine in methanol at 70 °C (Davidek et al., 2005).

## Conclusions

Recent studies continue to confirm the presence of highly reactive, chemically reducing substances in Maillard reactions. Study on the redox behavior of these compounds is helping to elucidate the as yet complex detailed mechanism of this reaction. Modern analytical techniques like cyclic voltammetry and pulsed amperometric detection are providing access to hitherto inaccessible highly reactive intermediates.

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## Protein Oxidation

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### Introduction

Protein oxidation is a complex phenomenon occurring quite often in protein-rich foods like meat, fish, milk, and eggs. Oxidation of proteins has been defined as a covalent modification induced by reactive species or a reaction with secondary products of oxidative stress. This process results in a wide variety of chemical modifications depending on the pathway and nature of the generated oxidation products, which involves the formation of carbonyl derivatives, amino acid side chain modifications, cleavage of protein backbone and protein cross-linking, among others. These modifications have been reported to imply negative effects on human health due to its participation in the pathogenesis of several diseases, as well as negative effects on foods from the sensory, nutritional and technological points of view.

### Chemical Mechanisms and Factors Promoting Protein Oxidation

Protein oxidation can be induced directly by reactive oxygen species (ROS) and reactive nitrogen species (RNS), or indirectly through reactions with secondary products of oxidative stress. Protein oxidation is initiated by many species including i) free radical species such as hydroxyl ( $\text{OH}^\bullet$ ), superoxide ( $\text{O}_2^{\bullet-}$ ), nitric oxide ( $\text{NO}^\bullet$ ), and peroxy ( $\text{PO}^\bullet$ ), ii) non-free radicals such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), singlet oxygen ( $^1\text{O}_2$ ), ozone ( $\text{O}_3$ ), and hypochlorous acid ( $\text{HOCl}$ ), and iii) reactive aldehydes and ketones. These reactive species are typically produced through the action of external agents such as ultraviolet light, irradiation (X-rays,  $\gamma$ -rays), metal ions ( $\text{Fe}^{2+}$ ,  $\text{Cu}^+$ ), ozone, air pollution, drugs, nanoparticles, and xenobiotics, or are derived from endogenous muscle sources including metal-catalyzed systems, oxidative enzymes, oxidizing lipids, reducing sugars, and pro-oxidative heme proteins such as myoglobin and hemoglobin.

The free radical-mediated pathway of protein oxidation can be initiated when a ROS abstracts a hydrogen atom from the protein molecule and generates a protein carbon-centered radical ( $\text{P}^\bullet$ ), which is readily converted in the presence of oxygen to an alkyl peroxy radical ( $\text{POO}^\bullet$ ). Afterwards, the abstraction of a hydrogen atom from another molecule, reaction with the reduced form of iron ( $\text{Fe}^{2+}$ ) or with the protonated form of superoxide radical can lead to the generation of alkyl peroxide ( $\text{POOH}$ ). Further reactions with free peroxy radicals ( $\text{HO}_2^\bullet$ ) or  $\text{Fe}^{2+}$  can yield alkoxy radical ( $\text{PO}^\bullet$ ) and its hydroxyl derivative ( $\text{POH}$ ). Additionally, alkyl peroxide and alkoxy radicals can lead to protein fragmentation by the  $\alpha$ -amidation or diamide pathways, generating amine derivative and N-carbonyl derivative, or diamide derivative and isocyanate derivative, respectively.

The nature of oxidation products depends on the target and oxidizing agents, intensity of oxidation conditions as well as the amino acids involved. Sulfur-containing amino acids such as methionine and cysteine are the amino acids more susceptible to ROS and oxidizing lipids, whereas tryptophan residues are quickly oxidized in the presence of metal ions. Other residues such as tyrosine, phenylalanine, proline, histidine, lysine, threonine, arginine, leucine and valine need more severe conditions to be oxidized. The position of these amino acids within the structure of the protein greatly affects their exposure to oxidant factors and thus, their modification. Oxidation of amino acids can imply the modification of essential amino acids such as arginine, lysine, proline and threonine through carbonylation, the hydroxylation of aliphatic amino acids, sulfoxidation of methionine residues, nitrosylation of sulfhydryl groups or hydroxylation and nitration of aromatic amino acids. As a result, a wide variety of oxidation products are generated, which are shown in [Table 1](#).

The formation of protein carbonyls (aldehydes and ketones) is the most common modification resulting from protein oxidation. The side chains of proline, lysine, arginine and threonine residues can be oxidized by metal ion-catalyzed reactions into carbonyl compounds. The reaction between transition-metal ions and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), namely Fenton reaction, is a source of highly reactive hydroxyl radicals ( $\text{OH}^\bullet$ ). So, the presence of ferric iron ( $\text{Fe}^{3+}$ ) can initiate the oxidative deamination from the intermediate radical molecule, converting lysine into  $\alpha$ -aminoadipic semialdehyde (AAS), arginine and proline into  $\gamma$ -glutamic semialdehyde (GGS) and threonine into  $\alpha$ -amino-3-keto butyric acid. The resulting  $\text{Fe}^{2+}$  can further oxidize other amino acids through reaction with  $\text{H}_2\text{O}_2$  and form hydroxyl radicals. Other metal ions and heme proteins, both activated by  $\text{H}_2\text{O}_2$ , are also able to promote the formation of AAS and GGS. Carbonyls can be formed to a lesser extent by other pathways including oxidative cleavage of peptide backbone, non-enzymatic glycation in the presence of reducing sugars, or covalent binding to non-protein carbonyl compounds from lipid oxidation. Thus, protein oxidation is related with other biochemical reactions occurring in food systems like lipid oxidation and Maillard reaction, as their products can further increase oxidation in a reciprocal manner. Finally, protein carbonyls can participate in advanced reactions, resulting in further oxidative degradation (such as AAS into  $\alpha$ -aminoadipic acid (AAA)) or formation of an aldol condensation product, Schiff-bases or Strecker aldehydes.

Oxidation can also lead to protein backbone cleavage, which is mainly induced by free radical oxidants. Radicals can react with a specific site of the polypeptide backbone resulting in its fragmentation and thus leading to conformational changes in the secondary and tertiary structures of the protein. The oxidation of proline, aspartyl and glutamyl residues of protein can lead to peptide bond cleavage generating derivatives of the oxidation process.

**Table 1** Oxidation products of the most susceptible amino acid residues

<i>Amino acids</i>	<i>Oxidation products</i>
Arginine	$\gamma$ -Glutamic semialdehyde
Cysteine	Cysteic acid, sulfenic acid, sulfinic acid, sulfonic acid, cystine
Glutamic acid	Oxalic acid, pyruvate adducts
Histidine	Aspartate, asparagines, aspartic acid, 2-oxohistidine, 3,4,5-hydroxyleucine, 4-hydroxyl glutamate
Leucine	3,4,5-Hydroxyleucine
Lysine	$\alpha$ -Aminoadipic semialdehyde
Methionine	Methionine sulfoxide, methionine sulfone
Phenylalanine	2-, 3-, 4-Hydroxyphenylalanine, 2,3-dihydroxyphenylalanine, nitrophenylalanine, <i>p</i> -, <i>m</i> - and <i>o</i> -tyrosine
Proline	$\gamma$ -Glutamic semialdehyde, 4,5-hydroxyproline, pyroglutamic acid, 2-pyrrolidone
Threonine	$\alpha$ -Amino-3-keto butyric acid
Tryptophan	2-, 4-, 5-, 6-, 7-Hydroxytryptophan, formylkynurenine, kynurenine, 3-hydroxykynurenine, nitrotryptophan
Tyrosine	3,4-Dihydroxyphenylalanine, 3-nitrotyrosine, dityrosine, Tyr- <i>O</i> -Tyr, 3-chlorotyrosine, 3,5-dichlorotyrosine
Valine	3-Hydroxyvaline

The formation of intra- and inter-molecular cross-links is another manifestation of protein oxidation, and results in the generation of different oxidation products as well as aggregation and polymerization of proteins. Metal ions and heme proteins can act as initiator factors of this modification, as well as two carbon-centered radicals that can react with each other and generate cross-linked derivatives in the absence of oxygen. In the presence of H<sub>2</sub>O<sub>2</sub>, proteins and peptides containing cysteine are highly susceptible to loss of sulfhydryl groups through several and complex reactions of oxidation, generating sulfenic acid, sulfinic acid, and cysteine (disulfide bonds) as oxidation products. On the other hand, tyrosine can be oxidized to tyrosyl radicals that can combine to form dityrosine. Both disulfide and dityrosine bonds result in the formation of cross-linked proteins.

### Effect of Processing on the Oxidation of Proteins

Protein oxidation is well recognized to have an impact on loss of quality during processing and storage of protein foods. Main oxidative modifications of proteins take place at the side chains of amino acids, including thiol oxidation, aromatic hydroxylation, and formation of carbonyl groups. **Table 1** shows the most usual products from amino acids oxidation, which can be formed during processing and storage and have impaired consequences in food. For instance, protein oxidation occurring during the aging and cold storage of meat has been linked to loss of functionality, giving poorer juiciness and increased toughness. The loss of functionality also affects the suitability of such meat to be further processed when functional properties (emulsifying, water-holding capacity, gelling, etc.) play a major role. Additionally, exudation and drip loss, upon thawing of frozen meat, may be due to changes in the water-holding capacity, in some extent related with the oxidation of proteins as frozen storage increases the susceptibility of meat proteins to undergo further oxidation during processing.

Methods to stabilize foods without the use of heat treatments may affect the oxidation of food proteins and further their final quality. So, high pressure treatment of fresh meat results in discoloration attributed in a large degree due to the oxidation of oxymyoglobin and denaturation of myoglobin protein. Different studies have also evaluated the effect of different pressure intensities, between 200 and 800 MPa, on functionality (solubility and water holding capacity) of myofibrillar proteins from pork meat.

The effect of sterilization using different irradiation technologies also induces oxidation in those proteins containing aromatic and sulfur amino acids, which are the most susceptible to be oxidized. Aromatic amino acids react with hydroxyl radicals formed during the radiolysis of water with the generation of three tyrosine isomers (*p*-, *m*- and *o*-tyrosine) from phenylalanine oxidation or  $\alpha,\alpha'$ -diamino acids after ionizing radiation. So, UV irradiation of milk can produce the breakdown of proteins while the application of  $\gamma$ -rays or low dose of <sup>60</sup>Co to chicken, fish or shrimps sample can generate *o*- and/or *m*-tyrosine products that are used as markers of irradiation dosimetry.

Curing process in meat products using a mixture of salts composed of nitrate, nitrite and sodium chloride, constitutes a very used preservation procedure. The curing agent nitrite is a preservative and color enhancer in meat products. This additive behaves as strong antioxidant but, at low pH and high temperature has a pro-oxidant effect through the formation of nitric oxide and peroxynitrite. These RNS are responsible of the nitration of certain amino acids such as tyrosine, which is converted into 3-nitrotyrosine that is considered as protein oxidation biomarker. Sodium chloride may exert a pro-oxidative effect by modifying the conformation, functionality and solubility of proteins, making them more susceptible to be oxidized.

Thermal methods are usual in the sterilization/pasteurization of milk and milk products. During thermal treatment of milk, proteins are oxidized as demonstrated by the increase in the number of carbonylation sites in pasteurized and UHT compared to fresh milk. This fact may reduce the nutritional value of milk and abolish protein functions supporting human health that is especially important for newborns. Oxidative degradation may be increased by other types of processing. For example, mechanical actions during meat grinding would lead to cell disruption and oxygen inclusion, thereby increasing the contact with ROS. Processing technologies involving high temperatures, either for the preparation of "ready to it" products or during cooking at home,



promote the occurrence of protein oxidation. The type of technology used (grilling, roasting, frying, boiling, traditional or microwave oven) has been described to have also an impact on protein oxidation, although at different degrees.

Finally, the exposure to oxygen and light during chilled display at points of sale can further increase oxidation processes. Different packaging systems such as the application of vacuum or the use of modified atmosphere have widely used to minimize oxidation effects in food during storage and later cooking.

### Effect of Protein Oxidation on Health

Chemical modifications produced in proteins by the oxidation of specific amino acids may have implications on human health and safety when consumed. Recent studies have related protein oxidation with many diseases. The investigation of the mechanisms of protein oxidation has been intensified in the last 20 years due to the growing evidence that aging and various human pathologies are correlated with the increase of protein oxidation. Moreover, the presence of abnormal amounts of oxidized amino acids has been observed in many degenerative and chronic diseases.

Most research has been focused on the possible implication of protein oxidation in muscle foods and its contribution to several human diseases. However, it is still pending to demonstrate the effect of extrinsic oxidized amino acids coming through the diet on health.

Given the proved relationship between the presence of lipid oxidation products, consumed or generated in the gastrointestinal tract, and health risks by oxidative stress, the effect of protein oxidation products on the overall oxidative stress and the onset of certain health disorders is under suspicion, especially in view of the association that has recently been made between the consumption of cured and red meat and colorectal cancer.

As described in a previous section, oxidized amino acids can be accumulated in foods during processing and storage, but also can be generated in the organism during gastrointestinal digestion. This last has been studied in the colonic phase during a simulated digestion of red meat, cured meat and cooked meat, where the presence of other food components like heme-iron and fats, can promote the extent of protein oxidation and the complexity in the different oxidative reactions. The biological significance of such reactions and their effects on health is still controversial. Changes in proteins may cause a reduced protein digestion and these non-hydrolyzed proteins can be fermented by colonic flora into mutagenic products. There are also numerous studies showing some kind of disturbance in the intestinal flora, changes in the redox state of intestinal tissues, all contributing to inflammatory bowel diseases that can give place to the onset of carcinogenic processes.

Since protein oxidation markers have been found in blood and organs, the systemic effect of such compounds should be studied for the added risk of incorporating these anomalous amino acids to the *de novo* synthesis of proteins that will not have the expected functionality or even could be toxic. One of the clearest cases is the neurotoxicity of tryptophan oxidation products such as kynurenine, which have been found in processed and stored milk and is absorbed upon digestion; but also oxidized forms of methionine, specially methionine sulfoxide, found in large amounts in dairy products; dityrosine, the main oxidized form of tyrosine, which has been reported to cause hepatotoxicity and fibrotic degeneration of liver and kidney in mice; and aminoadipic acid, the last oxidation product from lysine, which has been associated to diabetes constituting a marker of diabetes risk.

All studies that link the intake of oxidized proteins through diet with age-related diseases require a long time of observation with prolonged exposure in order to obtain conclusive results. In the meantime, a moderate protein-restricted diet is recommended to increase the protection against aging and cancer.

### Effect of Protein Oxidation on Nutritional Sensory and Technological Quality of Foods

Oxidation of proteins can affect nutritive, sensory and technological properties of protein foods. Protein oxidation produces a decrease in the nutritive value of food products due to a reduced digestibility and loss of bioavailability of the modified amino acids. This problem becomes more important because the amino acids most susceptible to oxidation are just the essential amino acids. Protein oxidation before digestion results in impaired proteolysis during digestion. Moreover, free radicals are responsible for the formation of dityrosine bonds during meat cooking or under photo-oxidative conditions, which may result in protein aggregates with reduced digestibility and nutritional quality.

The alteration of the protein structure by oxidative damage can alter its binding ability with volatile compounds affecting aroma. Nevertheless, the overall impact of protein oxidation on sensory attributes still needs to be elucidated.

Other changes due to oxidation lead to changes in hydrophobicity that alter protein aggregation and decrease protein solubility thereby affecting technological properties like gel forming ability, emulsification properties and water binding capacity. The presence of modified amino acids in a protein can prevent the activity of endoproteases in fresh meat and thus might negatively impact tenderness. Also these modifications might affect the activity of peptidases by altering its recognition of substrates, and thus modifying the final quality of processed foods.

## Antioxidant Strategies

The human body possesses an antioxidant defense system that includes enzymes such as superoxide dismutase, catalase and glutathione peroxidase; metal-binding proteins such as ferritin, transferrin and ceruloplasmin; systems to repair protein and DNA damages; and metabolites and cofactors such as glutathione, uric acid and bilirubin. Additionally, dietary antioxidants such as vitamins, minerals, phenolic acids, flavonoids, carotenoids and peptides present in fruits, vegetables, meat, fish, legumes and dairy products also participate in antioxidant mechanisms. However, oxidative stress is inevitable despite this protective system, so the addition of antioxidant compounds, either through dietary or technological strategies, would help to reduce or even prevent oxidation and its negative effects on human health and food quality. Most of the commonly used antioxidant strategies have been focused on preventing lipid oxidation in foods, whereas its use against protein oxidation is a recent trend. Nevertheless, many of these procedures have been shown to be also effective against protein oxidation, due to the relationship between both processes.

The addition of antioxidant compounds to the formulation of products is a common strategy to inhibit protein oxidation. Antioxidants can donate electrons to break and terminate the oxidation cycle at the propagation step and thus prevent the formation of radicals, or they can act as metal chelators to inactive radical initiators. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ) and propyl gallate have been long used to inhibit oxidation, but their possible adverse effects on health have led to a recent interest in the use of safer and natural antioxidant compounds. Plant extracts including spices, seeds, herbs and essential oils as well as fruit extracts have been traditionally used in foods for organoleptic purposes, but they are also good sources of antioxidants such as tocopherols and phenolic compounds. These substances can act as radical scavengers and metal ion chelators through linkages with food proteins, although these interactions depend on the chemical structure and amount of the antioxidant as well as the characteristics of the protein and intensity of oxidative reactions. On the other hand, plant and fruits extracts also contain vitamins and minerals, which are considered as natural antioxidants due to their function as co-factors of antioxidant enzymes. So, vitamins A ( $\beta$ -carotene as precursor), C (ascorbic acid) and E ( $\alpha$ -tocopherol), as well as minerals such as selenium and zinc, have been described to exert highly effective radical scavenging properties.

Peptides and amino acids derived from the hydrolysis of proteins can also act as antioxidant agents in food systems. Antioxidant peptides generally contain 4–16 amino acid residues and their properties are related to their structure, hydrophobicity as well as composition and location of amino acids. So, methionine, tyrosine, tryptophan, cysteine, lysine, phenylalanine and histidine are well-known amino acids with antioxidant potential, showing radical-scavenging activity, metal ion chelation, inhibition of lipid peroxidation and enhancement of the antioxidant defense system of the human body. Antioxidant peptides can be generated through i) hydrolysis of food proteins by endogenous/microbial enzymes during aging, processing or ripening, and storage, ii) controlled hydrolysis with commercial proteolytic enzymes or iii) release during gastrointestinal digestion after food ingestion. Numerous peptides derived from enzymatic hydrolysis of proteins from soy, corn, potato, and animal-derived proteins such as casein, whey, egg, muscle proteins, and collagen, have been described to exert antioxidant activity and could be used as food additives. Furthermore, the multifunctional nature of antioxidant peptides, being able to exert other bioactivities such as antihypertensive, antimicrobial, cholesterol-lowering effect, opioid, and anticancer, among others, makes them to be considered as novel and potential ingredients of functional foods and nutraceuticals.

The feeding regime of animals raised for food production has a major influence on the oxidative stability of the final food product. So, dietary strategies against muscle food oxidation usually imply reduction of polyunsaturated fatty acids in animal tissues and feed supplementation with tocopherols or carotenoids from plant extracts, which also improve organoleptic properties of foods. In this regard,  $\alpha$ -tocopherol is the most common feed additive because it not only exerts antioxidant effects but it can also maintain the color of meat products due to delay of oxidation of oxymyoglobin and inhibition of PUFA oxidation. Organic minerals, aromatic herbs and essential oils are also being used in animal feed to protect against oxidative damage and improve flavor and palatability of meat.

Other technological strategies used in food systems imply decreasing oxygen content in packaging or its replacement with nitrogen or carbon dioxide in modified atmosphere packaging to attenuate protein oxidation during storage. Furthermore, films and hydrogels would be a suitable food packaging material for preventing oxidation due to their barrier properties. In this regard, protein-based edible films such as those derived from gelatin, soy or casein proteins, can additionally reduce oxidation by their own antioxidant activity, as well as function as matrix carriers for other antioxidant agents such as phenolic compounds. Additionally, the encapsulation of antioxidants by nano-emulsions could be a suitable novel technique to improve the antioxidant capacity of natural ingredients. On the other hand, recent studies have suggested that the inhibition of several endogenous proteolytic enzymes such as calpain, cathepsins or caspase-3 during food storage could have a significant inhibitory effect on protein oxidation. Some examples of these dietary and technological strategies and their respective effects on protein oxidation are shown in [Table 2](#).

## Analytical Procedures

The detection of protein oxidation can be performed by different methodologies including chromatographic, electrophoretic, spectroscopic and spectrometric techniques, or through a combination of them, depending on the oxidative product to be measured.

**Table 2** Examples of dietary and technological strategies against protein oxidation in foods

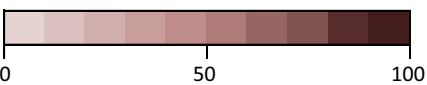
Strategy	Main active compound	Product	Oxidative process	Effects on oxidation compared to control samples	Reference
Addition of rosemary and sage essential oils	Phenolic diterpenes	Pork liver pâté	Refrigerated storage	Lower carbonyl content	Estévez et al. (2006)
Addition of black currant extract	Anthocyanins	Pork patties	Chilled storage	Lower carbonyl content and loss in sulfhydryl content	Jia et al. (2012)
Addition of gelatin hydrolyzates	Antioxidant peptides	Unwashed fish mince	Freeze-thawing process	Retarded carbonyl formation, lower loss in sulfhydryl content	Nikoo et al. (2015)
Supplementation of pig diet with avocado	Tocopherols	Pork loins	Cooking process	Lower carbonyl content and protein cross-linking	Hernández-López et al. (2016)
Use of modified atmosphere packaging	Mix of gases	Tilapia fish muscle	Iced storage	Lower carbonyl content, higher protein solubility	He et al. (2018)
Inhibition of endogenous proteolytic enzymes	Inhibitory compounds	Tilapia fish muscle	Iced storage	Lower carbonyl content, higher protein solubility	He et al. (2018)

Carbonylation is the most remarkable modification resulting from protein oxidation and thus, many analytical procedures are based on the quantification of these compounds. The 2,4-dinitrophenylhydrazine (DNPH)-method allows the quantification of protein carbonyls through their derivatization with DNPH to generate hydrazones, and subsequent spectrophotometric measurements at 370 nm. Carbonyls are also involved in protein cross-linking via Schiff base formation, and these structures are conjugated fluorophores with spectral properties that can be detected by fluorescence using excitation and emission wavelengths of 350 and 450 nm, respectively. These procedures measure the total amount of a variety of oxidation derivatives formed through unspecific pathways, limiting the evaluation of protein oxidation. In this regard, specific methods to determine particular protein carbonyls were developed, enabling a better understanding of the chemical structure and formation pathways of these compounds. High performance liquid chromatography with fluorescence detection (HPLC-FLD) allows the quantification of typical oxidation markers such as AAS and GGS after their derivatization with *p*-amino benzoic acid (ABA). Also compounds resulting from advanced oxidative reactions such as AAA can be identified by HPLC-FLD after a previous derivatization step. The introduction of mass spectrometry (MS) methodologies has allowed a fast, sensitive and accurate determination of these oxidation products. So, AAS and GGS can be detected by gas chromatography-mass spectrometry (GC-MS) in the form of their hydroxyl derivatives, or by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) after their derivatization with ABA.

Protein cross-linking is assessed using electrophoresis techniques and the analysis of these cross-linked bonds like disulfide or dityrosine bonds can be performed by RP-HPLC, LC-MS-MS, capillary electrophoresis, and spectroscopic methods including electron spin resonance (ESR) or nuclear magnetic resonance (NMR).

The loss or modification of amino acid residues due to oxidation processes can be assessed by using different chromatographic techniques. In HPLC methods, amino acids are derivatized before reversed-phase HPLC or after cation exchange HPLC separation to be better detected. When analyzed by GC-MS, amino acids are derivatized with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) or similar, to make them heat-stable and volatile. The loss of tryptophan residues due to oxidative processes can be determined due to its natural fluorescence recording the emission spectra from 300 to 400 after excitation wavelength of 283 nm. On the other hand, oxidized cysteines can be analyzed using the Ellman's reagent. So, protein thiol groups are derivatized with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and release a thiolate ion, which has a maximal absorbance at 412 nm and it is used to evaluate the concentration of thiols. Additionally, the analysis of the wide variety of oxidized residues derived from cysteine and methionine (see **Table 1**) can be performed by LC-MS despite the difficulty in the analysis due to the high reactivity of their thiol groups.

MS, usually combined with LC, are valuable proteomic tools to determine the specific site and nature of oxidative post-translational modifications in proteins such as oxidation of peptides and amino acids. These methodologies allow the identification and quantification of oxidized peptides due to modified amino acids are mass-shifted and thus identifiable in the fragmentation pattern. An example of peptides showing methionine oxidation identified and quantified by LC-MS/MS during the processing of Spanish dry-cured hams is shown in **Table 3**. The development of targeted MS methods that search for peptides containing ions markers of an oxidative modification such as methionine sulfoxide or chlorotyrosine, or the use of chemical reagents that react with oxidative modifications and are used as tags to label oxidized peptides or proteins, would facilitate the analysis of oxidative modifications compared to untargeted approaches. Nevertheless, the analysis of protein oxidation continues to be highly challenging due to the complexity of the oxidative mechanisms, the difficulty in detecting the wide variety of oxidation products as well as the low abundance and lability of some of them under the conditions of sample preparation and/or analysis.

**Table 3** Peptides showing methionine oxidation identified by nLC-MS/MS in dry-cured hams at different processing times and their relative quantification using a label-free approach based on peak intensity


Protein*	Peptide sequence	Processing times (months)					
		0	2	3.5	5	6.5	9
LDB3	VAIDGVNTDTM[Oxi]THL						
	IDGVNTDTM[Oxi]THL						
	FNM[Oxi]PLTISRITPGSKAAQSQL						
	FNM[Oxi]PLTISRITPGSKA						
	FNM[Oxi]PLTISRITPGSK						
	FNM[Oxi]PLTISRITPG						
	FNM[Oxi]PLTIS						
	M[Oxi]PLTISRITPGSKA						
	VVAIDGVNTDTM[Oxi]THL						
	DAIM[Oxi]DAIAGQAQAQGSDFSGSLPIKD						
	M[Oxi]DAIAGQAQAQGSDFSGSLPIKD						
	KPPDIPDSRVPIPTM[Oxi]PIR						
MYH1	M[Oxi]AIFGEAAPYLKSEK						
MYL1	EVKKVLGNPSNEEM[Oxi]						
NEBU	TM[Oxi]DPDVPQFIQA						
	DVSPGTAIGKTPEMM[Oxi]						
	VSPGTAIGKTPEMM[Oxi]						
	VSPGTAIGKTPEM[Oxi]						
	YKENVGKGTPPTVTPEM[Oxi]						
	VGKGTPPTVTPEM[Oxi]						
	MGKGTPLPVTPEM[Oxi]						
PDLIM3	APNIPLM[Oxi]ELPGVKIVH						
	M[Oxi]E[Dhy]LPGVKIVHAQF						
	M[Oxi]ELPGVKIVHAQF						
	M[Oxi]ELPGVKIVHA						
	M[Oxi]ELPGVKI						
TITIN	KPPDIPDSRVPIPTM[Oxi]PIRAVPP						

\* Protein name according to Uniprot database. LDB3: LIM domain-binding protein 3; MYH1: myosin heavy chain 1; MYL1: myosin light chain 1; NEBU: nebulin; PDLIM3: PDZ and LIM domain protein 3; TITIN: titin.

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## Coffee Flavor

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### Glossary

**Maillard reaction** Maillard reaction is a nonenzymatic browning reaction that is taking place between amino acids and reducing sugars.

**Strecker degradation** The deamination and decarboxylation of amino acids to imines and then to aldehydes or ketones, in the presence of a carbonyl compound.

**The degree of roasting** The intensity of the roasting of coffee, which is controlled by the roasting temperature and time. The degree of roasting could be classified as light, medium, or dark.

### Introduction

Coffee is the most traded commodity in the world just next to petroleum with annual production of more than 8.6 million tons (Getachew and Chun, 2017). The popularity of coffee as a beverage is as a result of its refreshing flavor and stimulating effect. The coffee flavor is a result of several stages and processes that involve from the raw bean to the cup. The flavor formation and profile of coffee is influenced by the species or variety of bean, the geographical origin of the bean, the type of raw coffee bean processing (dry or wet processing), the degree of roasting (light, medium, or dark), and the coffee brewing techniques, among others. The roasting process is thought to be the most important factor for the formation of coffee flavor. In this chapter, short and precise recent development on formation, detection, and quantification of coffee flavor compounds has been discussed.

### Formation of Coffee Flavor Compounds

As roasting is the most important factor affecting flavor formation, the focus of this section is on the formation of coffee flavor as result of the roasting process. Raw coffee bean has the pealike, green smell, and only after roasting, the smell of coffee can be converted into pleasant smell characteristic of roasted coffee because of the drastic formation of various flavor impact compounds (Czerny et al., 1999; Czerny and Grosch, 2000). Thus roasting has been the focus of much research in the coffee industries and in the academia (Baggenstoss et al., 2008; Poisson et al., 2009; Esquivel and Jiménez, 2012). The roasting time and temperature have a substantial influence on flavor formation. Roasting temperatures and time can vary between 180 °C and 240 °C and 8 to 15 min, respectively. During roasting, the heat transferred to the bean induces several thermal reactions in the coffee bean. Some of the heat-induced reaction produced during roasting includes Strecker degradation, Maillard reactions, degradation of trigonelline, amino acids, phenolic acids, lipids, and interaction between intermediate products (Sunarharum et al., 2014).

Although the formation pathways of most flavor compounds during the roasting of coffee are still not well understood (Akiyama et al., 2007), the initial chemical composition of raw coffee bean, its moisture content, roasting temperature, and the pressure developed inside the individual coffee beans are thought to be important factors for the formation of coffee flavor (Gloess et al., 2014). Nevertheless, Maillard reaction is believed to play the most significant role in the formation of major coffee flavor compounds.

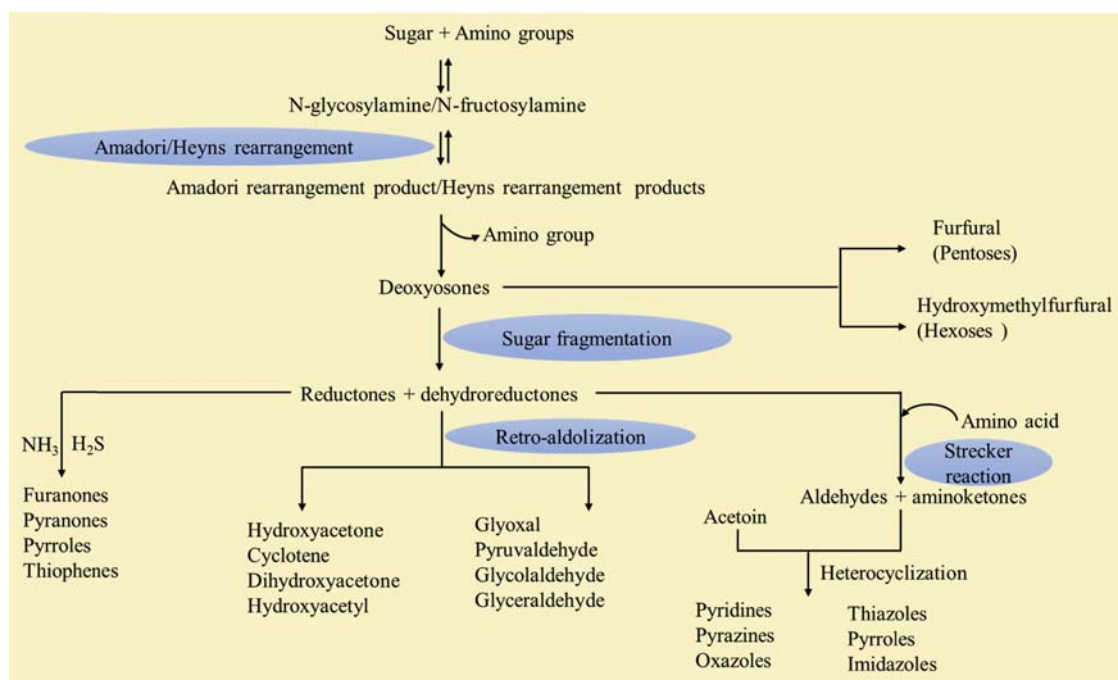
### The Role of Maillard Reaction for Flavor Formation

Most of the coffee flavor impact compounds are produced at a high temperature of roasting above 200 °C. The chemicals present in raw coffee beans such as carbohydrates, proteins, phenolic acids, and trigonelline participate as a precursor in flavor formation. Most importantly, during roasting process, Maillard-type reactions and other chemical transformations play a central role in the formation of coffee flavor. Some of the Maillard-derived aroma compounds produced during coffee roasting include thiols, diketones, pyrazines, furanones, thiazoles, pyrroles, and pyridines. Several researchers have studied the formation of these compounds in model systems under dry heating conditions (Grosch, 1999; Yaylayan and Keyhani, 1999; Poisson et al., 2009). Fig. 1 shows the details of the formation of flavor compounds during Maillard reaction.

### Coffee Flavor Impact Compounds

Since the first identification of few coffee volatile compounds by a German chemist Bernheimer in 1880, around 1000 different volatile compounds have been identified and reported in roasted coffee (Nijssen et al., 1996). Among these numerous volatile





**Figure 1** Flavor formation during Maillard reaction. Adapted from Van Boekel, M.A.J.S., 2006. Formation of flavour compounds in the Maillard reaction. *Biotechnol. Adv* 24, 230–233, as originally reported by Ho, C.-T., 1996. Thermal generation of Maillard aromas. John Wiley & Sons, Chichester, United Kingdom. Van Boekel, M.A.J.S., 2006 & Ho, C.T., 1996.

compounds, only a few of them have been considered as aroma impact compounds. The composition and concentration of the flavor impact compounds vary with the level of roasting, the coffee species, varieties, and the geographic origin (Baggenstoss et al., 2008). Some of the most potent classes of flavor impact compounds are discussed below.

### Furans

Furans are degradation products of sugars, unsaturated fatty acids, and ascorbic acids during the roasting of coffee (Crews and Castle, 2007; Ribeiro et al., 2009). They represent one of the most abundant groups of volatile compounds found in high concentration in both roasted powder and brewed coffee (Sunarharum et al., 2014). Furans have sweet and malty roasted aromas. As compared with other groups of volatile compounds in roasted coffee, they have higher sensory thresholds. However, because of their relatively high concentration, they are still important in coffee flavor (Sunarharum et al., 2014).

### Furanones

Furanones are produced through Maillard reaction following aldol condensation (Grosch, 2001). Furanones are a large group of compounds in coffee with regard to both abundance and potency. The sweet caramel aroma of roasted coffee is a result of different classes of volatile compounds (Sunarharum et al., 2014). The most important furanones in coffee are listed in Table 1.

### Phenolic Compounds

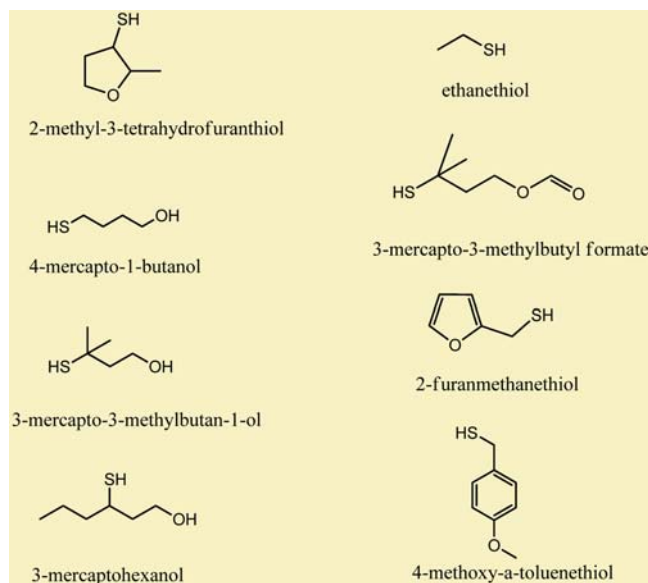
The roasting process generates some phenolic compounds that are part of the most important flavor compounds in coffee (Ribeiro et al., 2009). Among them, guaiacol, 4-ethylguaiacol, and 4-vinylguaiacol contribute for a spicy phenolic aroma (Akiyama et al., 2008; Mayer et al., 2000) and vanillin (Sunarharum et al., 2014). They are produced as a result of heat degradation of phenolic acids (such as quinic, ferulic, and caffeic acids). The concentration of these compounds greatly depends on their respective concentration in the original raw coffee. As the level of chlorogenic acids is high in Robusta coffee in relative to Arabica, consequently, the level of phenolic aroma compounds is higher in the earlier coffee variety (Sunarharum et al., 2014).

### Sulfur-Containing Compounds

Sulfur-containing compounds are among the most important classes of volatile compounds that are responsible for the coffee flavor (Caprioli et al., 2012; López-Galilea et al., 2006; Sunarharum et al., 2014). Despite their low concentration in coffee, volatile thiols

**Table 1** Most important flavor impact compounds in coffee, flavor notes, and their formation pathways

<i>Flavor compound</i>	<i>Flavor contribution</i>	<i>Formation pathway</i>	<i>References</i>
<b>Pyrazines</b>		Maillard reaction	Amrani-Hemaimi et al. (1995) Cheong et al. (2013)
2,5-Dimethylpyrazine	Cocoa, roasted nuts, roasted beef, woody, grass, medical		
2,6-Dimethylpyrazine	Cocoa, toasted nuts, roasted meat		Blank et al. (1992)
2-Ethylpyrazine	Peanut butter, musty, nutty, woody, roasted cocoa		Blank et al. (1992)
2,3-Dimethylpyrazine	Nutty, coffee, peanut butter, walnut, caramel, leather		Cheong et al. (2013)
<b>Thiols</b>		Maillard reaction and heat-induced cysteine or glutathione degradation	Zhang and Ho (1991) and Silva Ferreira et al. (2003)
3-Mercapto-3-methyl butylformate	Green blackcurrant, herbal, fruity, roasted, sweaty		Akiyama et al. (2007)
2-Furfurylthiol	Roasted (coffeelike), sulfurous		Blank et al. (1992)
2-Methyl-3-furanthiol	Sulfurous, meaty, fishy, metallic, boiled		Blank et al. (1992)
3-Mercapto-3-methylbutylacetate	Roasty, fruity, sulfurous, sweet		Kumazawa and Masuda (2003a)
3-Methyl-2-butene-1-thiol	Sulfurous, smoky, leek, onion		Blank et al. (1992)
Methanethiol	Rotten eggs, meat or fish, cabbage, garlic, cheesy		Akiyama et al. (2008)
<b>Furanones</b>		Maillard reaction and subsequent aldol condensation	Grosch (2001) and Sunarharum et al. (2014)
Furfural	Sweet, brown, woody, bread, caramellic	Thermal degradation of carbohydrates, ascorbic acids, or unsaturated fatty acids	Grosch (2001)
Furfuryl acetate	Onion, garlic, sulfurous, pungent, vegetable, horseradish		Crews and Castle (2007)
5-Methylfurfural	Caramel, burned sugar, aromatic, sweet		Grosch (1998)
5-Methylfuran-2-carboxylic acid, methyl ester	Sweet, caramellic, bready, brown, coffeelike		Grosch (1998)
5-Hydroxymethylfurfural	Sweet, caramel, bread, maple, brown sugar, burnt		Grosch (1998)
<b>Phenols</b>		Degradation of phenolic acids	Bicho et al. (2013)
2-Methoxyphenol (Guaiacol)	Phenolic, burnt, smoke, spice, vanilla, woody		Akiyama et al. (2007)
2-Methoxy-4-vinylphenol	Spicy, peanut, winelike, or clove and curry	Thermal degradation of ferulic acid	Buffo and Cardelli-Freire (2004)
4-Ethylguaiacol	Spicy		Blank et al. (1992)
Vanillin	Vanilla-like		Blank et al. (1992)



**Figure 2** Thiols in coffee flavor impact compounds.

are considered to be the most influential sulfur-containing compounds in sensory profile of coffee having very low sensory thresholds (Sunarharum et al., 2014). Fig. 2 shows some of the key aroma impact sulfur-containing volatile compounds in roasted coffee, which are usually associated with roasty, sulfurous coffee flavor (Kumazawa and Masuda, 2003b; Mayer et al., 2000; Sunarharum et al., 2014). As it has been confirmed by different model systems, formation pathways of thiols such as 2-furfurylthiol (FFT) are the reaction of pentose and hexose sugars and cysteine under roasting conditions (Cerny and Davidek, 2003). Other model system comprising arabinogalactan/cysteine showed that arabinogalactan is a key precursor for the formation of FFT (Poisson et al., 2009).

### Pyrazines

Pyrazines are formed during the Maillard reaction, preferably at temperatures above 100 °C. They are frequently found in foodstuffs prepared by a heating process (Amrani-Hemaimi et al., 1995). They contribute to the earthy-roasty character of roasted coffee. Different studies in model systems demonstrated that alkylpyrazines are formed from Strecker degradation of C<sub>6</sub> and C<sub>5</sub> sugar compounds and  $\alpha$ -amino acids through condensation reaction and oxidation of the resulting condensation product (Amrani-Hemaimi et al., 1995).

Table 1 summarizes some of the most important flavor impact classes of compounds, their flavor notes, and formation pathways.

### Influence of Degree of Roasting on Flavor Formation

As discussed in section **Formation of Coffee Flavor Compounds**, roasting is the key factor for the formation of coffee flavor. The degree of roasting also plays an important role for the formation of the flavor profile that better suits the type of coffee, its final use, and the personal preference of the consumers (Bhumiratana et al., 2011). Different roasting level produces different flavor profile. For instance, light roasting produces cocoa, nutty, and sweet aroma and dark roasting produces sour, ashy, and pungent aroma, whereas medium roasting is responsible for the formation of complex aroma profile (Bhumiratana et al., 2011). Furfural derivatives and furanones are yielded in relatively high concentrations under mild roasting conditions and then reduced at higher roasting intensities. More pyridines and pyrroles are formed by high roasting degree than by mild roasting intensities. Chlorogenic acid degradation products, phenols, and lactones are produced more by high roasting degree than by low roasting degree (Moon and Shibamoto, 2009).

### Detection and Quantification of Coffee Compounds

As it is mentioned in section **Coffee Flavor Impact Compounds**, to date, more than 1000 coffee volatile compounds have been identified. The nature of the compounds and their interaction has always created difficulty in detection and quantification. Since the first attempt to isolate and quantify the compounds responsible for coffee aroma by Bernheimer in 1880, there have been several advancements in extraction, isolation, and quantification. This is a result of understanding the nature of the formation of coffee

flavor compounds and the development of advanced analytical instruments. Some of the recent developments in coffee flavor detection and quantification are discussed below.

### Recent Development in Detection and Quantification of Coffee Flavor

The advancement of chromatographic techniques, more specifically gas chromatography coupled with mass spectroscopy, in recent decades has dramatically changed the quantification speed and the accuracy of flavor detection. The recent development of technology is also used to differentiate the geographic source and authenticity of roasted coffee beans based on their signature flavor profiles.

Recently, Quintanilla-Casas et al. (2015) developed a fast analysis method for volatile thiols in brewed coffee. Their developed method involves a simultaneous derivatization/extraction procedure followed by liquid chromatography–electrospray high-resolution mass spectrometry. They identified and quantified seven targeted and nine nontargeted thiols in the coffee brew, and most of them are reported for the first time in brewed coffee. In another study, Yener et al. (2015) used proton transfer reaction–time-of-flight mass spectrometry (PTR/TOF-MS) for the first time to trace the origin of both roasted and brewed coffee using their volatile flavor compounds. In their study, they reported that the switching reagent ion system in PTR-MS instrumentation was applied for the first time to coffee not only with commonly used  $\text{HO}_3^+$  but also with  $\text{NO}^+$  and  $\text{O}_2^+$  as precursor ions to minimize the classification errors of the volatile compounds. For authentication of specialty coffees based on their volatile compounds profile, Özdeştan et al. (2013) used high-sensitivity proton transfer reaction mass spectrometry (HS PTR-MS). They showed that the different coffee samples could be distinguished by their volatile compounds profile obtained from PTR-MS with the help of chemometrics. The basic principles and in-depth discussion of the PTR-MS can be found in Cappellin et al. (2013) and Blake et al. (2009).

### Conclusion

The cup of coffee that millions of people enjoying every day around the world has a complex array of flavors, which is completely different from its original raw material, the raw coffee bean. To get its refreshing flavor, the coffee bean must pass through the roasting process. The roasting process is the most important factor for the formation of coffee flavor along with the chemical composition of the original raw coffee bean. During roasting, several heat-induced reactions take place in the so-called “tiny reactors,” the individual coffee beans. The chemical reactions include Maillard reaction, Strecker degradation, decomposition, and degradation of phenolic acids such as chlorogenic acid and ferulic acids, degradation of trigonelline, and sugars. Thus the formation pathways of the compounds are significantly affected by the composition of the raw coffee bean (which is also affected by its geographic origin), the degree of roasting, the roasting temperature, and time. These heat-induced reactions produce several classes of compounds such as thiols, pyridines, pyrazines, and furans with sensory thresholds ranging from few parts per billion to several hundreds of parts per billion. The advancement of modern analytical instruments and techniques significantly improved the quality and accuracy of flavor analysis.

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## Configuring Phenolic Antioxidants for Frying Applications

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### Glossary

**Acidolysis** It is an acyl-transfer chemical reaction involving an ester and a free fatty acid.

**Alcoholysis** It refers to an acyl-transfer chemical reaction involving an ester and a free fatty alcohol.

**Amphiphilic** A term that describes molecules that possess both hydrophilic and lipophilic moieties within their chemical structures.

**Antioxidants** These are compounds with the ability to inhibit oxidation when present in food or biological systems.

**Carry-through** It refers to the ability of antioxidants to survive frying conditions and get transferred into the fried foods.

**Fractionation** It is the controlled separation of oil/fat into different fractions by either low temperature (dry fractionation) or solvents (solvent fractionation). Palm oil, for example, is fractionated into palm olein and palm stearin.

**Hydrogenation** A process of adding hydrogen atoms across the double bonds of unsaturated triacylglycerols, usually in the presence of catalysts such as nickel and under controlled temperatures. The process decreases the degree of unsaturation of the vegetable oil but may generate *trans* fats.

**Hydrolysis** The reaction between triacylglycerol and water molecule resulting in the release of free fatty acid from the glycerol backbone, resulting in diacylglycerol, monoacylglycerol and eventually, free glycerol.

**Hydrophilic** A term that describes chemical species (polar molecules) that positively interact with water and are dissolved by it.

**Interesterification** A process whereby the fatty acids on the glycerol backbone of triglycerols are by rearrangement. This could be affected by chemical means or by the introduction of enzymes.

**Isomerization** Heat-enhanced reaction whereby the natural *cis*-configured fatty acid is transformed into the *trans* isomer.

**Lipophilic** A term that describes chemical species (nonpolar molecules) that positively interact with and are soluble in fats and oils.

**Lipophilization** A chemical or enzymatic process for transforming a hydrophilic molecule into a lipophilic species.

**(Poly)phenolic (also polyphenols)** A class of chemical compounds consisting of a hydroxyl group bonded directly to an aromatic hydrocarbon group. They are classified as simple phenols or polyphenols based on the number of phenol units in the molecule.

**Polymerization** This is a heat-driven aggregation of triacylglycerol monomers that can be connected by carbon–carbon, carbon–oxygen–carbon, or carbon–oxygen–oxygen–carbon linkages.

**Prooxidants** These are components or compounds, which encourage oxidative deterioration of edible oils.

**Regiospecific** This refers to the habitual tendency of some enzymes to direct reaction to a particular site on a molecule.

**Thermooxidation** A heat-enhanced reaction between atmospheric oxygen and unsaturated lipids.

**Triacylglycerols** Also known as triglycerides, triacylglycerides, abbreviated TAG or TG are esters obtained from glycerol and three fatty acids.

**Unsaponifiables** These are nontriglycerol minor components of oils, which are not saponified by alkali hydroxides and are extractable into ether.

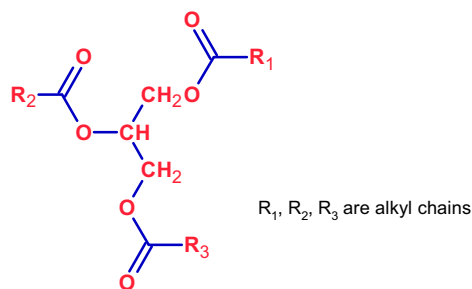
**Unsaturated** An organic compound (e.g., fatty acids, triacylglycerols) is referred to as “unsaturated” if it possesses one or more carbon–carbon double bond within its chain. It is monounsaturated if it contains one and polyunsaturated if it has more than one carbon–carbon double bond.

### Introduction

Frying is a food preparation technique where the foods are immersed in edible oil at a temperature above the boiling point of water, usually between 150 and 200 °C. It is one of the oldest food preparation methods known to man, and although popularly and routinely used by an average household, the art of frying is hardly simple, and the science is simply hard. The complexity of frying, as a food preparation method, stems from the need to delicately balance many interrelated factors, such as frying time, temperature, fryer types/dimensions, and surface-to-volume ratio, known to modulate the frying process and also sufficiently control the self-sustained physicochemical reactions occurring during the process.

Compositionally, the frying oil is essentially a mixture of triacylglycerols (c. 96%), and the changes observed during frying are primarily related to the chemical structures of the constituent triacylglycerols (Fig. 1). The predominant triacylglycerols of frying oils are unsaturated; and while this may offer some advantages with regards to fluidity, mouthfeel, heat transfer, and health benefits,





**Figure 1** Triacylglycerol composing of three molecules of fatty acid esterified to a glycerol backbone.

unsaturated fatty acids are predisposed to thermal and oxidative degradative reactions prevailing during frying (Choe and Min, 2009).

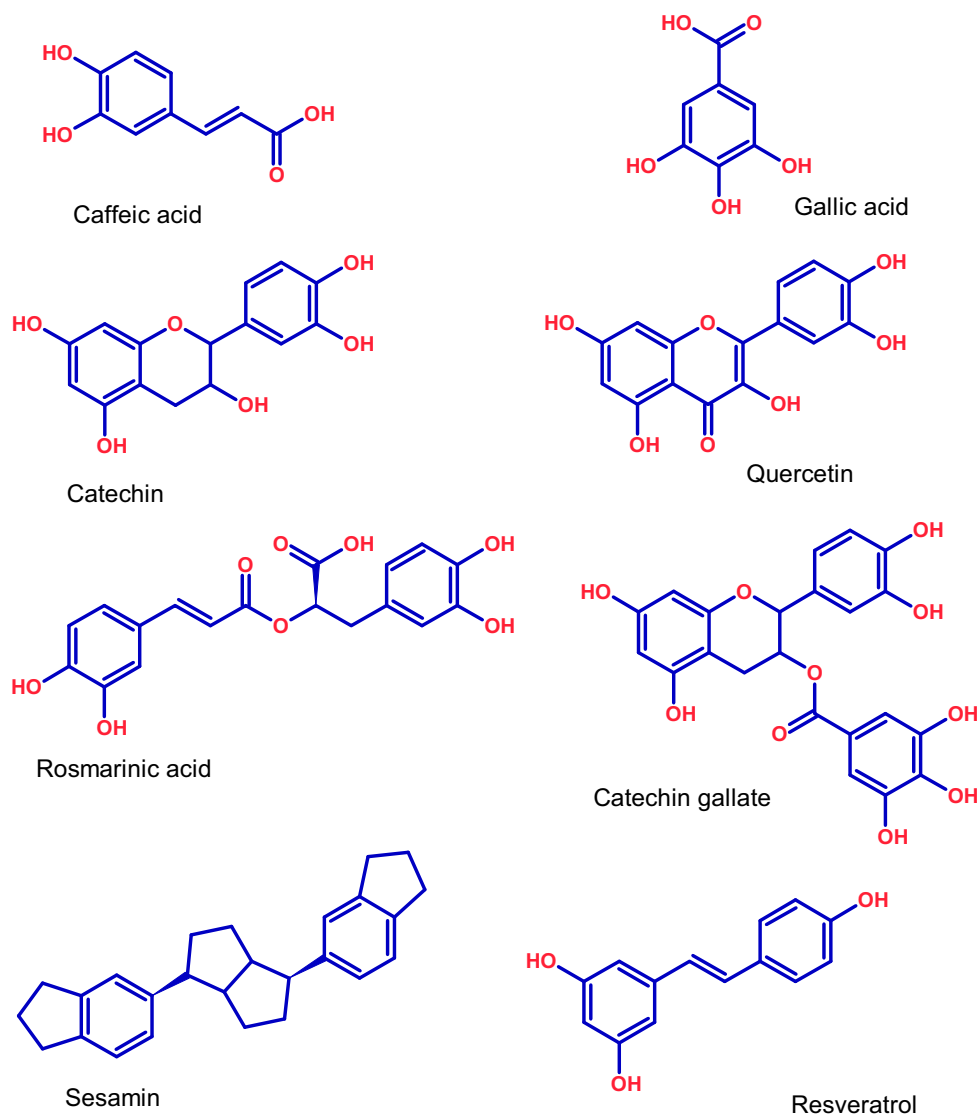
The high operating temperature (in excess of 170°C) utilized for frying processes inevitably instigates and accelerates a series of complex chemical reactions, involving dissolved oxygen (from air), moisture (from the food and air), the frying oil, and components of the fried foods, resultantly altering the structural composition and sensory qualities of the frying oil and the food prepared in it. Thus, the frying oil is transformed from an almost pure triacylglycerol to a mixture of hundreds of chemical compounds (Blumenthal, 1987). Currently, over 500 chemical compounds have been identified in degraded frying oils with a number of them potentially toxic above certain thresholds (Gertz, 2001). It must be mentioned that at reasonable levels, these chemical compounds are directly and indirectly responsible for the unique golden color and delightful deep-fried flavor of fried foods.

Thermal oxidation (thermooxidation), thermal hydrolysis, polymerization, and isomerization are the most noteworthy reactions occurring during frying, with thermooxidation being the most prevalent and most destructive. The stability of a frying oil is a measure of its ability to offer resistance against any of these degradative reactions, and it is related to the inherent composition of the frying oils (internal factors) and the applied frying conditions/managements (external factors). With regards to the fatty acid composition (triacylglycerol compositions), generally, the higher the level of carbon–carbon unsaturation, the more susceptible the oil is to thermooxidative degradations, and the methods for enhancing the stability along this line have been toward reducing the fatty acids unsaturation through methods such as fractionation, hydrogenation, interesterification (chemical and enzymatic), conventional seed breeding, and genetic engineering (Holm and Cowan, 2008; Clemente and Cahoon, 2009; Dijkstra, 2009; Jhala et al., 2009; Martin et al., 2010; Hayes and Pronczuka, 2010; Kontkanen et al., 2011). Enhancing the stability of frying oils by altering the saturation level is not without controversies and challenges as previously discussed (Lichtenstein et al., 2006; Wang et al., 2010; Turan et al., 2007; Maduko et al., 2008; Jennings and Akoh, 2009; Matthäus, 2007), and the use of antioxidants is often more favored.

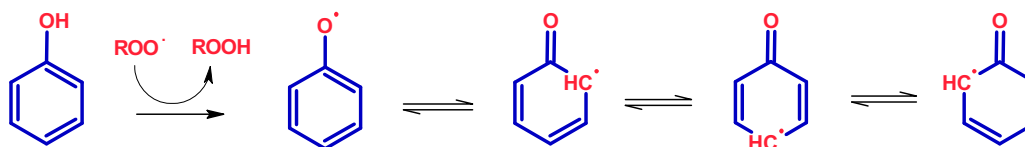
As mentioned earlier, the frying oil is almost a pure mixture of triacylglycerols, representing an average of 96% of the oil's composition. Nevertheless, the minor components of frying oils (<5%), also referred to as "unsaponifiables," can and do exert significant influence on the performance of frying oils with respect to their thermooxidative stability. Components of the unsaponifiables, depending on the relative amounts and structural identities, can either enhance the frying stability by limiting lipid oxidation and subsequent polymerization (antioxidant) or destabilize the oil during frying (prooxidant). Generally, for a typical commercially processed frying oil, the influence of the antioxidative compounds (primarily, tocopherols, phytosterols, lignans, phenolics, and to a lesser extent, carotenoids) significantly outweighs the effects of prooxidative compounds (mainly, free fatty acids, hydroperoxides, and metals) in the unsaponifiables, tending the oil's minor components cumulatively toward antioxidative activities.

Although antioxidative minor components such as tocopherols (tocopherols and tocotrienols) offered marked protection to oil during storage and other ambient temperature applications, they offer relatively little protection during frying, since they are quickly decomposed, volatilized, or inactivated, due to the extreme processing conditions utilized for frying operations. Hence, to improve the stability of the frying oils, external antioxidative compounds are generally added (Aladedunye, 2014; Aladedunye et al., 2017). Unlike the complicated, time-consuming, and highly technical procedures required for the modification of fatty acid compositions of frying oils, the addition of external antioxidants is rather simple, economical, and often requires relatively smaller amounts (<0.5%) of the applied antioxidants.

Potentially, naturally occurring (poly)phenolic compounds are excellent antioxidants (Fig. 2), inherently possessing the ability to easily donate hydrogen radicals to hydroperoxy radicals, thereby inhibiting the propagation stage of radical-mediated lipid oxidation (Fig. 3) that is a prominent mechanism for frying oil's deterioration. However, the same structural moiety (phenolic OH functional groups), which confers this antioxidative advantage on polyphenolic compounds, is also the limiting factor to their applications in frying oil. In general, the presence of phenolic OH in a compound decreases its lipophilicity (solubility in oils/fats), and there is an inverse relationship between the number of the phenolic OH and the compound's solubility in oils/fats. Consequently, polyphenolic compounds are poorly soluble in edible oils. While the poor lipophilic nature may be advantageous under storage conditions where lipid oxidative deterioration is localized at the oil–air interface (Porter, 1993; Frankel et al., 1994; Frankel, 2005), it does limit its effectiveness during frying where active agitation (arising from bubbling at the introduction of fresh



**Figure 2** Selected structural diversities in polyphenols ranging from simple phenolic acids (caffeic and gallic), flavonoids (catechin, quercetin, catechin gallate), lignin (sesamin), and Stilbene (resveratrol).



**Figure 3** Typical radical scavenging mechanism of a phenolic compound ( $\text{ROO}^\bullet$  is a lipid hydroperoxy radical).

food and convective stirring as the frying oil bounces back to the preset frying temperature) continually “delocalizes” thermooxidative reactions, requiring a good degree of mixability between the oil and the applied antioxidative compound. In other words, for an antioxidant to be practically effective, it must be located or easily deployable to the site of thermooxidative deterioration. To overcome the challenge of high hydrophilicity in polyphenolics and thereby broadening their applicability as frying oil antioxidants, a restructuring or modification of the moiety is required. Interesterification, a process commonly employed for this purpose, is hereby discussed in relation to the frying process.

## Interesterification—Lipophilization of (Poly)phenolics

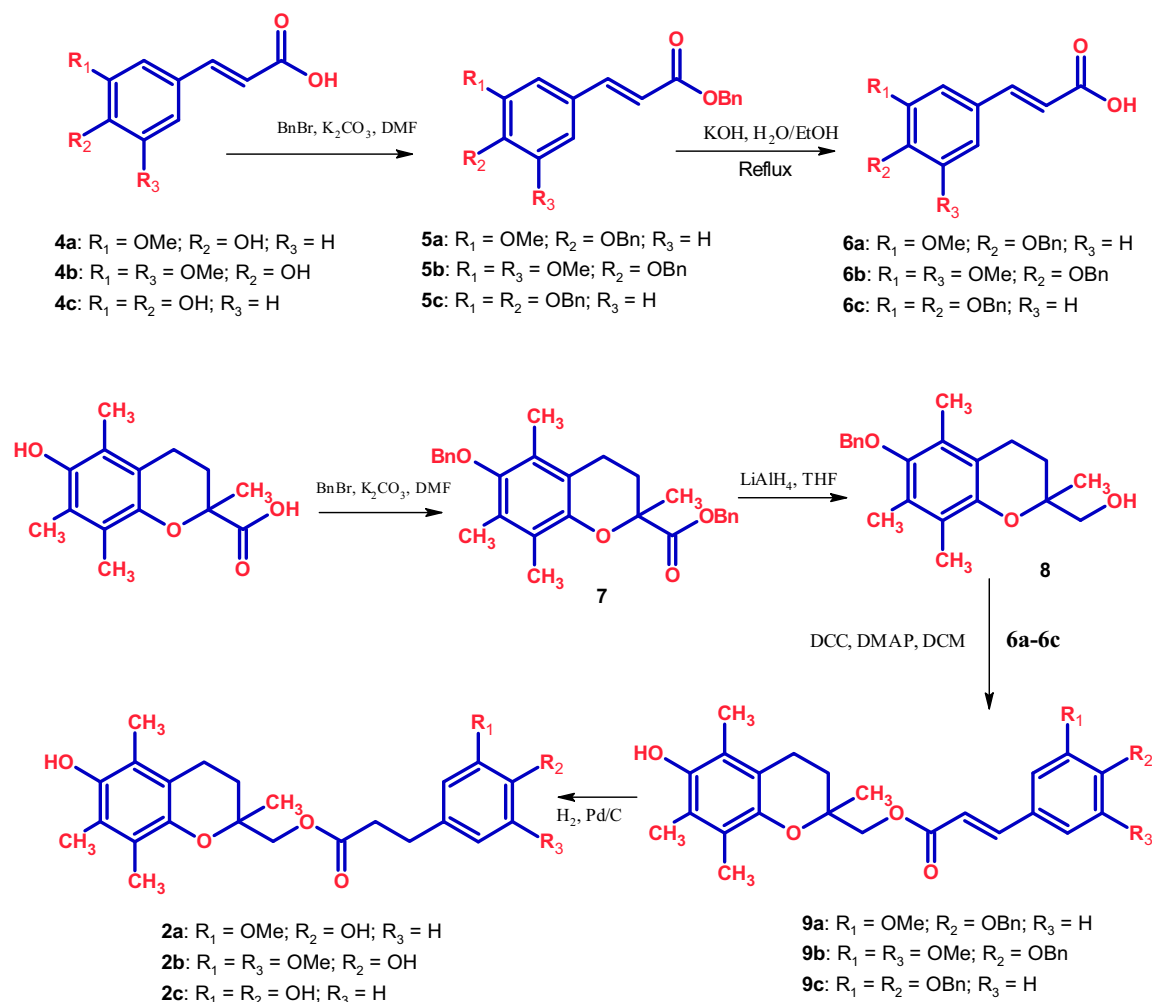
Basically, esterification is a reaction between an alcohol and an acid to form an ester as the reaction product. Depending on the functional group of interest in the phenolic compound, a fatty acid moiety can be added by reacting with a fatty alcohol (for phenolic acids) or a fatty acid (for polyphenols), resulting in an amphiphilic molecule with relatively unchanged functional properties such as antioxidant, antimicrobial, UV filter, etc. (Figuerola-Espinoza and Villeneuve, 2005). The reaction can also be an ester exchange between the alkyl ester of the phenolic acid and (1) a fatty acid ester, including triacylglycerols (interesterification, transesterification), (2) a fatty acid (acidolysis), or (3) a fatty alcohol (alcoholysis). This process is technically referred to as lipophilization, since it essentially transforms the hydrophilic polar phenolic compounds to lipophilic (lipid soluble) molecules.

The addition of alkyl chain to the relatively compact hydrophilic phenolic compounds can be achieved by (1) chemical, (2) enzymatic, and (3) chemoenzymatic processes. In chemical lipophilization, the esterification reaction is effected by chemical catalysts; however, chemical modification is not selective and, unless controlled, the entire active phenolic OH sites on the polyphenol can be “blocked” rendering the polyphenolic compound antioxidatively inactive (Danihelova et al., 2012). Furthermore, chemical restructuring of polyphenols often requires relatively severe reaction conditions, including toxic solvents and high temperature, resulting in poor yields, unwanted side products, and unwarranted decomposition of the thermally labile phenolic compounds and the acyl or alkyl donor substrates that often contain unsaturated C—C bonds (Figuerola-Espinoza and Villeneuve, 2005; Chebil et al., 2006). In cases where the phenolic compounds are esterified to the glycerol backbone of a natural edible oil, it has been reported that the acidolysis chemical reaction often results in depleted natural tocopherol contents of the edible oil due to the side reaction between tocopherols and the free fatty acid present in the medium, under the reaction conditions (Hamam and Shahidi, 2006). Consequently, chemical modification of phenolic compounds is not viewed as a practical, economical, sustainable, and viable modification protocol for the lipophilization of phenolic compounds.

Unlike chemical modifications, enzymatic restructuring of polyphenolics, although requiring relatively longer reaction time, is carried out under relatively mild conditions, thereby protecting the structural integrity of both the reactants and products. The yields are generally high, and the side products are minimal, especially since enzymatic restructuring of polyphenolics can be regiospecific, depending on the choice of enzymes (Figuerola-Espinoza and Villeneuve, 2005; Chebil et al., 2006). For wholesome edible oils, enzymatic modification does not result in flavor reversion and the oxidative stability of the oil is not compromised. These advantages and opportunities presented by enzymatic restructuring of polyphenols have resulted in renewed interests in the subject in the last decade.

The effectiveness of enzymatic modification of polyphenol is governed by several parameters, principally, type of solvents, choice of enzymes, operational temperature, and water activity of the system. Consequently, these parameters must be optimized for improved yields and economics (Chebil et al., 2006). Excellent reviews on the enzymatic lipophilization of phenolics up till 2017 are available (Figuerola-Espinoza and Villeneuve, 2005; Chebil et al., 2006; Viskupicova et al., 2009; Gonzalez-Sabin et al., 2011; Laguerre et al., 2013; Crauste et al., 2016; de Araujo et al., 2017; Aladedunye et al., 2017). Several solvents such as acetonitrile, acetone, tetrahydrofurane, *tert*-amyl alcohol, hexane, *tert*-butanol, dioxane, DMF, 3-pentanone, THF, chloromethane, and their binary mixtures have been evaluated for enzymatic lipophilization of phenolic compounds. Solvent-free systems have also been used, and recently, deep eutectic solvent–water binary mixture and ionic solvent have been evaluated and optimized (Lopez-Giraldo et al., 2007; Yang et al., 2012; Durand et al., 2013). Various types of enzymes, notably, lipases, proteases, cellulases, and transferases are being used to effect restructuring reactions with lipases, principally those from *Candida* sp., *Pseudomonas* sp., and *Mucor* sp. or *Rhizopus* sp., being the most widely used. The lipases can be free or immobilized on a support, with immobilized enzymes being the most preferred. Indeed, not only does immobilization makes for easier product recovery/clean up and easier substrate’s access to catalytic sites of enzymes, but it has also been reported that it enhances enzyme’s resistance to inactivation, resulting in extended usage (Chebil et al., 2006).

Virtually all classes of phenolic compounds ranging from the simple phenolic acids (ferulic, caffeic, dihydrocaffeic, coumaric, sinapic, phloretic, protocatechuic, gentisic, gallic, vanillic, syringic, etc.) to relatively complex polyphenols (chlorogenic acid, rutin, catechin, epicatechin, quercetin, phloridzin, naringin, hesperidin, quercitrin, isoquercitrin, luteolin, epigallocatechin, epigallocatechin gallate, callistephin, esculin, delphinidin, cyanidin, pelargonidin, etc.) have been chemically, enzymatically, or chemoenzymatically modified to increase lipophilicity and functionality (Figuerola-Espinoza and Villeneuve, 2005; Chebil et al., 2006; Viskupicova et al., 2009; Gonzalez-Sabin et al., 2011; Laguerre et al., 2013; Crauste et al., 2016; de Araujo et al., 2017), albeit with widely varying results. However, despite the hundreds of available literature and novel studies in this important research area, there are only a handful of studies available where phenolic compounds were configured and evaluated as antioxidants for frying applications (Gertz, 2004; Catel et al., 2010, 2012; Aladedunye et al., 2012, 2015; Gruczynska et al., 2015; Aladedunye and Matthaeus, 2016). The reasons for these limited interests in the assessment of restructured or lipophilized polyphenolics for frying applications may be related to the erroneous extrapolation of data obtained for radical scavenging activity in chemical tests (DPPH, ORAC, etc.) or antioxidant activities under oxidative conditions (Schaal oven test, Rancimat, etc.), whereby the relatively bulkier, less polar, and more sterically hindered modified polyphenolic compounds understandably performed worse than their native counterparts (Lorentz et al., 2010; Ma et al., 2012; Milisavljevic et al., 2014). Indeed, it has been established that the activity of antioxidative compounds under ambient conditions using chemical assessments or during accelerate storage tests cannot correctly depict its performance during frying, considering the wide differences in operational conditions including: (1) temperatures; (2) oxygen pressure; (3) water content; (4) thermooxidative degradation of components and their interactions with antioxidative compounds; (5) volatilization, degradation, and thermal inactivation of antioxidants; (6) continuous changes in relative



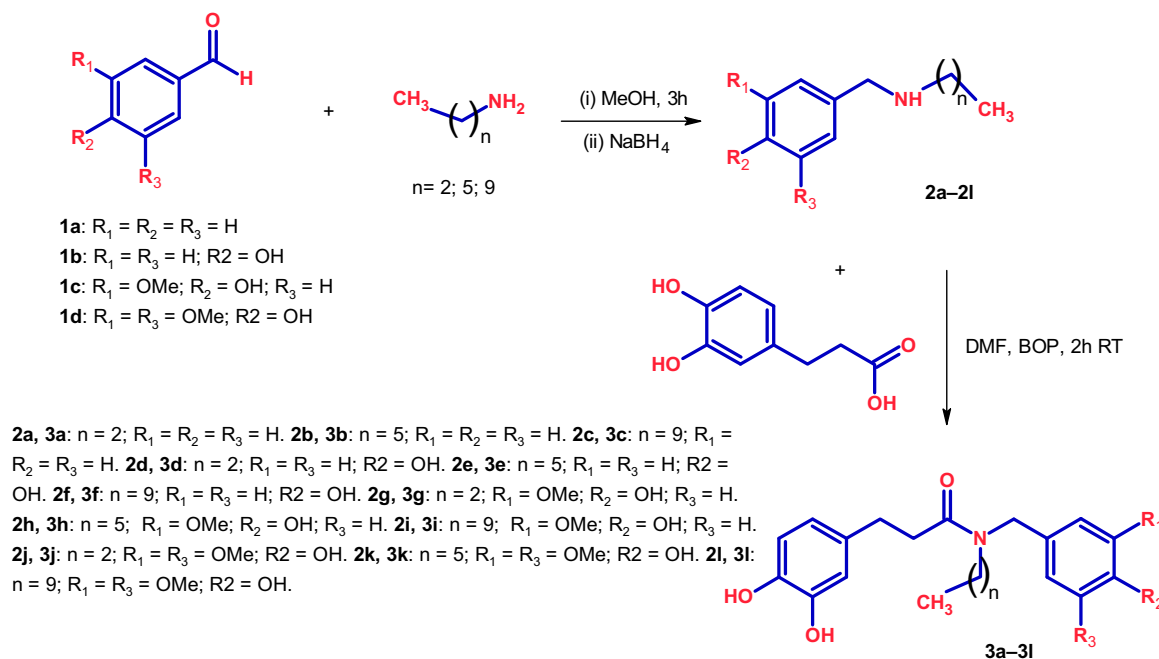
**Figure 4** Synthetic modification of phenolic compounds to improve activity, lipophilicity, and thermal stability. Adapted from Catel, Y., Aladedunye, F.A., Przybylski, R., 2010. Synthesis, radical scavenging activity, protection during storage, and frying by novel antioxidants. *J. Agric. Food Chem.* 58, 11081–11089.

locations of antioxidant, oxidant, and substrates due to constant agitation during boiling; and (7) occurrence of parallel reaction mechanisms during frying that may as well be more important than radical scavenging mechanism, an activity solely assessed by DPPH and oxidative storage tests (Gertz et al., 2000; Gertz, 2004; Kochhar and Gertz, 2004; Aladedunye et al., 2012).

With regards to configuring polyphenolics for frying applications, Catel et al. (2010, 2012) reported the chemical synthesis of a series of trolox derivatives of selected hydroxybenzoic acids (p-hydroxybenzoic, syringic, gallic, vanillic, and protocatechuic) and dihydrocaffeic acid (Fig. 4) by esterifying the phenolic acids with trolox chromanol ring, which is regarded as the primary antioxidant active center for tocochromanols, the most abundant natural antioxidants in frying oils.

The new sets of antioxidants were evaluated for frying performance and were recorded to be significantly better than  $\alpha$ -tocopherol as measured by the amount of total polar compounds (TPC) formed under the experimental frying conditions. Further, the configured phenolic compounds were also reported more thermally stable than the natural chromanol-bearing  $\alpha$ -tocopherol. This is particularly important because one of the limitations to the application of phenolic compounds, especially simple phenolic acids, is their thermal lability, as they are readily thermally degraded or volatilized under the stringent conditions used for frying operations. The study, however, is limited in that it did not compare the performance of the newly composed antioxidants with their parent phenolic acids focusing more on the chromanol moiety rather than the phenoxyl unit.

In a related study, Aladedunye et al. (2012) reported the synthesis of 12 novel dihydrocaffeic acid amides (Fig. 5). The dihydrocaffeic acid derivatives were prepared in two-step synthesis from the corresponding aldehydes. Briefly, the aldehydes were reacted with the appropriate alkylamine in methanol, yielding the expected imines that were subsequently reduced with sodium borohydride to afford the corresponding amines. The amines were then coupled with dihydrocaffeic acid using (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate as a coupling reagent, generating a series of new dihydrocaffeic

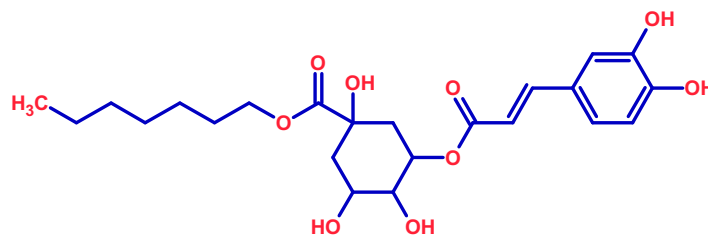


**Figure 5** Synthetic modification of phenolic compounds to improve activity, lipophilicity, and thermal stability. Adapted from Aladedunye, F., Catel, Y., Przybylski, R., 2012. Novel caffeic acid amide antioxidants: synthesis, radical scavenging activity and performance under storage and frying conditions. Food Chem. 130, 945–952.

acid amides, each as a mixture of the two rotamers (Fig. 5). The length of the alkyl chain was adjusted to moderate the antioxidant's lipid solubility. In addition to being significantly more active at protecting frying oil from thermooxidative degradation, compared to  $\alpha$ -tocopherol, butylated hydroxytoluene (BHT), and, depending on the derivatives, the parent dihydrocaffeic acid, the newly configured phenolic antioxidants were much more thermally stable. For instance, at the end of the frying experiment, the TPC content of canola oil triacylglycerols fortified with dihydrocaffeic acid was at 18%, whereas the amount found in the configured phenolic compound 3a (Fig. 5) was 14%, and while no dihydrocaffeic acid was detected in the oil at the end of the frying period, about 20% of 3a still remained in the counterpart oil. Furthermore, a number of the structured antioxidants were found to possess better anticancer activity, effectively killing breast cancer cells, including on chemotherapy-resistant breast cancer cells and on oral squamous carcinoma cells without any negative effects on morphology and gene expression of normal human oral and gingival epithelial tissues (Kovalchuk et al., 2013).

In a study similar to Choo et al. (2009), Gruczynska et al. (2015) described the preparation and frying performance evaluation of structured canola oil, incorporating ascorbic acid and selected phenolic acids (gallic, ferulic, caffeic, and *p*-hydroxyphenylacetic) into the triacylglycerol backbone through enzymatic interesterification using Lipozyme RM IM lipase from *Rhizomucor miehei* as a biocatalyst. Apart from the gallic acid derivative, the authors reported up to a threefold decrease in the rate of TPC formation in canola oil sample fortified with the structured phenolic compounds. The unexpectedly high level of TPC and polymers observed for the structured gallic acid canola triacylglycerols was attributed to the potential of galloyl moiety for self-polymerization, noting that none of the stability indices (TPC and polymers) could sufficiently differentiate between polymerized triacylglycerols and polymerized galloyl groups.

To circumvent the high cost of pure polyphenolic compounds, especially considering the relatively high amounts of antioxidants required per kg of frying oil, the direct modification of crude or partially purified polyphenolic extracts have been attempted (Stevenson et al., 2006; Hernandez et al., 2009; Ma et al., 2012; Aladedunye et al., 2015; Aladedunye and Matthaeus, 2016; Bernini et al., 2017). Using immobilized *Candida antarctica* B, Stevenson et al. (2006) reported a direct lipophilization of Penglai apple and blueberry polyphenolic extracts with palmitic, cinnamic, and phenylpropionic (PPA) acids and hydroxylated derivatives of PPA as acyl donors in the presence of 4 Å molecular sieves, using 2-methyl-2-propanol as solvent. The author reported the presence of several lipophilized anthocyanin glycosides, such as delphinidin-3-*O*-galactoside, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-galactoside, petunidin-3-*O*-galactoside, petunidin-3-*O*-glucoside, malvidin-3-*O*-galactoside, and malvidin-3-*O*-glucoside from the blueberry extract, based on LC–MS analysis. Phloridzin esters were reported as the major lipophilized products from apple polyphenolic esters with palmitic acid being the best acyl donor, yielding up to 80% conversion rate. Hernandez et al. (2009) directly lipophilized coffee pulp's methanolic extracts using immobilized *C. antarctica* lipase B in supercritical  $CO_2$ /*t*-butanol solvent and heptanol as aliphatic chain donor. Heptyl chlorogenate (Fig. 6) was identified as the only product, recording a 65% conversion.



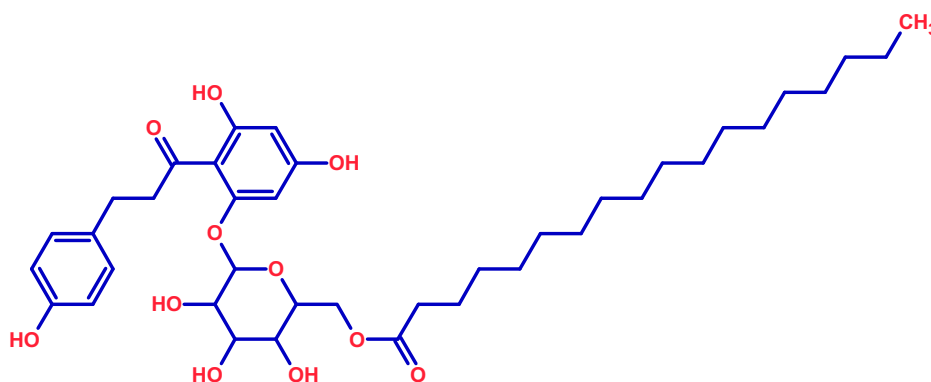
**Figure 6** Heptyl chlorogenate. Hernandez, C.E., Chen, H.H., Chang, C.I., Huang, T.C., 2009. Direct lipase-catalyzed lipophilization of chlorogenic acid from coffee pulp in supercritical carbon dioxide. *Ind. Crop Prod.* 30, 359–365.

Although none of these studies were applied to frying oils, they did provide the inspiration for subsequent studies by Aladedunye and his research group on the Canadian small fruits (Aladedunye et al., 2015; Aladedunye and Matthaeus, 2016). Enzymatic alkylation of rowanberry (*Sorbus aucuparia*) ethyl acetate extract was carried out in 2-methyl-2-butanol using octadecanol as the long chain alkyl donor and *C. antarctica* lipase B as a biocatalyst. Reaction bioconversion was monitored periodically by HPLC and the reaction was terminated at the end of the sixth day. Chlorogenic acid, which represented 87% of the polyphenolics in the rowanberry extract, was effectively lipophilized with about 70% conversion at the end of the fifth day. Assessments of the lipophilized polyphenolic extract for radical scavenging activity by DPPH assay did show them as poorer radical scavenger compared to the native extract containing unmodified chlorogenic acid, in agreement with other studies (Lorentz et al., 2010; Ma et al., 2012); however, the lipophilized extract containing predominantly octadecyl chlorogenate significantly outperformed the native extract by a factor of 1.3 as measured by the ability to limit polar and polymeric triacylglycerols formation during frying.

Furthermore, the stability and enhanced lipophilic nature of the structured chlorogenate was practically demonstrated by measuring its carry-through property that is related to the ability of the antioxidant to survive the stringent conditions of frying and gets transferred to the fried food, considering the extensive mass exchange between the food and the frying oil. The authors reported that the level of octadecyl chlorogenate found in the French fries (55.4 µg/g French fries, wet basis) was seven times higher than the amount of chlorogenic acid (8.1 µg/g French fries, wet basis) after deep frying in canola oil fortified with lipophilized and native phenolic extracts, respectively. It was also observed that due to its poor solubility in the frying oil, the major portion of the available chlorogenic acid did not dissolve in the frying oil or make it into the fried products, possibly “trapped” within the extraneous materials of the rowanberry extract.

Using the same conditions as in the above publication (Aladedunye et al., 2015), the authors extended the study to Canadian crabapple (*Malus baccata*), isolating phloridzyl octadecanoate (Fig. 7), which was reported to offer better protection against thermal and oxidative degradation of refined, bleached, and deodorized rapeseed frying oil, compared to the native unmodified polyphenolic extract (Aladedunye and Matthaeus, 2016).

Beside esterification reactions, enzymatic polymerization is another promising technique for configuring polyphenolic compounds to potentially improve their antioxidant/antipolymerization effects during frying. Unlike in esterification reactions where lipases are the most employed, peroxidases, laccases, tyrosinase, oxidases, and oxidoreductase are the typical biocatalysts for polymerizing polyphenolics (Racicot et al., 2012; Miao et al., 2015; Zheng et al., 2015; Sánchez-Mundo et al., 2016; Kocak et al., 2016; Gao et al., 2016; Reano et al., 2016; Muniz-Mouro et al., 2017). For instance, Racicot et al. (2012) reported the horseradish peroxidase-catalyzed synthesis and purification of polyepicatechin with improved antioxidant activity and thermal stability. Disentis-Mendoza et al. (2006) bioconfigured quercetin and Kaempferol using laccase and tyrosinase from *Ustilago maydis*, generating relatively low molecular weight polymers with improved antioxidant activity. Zheng et al. (2015) enzymatically polymerized



**Figure 7** Phloridzyl octadecanoate. Aladedunye, F., Matthaeus, B., 2016. Effective lipophilic antioxidant enzymatically derived from Canadian crabapple. *Eur. J. Lipid Sci. Technol.* 118, 919–927.



pyrogallol acid, resulting in sterically hindered phenolic antioxidants with markedly improved thermal and antioxidant activity against thermal degradation of polypropylene. Sánchez-Mundo et al. (2016) described the enzymatic polymerization of epicatechin, ferulic acid, quercetin, and phenolic mixtures isolated from fresh bell pepper using polyphenol oxidase isolated from bell pepper. According to the authors, regardless of aggregate sizes, all polymerized phenolics showed antioxidant activity better than their respective monomers.

Now, despite this opportunity, neither the technique nor the polymerized polyphenols obtained therefrom has been applied to the frying system/operation. Indeed, a natural nonextractable tannin-rich carob fiber has been reported to limit TPC and polymers formation in frying oil by up to 50% (Botega et al., 2009), highlighting the potential of polymerized polyphenols. Polymerization of polyphenols affects both the structure, texture, and other physical properties of the polyphenols, providing opportunity for a protective mechanism (antioxidant, antipolymerization, heat dispersal, oxygen barrier, etc.) different than that of the original monomer. Indeed it is highly likely that, with just the right aggregate sizes, polymerized polyphenolics may be able to protect frying oil with similar mechanism currently acknowledged for polydimethylsiloxane (PDMS), namely, creating oxygen barrier at the air-oil interface, in addition to other protective mechanisms.

## Conclusions

Indeed, frying is a unique method of food preparation, principally because the processing conditions are relatively more drastic, compelling unbridled physicochemical interactions among multitudes of components both from the frying oil and the food being fried in it. These stringent processing conditions often overwhelm conventional endogenous antioxidants such as the tocopherols and carotenoids, requiring external antioxidant fortification. Such exogenous antioxidants are expected to be effectively soluble in the oil, thermally stable, operating by more than one mode of antioxidative mechanisms, and preferably, naturally occurring. But for their poor lipophilicity and thermal lability, phenolic compounds are uniquely structurally designed to fit this role. Evidently, chemoenzymatic esterification and polymerization have the potential to expand the applications of phenolic compounds as frying oil antioxidants by enhancing lipophilicity and thermal stability. Although opportunities abound, currently, available studies in these directions are limited and localized within a very few research groups.

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# Milk Protein Interactions

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## Glossary

$\alpha$ -La	$\alpha$ -Lactalbumin
$\beta$ -Lg	$\beta$ -Lactoglobulin
AGE	Advanced glycation end product
BSA	Bovine serum albumin
CCP	Colloidal calcium phosphate
CMP	Caseinomacropptide
DF	Diafiltration
GMP	Glycomacropptide
Ig	Immunoglobulins
MF	Microfiltration
MFGM	Milk fat globule membrane
MPC	Milk protein concentrate
SMP	Skimmed milk powder
TS	Total solids
UF	Ultrafiltration
UHT	Ultra high temperature
WPI	Whey protein isolate
WPNI	Whey protein nitrogen index

## Introduction

Bovine milk contains about 3.5%–4% proteins and their interactions govern the behavior of milk and form the basis for the manufacture of different milk products and their properties. Milk proteins are present mainly in the aqueous phase of milk either in soluble form or as colloidal suspensions. Although the proportion of these forms of proteins varies greatly among different species, their structure, general properties, and interactions remain largely similar. The following sections discuss some key chemical and physical properties of milk proteins and their interactions during milk processing.

## Caseins

The caseins form nearly 80% of the total protein in milk and are defined as phosphoproteins that precipitate out upon the acidification of milk to pH 4.6. Bovine milk consists of four major types of casein:  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ -, and  $\kappa$ -casein. Some of their key characteristics are shown in **Table 1**. The casein in milk exists as three-dimensional colloidal supramolecular structures called micelles. Three of the caseins,  $\alpha_{s1}$ -,  $\alpha_{s2}$ -, and  $\beta$ -casein, act as block copolymers and interact via noncovalent interactions into small nanoclusters, which in turn form the building blocks of micelles. The phosphate groups on the caseins from multiple nanoclusters bind calcium, resulting in calcium phosphate bridges between the nanoclusters resulting in a three-dimensional network or a micelle. The N-terminal region of  $\kappa$ -casein links in to the nanoclusters by hydrophobic interactions but, unlike other caseins,  $\kappa$ -casein does not have any phosphoserine cluster in its structure due to low levels of phosphorylation and therefore is unable to form the calcium phosphate nanocluster link. The C-terminal region of  $\kappa$ -casein is highly charged and instead contains carbohydrate moieties that extend into the aqueous medium, giving the micelle surface a 'hairy' appearance. This stabilizes the micelle by electrostatic repulsion and by steric effects arising from the bulky hairy layer. Fully assembled casein micelles are approximately 40–150 nm in size and hold up to 3.5–4 g water per g of casein. The stability of the casein micelle structure is governed by conditions that impact the protein–protein interactions and protein–calcium phosphate nanocluster cross-links. Factors that destabilize the micellar structure of caseins include temperature, lowering of the pH, sequestering of calcium ions, and the presence of cosolvents, such as ethanol, in the aqueous medium.

**Table 1** Key characteristics of different casein fractions in bovine milk

Parameter	$\alpha_{s1}$ -Casein	$\alpha_{s2}$ -Casein	$\beta$ -Casein	$\kappa$ -Casein
Content of total protein (g.L <sup>-1</sup> )	1.2–1.5	0.3–0.4	0.9–1.1	0.2–0.5
Fraction of total casein (%)	40	10	35	15
Amino acid residues	199	207	209	169
Molecular weight (Da)	23,599	25,206	23,973	19,052
Number of proline residues	17	10	35	20
Number of lysine residues	14	24	11	9
Isoelectric point (pH units)	4.42	4.95	4.65	5.6
Phosphorylated serines	8	11	5	3
Polymorphic forms	8	4	12	11
Disulfide bridges	-	2	-	2
Calcium sensitivity	++	+++	+	-
Unique structural features	Largely unstructured	Largely unstructured	Highly hydrophobic  Partial amphiphilic character	Glycosylated  Glycans of galactose, N-acetylglucosamine, N-acetylneuraminic acid

### Structural Properties of Caseins

Caseins have unique structural properties that make them particularly stable during processing. An important structural attribute of all casein fractions is their high content of proline residues, which are uniformly distributed throughout their sequence, limiting the formation of ordered secondary and tertiary structures. Hence, caseins are considered to be naturally denatured proteins or intrinsically unstructured proteins. The relatively open structure, in turn, imparts conformational flexibility or molten-globule-like character to caseins.

The primary structures of  $\alpha_{s2}$ - and  $\kappa$ -caseins have cysteine (Cys) residues that can form strong covalently linked disulfide bonds, which assist in polypeptide folding or the formation of cross-links with other proteins.  $\alpha_{s2}$ -Casein possesses two cysteine residues (Cys36 and Cys40) in its sequence, which form inter- and intramolecular disulfide bonds with other  $\alpha_{s2}$ -casein monomers. In comparison,  $\kappa$ -casein possesses two cysteine residues (Cys11 and Cys88), which participate in the disulfide-linked oligomerization of  $\kappa$ -casein in untreated raw milk. Upon the heat treatment of milk, the Cys residues of  $\kappa$ -casein react with those of whey proteins, which affects the quality of processed milk for the manufacture of different dairy products.

A structural feature that is common to all caseins is the proximity of amino acid residues with similar properties in their primary structure. This results in the formation of clusters of hydrophobic, charged, and hydrophilic regions on the polypeptide chain, which are responsible for several important properties of caseins, such as their ability to form micelles, their association tendency, their interactions with calcium, and their unique functional properties, including emulsification and foam stabilization. One of the key clusters that imparts unique properties to caseins is formed by phosphates that are bound to the serine and threonine residues of caseins. These phosphorylated clusters increase the negative charge density in the region and contribute to the structural stability of caseins because of high electrostatic repulsion. The phosphoserine clusters also form bridges with calcium resulting in a three-dimensional casein micelle structure and during aggregation, such as during the second phase of rennet action in cheese manufacture.

The clusters of hydrophobic and charged hydrophilic regions result in a strong tendency for the caseins to self-associate into multimers or oligomers via noncovalent interactions. The self-association tendency of  $\beta$ -casein has important implications for the properties of the casein micelle structure, as discussed below in the chilling and thermal processing of milk. The hydrophobic patches, together with the phosphoserine clusters, impart an amphiphilic character to caseins, which is responsible for their emulsifying, foam-stabilizing, and chaperone-like properties. The chaperone-like activity of proteins is defined as their ability to assist in the noncovalent assembly or folding of other proteins without being a part of their structure or interactions during their biological action. Different forms of casein, such as sodium caseinate, micellar casein, and pure casein solutions, possess chaperone-like activity by preventing the aggregation of several enzymes.  $\beta$ -Casein and  $\alpha_s$ -casein have been shown to bind to the hydrophobic surfaces of unfolded globular proteins and to prevent their heat-induced aggregation.

### Sensitivity of Caseins to Calcium

The negatively charged clusters of phosphoserine residues in caseins can bind to calcium, which can affect the stability of micelles by two different phenomena. The calcium ions first bind to the phosphoserine residues of caseins by electrostatic interactions, lowering the stabilizing electrostatic repulsion. This may induce a conformational change in the casein polypeptide ( $\alpha_{s1}$ -casein) structure. In the absence of low electrostatic repulsion between monomers, the casein–calcium complexes reversibly interact with each other by noncovalent interactions such as hydrogen bonding and hydrophobic interactions, resulting in their aggregation.

The different casein fractions possess various sensitivities to calcium-induced precipitation, with  $\kappa$ -casein being the least sensitive. The  $\alpha_{s1}$ - and  $\alpha_{s2}$ -caseins are particularly sensitive to calcium and can precipitate from solution at calcium concentrations from 3 to 8 mM and <2 mM, respectively. The ability of these caseins to bind calcium has important implications during the processing of milk and milk products, e.g. during the manufacture of concentrated milks, dried milks, and fermented milks and in the second stage of rennet action during cheese manufacture. In comparison,  $\beta$ -casein displays temperature-dependent stability in the presence of calcium, with up to 400 mM calcium required for its precipitation at low temperature (<18 °C) but only up to 15 mM calcium required at 37 °C. As the binding of caseins to calcium is electrostatic in nature, factors that screen charges, such as pH, ionic strength, dephosphorylation of phosphoserines, and deamination of arginine residues in the primary structure, greatly affect the calcium–casein interactions.

## Whey Proteins

The whey proteins represent a heterogeneous group of proteins in milk that remain soluble after its acidification to pH 4.6 or after rennet coagulation. They have a well-defined globular structure and include  $\beta$ -lactoglobulin ( $\beta$ -Lg),  $\alpha$ -lactalbumin ( $\alpha$ -La), bovine serum albumin (BSA), and immunoglobulins (Ig). Whey proteins form up to 20% of the total protein in milk and some of their key characteristics are listed in Table 2. Unlike caseins, the whey proteins are devoid of any bound phosphorus or glycan chains in their structure. Some key properties of whey proteins that govern their interactions are discussed below.

### Globular Structure of Whey Proteins and Factors Affecting Their Structural Stability

The unique composition of the amino acid residues in the polypeptide allows it to assume specific conformations with well-defined secondary and tertiary structures. In a globular structure, the hydrophobic residues are buried deep in the core whereas the hydrophilic residues cover the surface. The formation of the globular structure is facilitated by hydrogen bonding, van der Waals' bonds, electrostatic interactions, and disulfide bonds; its stability is maintained by the interactions of the side-chain residues with solvent molecules (termed preferential interactions) and is a net result of forces that oppose unfolding and forces that induce a conformational change. Any alteration to this delicate balance between the forces that maintain stability results in non-native conformations that minimize the free energy. These non-native structures of a globular protein under the new equilibrium conditions are called the denatured state of the protein. The common unit operations that denature whey proteins are thermal processing, high shear, and high pressure treatment. Although the denaturation of globular proteins can theoretically be considered to be a reversible process, this is seldom true in practice because the denatured proteins rapidly assemble into aggregates. Thus, unless stated otherwise, the term denaturation or denatured protein, used henceforth in this chapter, refers to the irreversible denatured state of the protein.

### Disulfide Exchange Reactions of Whey Proteins

The unfolding of whey proteins results in the opening-up of the structure, giving a highly reactive molten-globule state that can aggregate readily via different pathways. At neutral pH, the unfolded polypeptide aggregates mainly via intermolecular thiol–disulfide exchange reactions and, to a lesser extent, by hydrophobic interactions. The unfolded polypeptides of the whey proteins  $\beta$ -Lg and  $\alpha$ -La readily interact with  $\kappa$ -casein, resulting in disulfide-linked whey protein– $\kappa$ -casein complexes, although BSA and lactoferrin have also been reported to form disulfide linkages with  $\alpha_{s2}$ -casein in ultra-high-temperature (UHT)-processed milk. The disulfide exchange reactions of whey protein and  $\kappa$ -casein are of technological importance because they affect several processes, including the UHT processing of milk, cheese and yogurt manufacture, and the fouling of evaporators during concentration.

**Table 2** Key characteristics of whey proteins in bovine milk

Parameter	$\beta$ -Lactoglobulin	$\alpha$ -Lactalbumin	Bovine serum albumin	Immunoglobulins
Content of total protein (g/mL <sup>-1</sup> )	0.32–0.35	0.12–0.15	0.04–0.06	0.08–1
Fraction of total whey proteins (%)	50	20	8	10
Amino acid residues	162	142	582	–
Cysteine residues in sequence	2 (1)	4	35	–
Molecular weight (Da)	18,281	14,176	66,267	150,000–900,000
Isoelectric point (pH units)	5.4	4.8	5.4	
Thermal denaturation temperature (°C)	75	60–63	85–87	80
Unique features	Absent in human, rat and guinea pig milks	Aids in lactose biosynthesis in golgi apparatus of cells	Also present in blood	Higher content in colostrum (up to 10%, w/v)

The heating of whey proteins ( $>60^{\circ}\text{C}$ ) at low pH ( $\text{pH} < 3$ ) results in the formation of highly ordered amyloid-like nanofibrils. The disulfide exchange reactions are significantly reduced mainly because of the protonation of free sulfhydryl groups; the main pathway for the aggregation of unfolded whey proteins under these conditions is by hydrophobic interactions.

## **Milk Protein Interactions During Unit Operations of Milk Processing**

The casein micelle structure is remarkably stable during routine processing operations, except during cheese and yogurt manufacture. The technology for the manufacture of milk products is largely a manifestation of a change in the equilibrium conditions that stabilize the micelles. In comparison, the whey proteins undergo extensive changes during milk processing, as discussed above, and their interactions with the casein micelles affect the suitability of milk for the manufacture of dairy products, affecting their yield and their final properties. The following sections discuss the interactions of milk proteins during the manufacture of milk products.

### **Chilling and Thermal Processing of Milk**

Chilling is one of the most common operations to which milk is subjected on farms and in processing plants, to arrest bacterial growth. Chilling brings about changes in the salt equilibrium and causes solubilization of micellar calcium phosphate, affecting the stability of the micelles. This may affect further processing of the milk, e.g., an increase in the rennet coagulation time during cheese manufacture.

At temperatures employed for the chilling of milk,  $\beta$ -casein undergoes a highly cooperative and reversible temperature-dependent self-association. At low temperatures ( $0$ – $5^{\circ}\text{C}$ ) and in relatively dilute solutions ( $0.03$ – $0.07\%$ ),  $\beta$ -casein exists as a monomer but gradually self-associates into multimeric structures as the temperature is increased to  $20^{\circ}\text{C}$ . These structures have been referred to as micelles that are similar to those in detergent solutions. In chilled milk ( $0^{\circ}\text{C}$ ), approximately 50% of the total  $\beta$ -casein in the micelles has been known to reversibly dissociate from the casein micelles, which increases the viscosity of milk. This effect is reversible and, upon heating to approximately  $40^{\circ}\text{C}$ , the  $\beta$ -casein migrates back into the casein micelles. The dissociation of  $\beta$ -casein from the casein micelle has important implications for the quality of untreated milk because dissociated  $\beta$ -casein is prone to proteolytic cleavage by the enzyme plasmin (EC 3.4.21.7), resulting in  $\gamma$ -casein (residues 29–209). The  $\gamma$ -casein fraction has been reported to play a detrimental role in the stability of UHT milk.

The heating of milk at  $50$ – $60^{\circ}\text{C}$  results in the interaction of  $\beta$ -Lg with plasmin, blocking its action on the caseins and thus improving the quality of milk for the manufacture of UHT milk. The heating of raw whole milk to  $60$ – $65^{\circ}\text{C}$  results in denaturation of the proteins associated with the milk fat globule membrane (MFGM) and their subsequent interactions with the whey proteins by thiol–disulfide exchange reactions. The whey proteins are relatively stable at  $< 65^{\circ}\text{C}$  and the free-thiol-mediated disulfide exchange reactions are driven primarily by the thiol groups of the denatured MFGM proteins, which may cross-link with  $\kappa$ -casein during more intense heating. Alternatively, some  $\kappa$ -casein on the casein micelle surface may also interact with the MFGM proteins.

The most important changes in milk occur during heating in the temperature range  $80$ – $90^{\circ}\text{C}$ . At these temperatures, the thiol-mediated reactions cause the generation of compounds that are responsible for the cooked flavor of heated milk. The whey proteins  $\beta$ -Lg and  $\alpha$ -La unfold and interact with  $\kappa$ -casein by thiol–disulfide exchange reactions, forming complexes on the casein micelle surface. A more severe heat treatment results in a greater extent of whey protein– $\kappa$ -casein complexation and thus a stronger anchoring of the complex to the micelles. The formation of the whey protein– $\kappa$ -casein complex affects the properties of milk and milk products during subsequent processing, e.g. a delay in the rennet clotting time, change in rheological properties of yogurts and milk powders.

Another major effect of the heat treatment of milk is Maillard or nonenzymatic browning because of the interactions of the milk proteins with lactose. The  $\epsilon$ -amino groups of lysine and arginine residues interact covalently with the carbonyl group of lactose to form a Schiff base that undergoes a series of degradation reactions to form complex advanced glycation end products (AGEs). The Maillard reaction translates into the loss of color and flavor, nutritional value of the product, changes in the functional properties of the proteins, and the formation of AGEs that have detrimental effects on human health.

Continued heating at high temperatures ( $120^{\circ}\text{C}$  for 90 min) results in dephosphorylation of the caseins, with as much as 65% of the casein phosphate being solubilized by a heat treatment of  $130^{\circ}\text{C}$  for 60 min. In addition, the heating of milk decreases its pH because of the release of organic acids resulting from the thermal degradation of lactose, the precipitation of calcium phosphate from the micelles with a concomitant release of  $\text{H}^{+}$  ions, and dephosphorylation of the caseins. In milks with  $\text{pH} > 6.7$ , heating results in  $\kappa$ -casein dissociation from the micelle surface, which exposes the calcium-sensitive  $\alpha_s$ - and  $\beta$ -caseins to the calcium-rich aqueous phase. A combination of one or more of these effects lowers the heat stability of milk and results in coagulation of the caseins upon prolonged heating.

The interactions of whey proteins with caseins during heating at low pH is an area that has largely remained unexplored, perhaps because of the disintegration of the micellar structure and the poor stability of the caseins under these conditions. The high acid and high temperature conditions of heating bring about the heat-induced acid hydrolysis of whey proteins at peptide bonds involving aspartic acid (Asp) residues. In pure protein solutions and at relatively dilute concentrations, the interaction between  $\beta$ -Lg and  $\beta$ -casein during heating ( $80^{\circ}\text{C}$ ) at low pH ( $\text{pH} 2$ ) was recently reported to be minimum except at lower molar ratios.



## Renneting of Milk

The manufacture of cheese exploits the structural features and the properties of the casein micelles, discussed above. Gelation of the caseins during cheese manufacture is a result of two separate reactions: enzymatic cleavage of the 'hairy'  $\kappa$ -casein layer stabilizing the micelle, followed by aggregation of the casein micelles into a three-dimensional network. The primary phase of rennet coagulation consists of enzymatic cleavage of  $\kappa$ -casein by the enzyme chymosin (EC 3.3.23.4). This enzyme cleaves the peptide bond between residues 105 (Phe) and 106 (Met), resulting in the removal of the hydrophilic region that is referred to as caseinomacropeptide (CMP) or glycomacropeptide (GMP) (because of the presence of glycan chains). The optimum pH for the activity of chymosin is 5.5–5.3; at lower pH, the proteolysis becomes more and more nonspecific, resulting in cleavage of the  $\alpha_{s1}$ -,  $\alpha_{s2}$ -, and  $\beta$ -caseins, an important phenomenon that occurs during the ripening of cheese.

In the second phase of rennet coagulation, the para-casein forms a three-dimensional gel via calcium-mediated interactions. The cleavage of GMP from the casein micelles reduces their net charge (by up to 50%) and the steric-stabilizing effect of GMP, resulting in the aggregation of para-casein by calcium, van der Waals' forces, and hydrophobic interactions of the  $\alpha_s$ - and  $\beta$ -caseins. Nearly 70% cleavage of the  $\kappa$ -casein is necessary for the para-casein micelles to interact with each other and coagulation occurs only after nearly all of the GMP has been released from the micelles. These extensive changes in the structure of the casein micelles leads to the formation of clusters that aggregate through hydrophobic and electrostatic interactions to form a three-dimensional gel network.

The rennet gel physically entraps a considerable amount of water, which is released rapidly during cutting; disturbing the gel causes shrinkage of its volume by up to 2–2.5 times the initial volume. During subsequent processing operations, the casein particles undergo a complex and dynamic process of disruption and reformation of casein–casein linkages, with the expulsion of water resulting in their fusion. The fused particles are interlinked by hydrophobic and calcium bridges and envelop the fat phase in the matrix.

The pH of the cheese curd, which affects the colloidal calcium phosphate (CCP) content (the lower is the pH, the lower is the CCP content), greatly affects the microscopic and macroscopic properties of the matrix, affecting subsequent operations such as shreddability, slicing, stretchability, chewiness, and melting of the cheese. Transformation of the para-casein curd network into a cheese mass with a characteristic flavor occurs during ripening or curing. The residual rennet and proteases of microbial origin hydrolyze the para-casein into peptides and amino acids. The simultaneous action of lipases on the fat, producing free fatty acids, aldehydes, and alcohols, imparts a characteristic flavor to the matured cheese. In addition, the continued drop in pH alters the calcium phosphate levels within the para- $\kappa$ -casein network, which may in turn affect the water-holding capacity and result in swelling of the network.

The preheat treatments of the cheese milk prior to renneting greatly affect the gelation time and the characteristics of the gel. The whey protein– $\kappa$ -casein complexation in high-heat-treated milk blocks access of the enzyme to its site of action (Phe105 and Met106), resulting in delayed rennet coagulation. The whey-protein-coated casein complexes pose challenges during fusion of the casein micelles because of steric effects, resulting in a poor texture in the final product. In addition, high heat treatment of cheese milk causes solubilization of CCP from the casein micelles. The net effect of these phenomena affects important properties of the cheese curd, including reduced shrinkage of the curd upon cutting, poor matting and fusion of the curd particles, and a higher moisture content in the final product.

## Acidification of Milk

The acidification of milk, either by the addition of acid externally or by the generation of lactic acid by lactose-fermenting starter cultures, reduces the net charge on the casein micelles (–20 mV at pH 6.7) and collapses the 'hairy' layer of  $\kappa$ -casein. At pH 5.5–5.0, the voluminosity of the casein micelles and their dissociation is greatly increased by the irreversible loss of CCP. With nearly all the CCP in micelles being solubilized at pH 4.9, the micelles lose their integrity. The dissociation of the caseins during acidification is markedly temperature dependent, with virtually no dissociation at 30 °C but up to 40% of the micelles being disrupted at 4 °C and pH 5.5. This facilitates protein–protein interactions between casein particles, resulting in clusters that form a three-dimensional gel network. Acid gelation involves mainly noncovalent linkages rather than hydrophobic interactions, because the storage modulus of the gel increases at low temperature, where hydrophobic interactions are weaker.

The formation of gel networks is greatly influenced by the interaction of whey proteins and  $\kappa$ -casein on the micelle surface. Preheat treatment (85–95 °C for 10–15 min) of milk modifies the characteristics of the casein micelle surface by the complexation of whey proteins with  $\kappa$ -casein. As whey proteins have a higher isoelectric point than caseins, gelation in such milks occurs at higher pH. In addition, the strength of the resulting networks is enhanced by additional hydrophobic interactions involving whey proteins and disulfide cross-links. The whey protein– $\kappa$ -casein complexes bind significant quantities of water, preventing 'wheying off' or syneresis in yogurts.

## Concentration of Milk

Commercially, the concentration of milk is achieved either by thermal concentration or by membrane processing. Thermal concentration, or evaporation, involves heating milk at temperatures between 50 and 70 °C under vacuum in a falling film multi-effect evaporator to obtain a final total solids (TS) content of 50% (w/v). Under these conditions, the risk of heat denaturation of the

wey proteins is minimized, and the extent of denaturation of the whey proteins in the final condensed product is essentially a result of preheat treatments employed prior to concentration.

However, the concentration of milk brings about changes in the salt equilibria by converting the soluble calcium phosphate into a colloidal form. In addition, there is a simultaneous release of the hydrogen ions during concentration, which decreases the pH from pH 6.7 in un-concentrated milk to 6.3 in the concentrate at approximately 20% TS. All these changes affect the characteristics of the casein micelle, such as an increase in the size of the casein micelles and an increased tendency to aggregation. A decrease in the pH during the concentration of milk solids will also decrease the negative charge on the casein micelles and whey proteins, lowering the electrostatic repulsion between the two groups of proteins and facilitating their interaction via noncovalent interactions. Many of these changes are reversible upon dilution of the milk.

An alternative process for the concentration of milk is to pass it through membranes of different pore sizes to separate different components; this is widely used to produce milk protein ingredients, such as milk protein concentrates (MPCs) and whey protein isolates (WPIs). Under the influence of a pressure gradient, the milk is separated into permeate, which flows through the membrane, and retentate, which is retained after release of the permeate. As membrane processing involves minimal thermal treatment, the casein micelles and the whey proteins retain their respective structural features prior to concentration. In addition, the extent of Maillard browning reactions is greatly reduced in membrane-concentrated milks.

The interactions of caseins and whey proteins can be exploited to concentrate different fractions in the retentate. Skimmed milk, when subjected to ultrafiltration (UF), allows concentration of the micellar casein and the whey proteins, retaining the same ratio as that in milk, resulting in MPCs. The size of the casein micelles in ultrafiltered milk is largely similar to that in the original milk and the different salts associated with the micelles are retained in the concentrate. In comparison, diafiltration (DF) causes the dissociation of CCP, loosening of the micellar structure, and swelling of the micelles. The whey proteins may be separated from the casein fractions by the microfiltration (MF) of skimmed milk at 50 °C. The casein-rich fraction may be subjected to cooling to < 4 °C, to allow  $\beta$ -casein dissociation from the micelles, and then subjected to a second MF to obtain  $\beta$ -casein-depleted casein micelles. Similarly, when subjected to MF, heated skimmed milk will result in a casein- $\beta$ -Ig-rich retentate and a permeate that is rich in  $\alpha$ -La.

### Drying of Milk

The dehydration or drying of milk is widely used commercially for the storage and shipping of milk solids and is commonly achieved by spray drying. As the drying of milk is essentially a process of water removal, the changes brought about during drying are akin to the effects of concentration, i.e., the conversion of soluble calcium phosphate into a colloidal form and a lowering of the pH. The characteristics of the casein micelle are largely conserved during drying, implying that the properties of reconstituted milk are similar to those of the milk before drying. This implies that changes to the proteins and their interactions are governed by the pre-treatments given to the milk before concentration.

For skimmed milk that is intended for drying, it is common to preheat the milk to temperatures ranging from 72 °C for 15 s to 120 °C for 2 min before concentration to 48–50% (w/v) TS. These conditions cause denaturation of the whey proteins and their complexation with the casein micelles, as discussed earlier. The temperature of the concentrated milk droplet, which undergoes heat and mass transfer during drying, is maintained at < 70 °C and this, combined with its sufficiently low residence time in the drying chamber, ensures that any additional denaturation of the whey proteins during the drying process is minimal.

The extent of denaturation of the whey proteins in the final dried milk powder [skimmed milk powder (SMP)] can be measured in terms of the whey protein nitrogen index (WPNI), which is defined as the amount (mg) of denatured whey proteins per g of milk powder. Although the WPNI does not account for the initial whey protein concentration in the milk, which varies with the season and with the stage of lactation, it is a useful guide for deciding the suitability of a milk powder for a given application. The heat classification of SMPs based on their WPNI and their applications is shown in Table 3.

Although the casein micelles in spray-dried milk powder retain native-like characteristics upon reconstitution, in high protein powders, such as MPCs, the casein micelles have been reported to fuse with each other and to form a shell around the dried powder. This prevents the penetration of water to the interior of the powder particle, resulting in poor solubility at low temperatures.

**Table 3** Heat classification of skimmed milk powders (SMPs), their functional properties, and their applications

WPNI <sup>a</sup>	Heat classification	Pre-warming conditions	Functional properties	Applications
> 6	Low heat	71–74 °C for 15 s	Solubility, flavor	Cheese manufacture, recombined milk
1.5–6	Medium heat	85–90 °C for 30–60 s	Emulsification, foaming, water absorption, viscosity, color and flavor	Recombined evaporated milk, UHT milk, ice cream
< 1.5	High heat	120 °C for 2 min, 90 °C for 5 min	Heat stability, water binding, gelation and texture, viscosity	Bakery and confectionery applications

<sup>a</sup>Whey protein nitrogen index.

## Concluding Remarks

This chapter has discussed the key properties of milk proteins, casein and whey proteins, that form the basis for the manufacture of different dairy products. The in-depth understanding of the structural aspects of milk proteins and their interactions has opened up new opportunities for the development of milk protein-based ingredients. Recently a new casein-based ingredient Ferri Pro™ fortified with iron was developed using the structural principles of casein micelles. Similarly, by careful selection of membrane separation it was possible to manufacture dairy ingredients with protein composition similar to that in human milk (a lower casein: whey protein ratio) from skimmed milk. The denaturation conditions of whey proteins have been explored in detail to produce ingredients such as microparticulated whey proteins (Simplese™) that have been used as fat replacers in different food products. In addition, approaches such as enzyme cross-linking of milk proteins have a huge potential to develop new functional ingredients. Another avenue that has been explored to modify the properties of milk proteins for commercial applications is the use of novel processing techniques. High-pressure treatment or high-pressure homogenization of milk improve the texture of yoghurts by their effect on casein micelle structure and resulting interactions during gelation.

Thus, interactions of milk proteins continue to be explored for improving the functional properties of conventional dairy products and manufacture of protein-based ingredients with novel functionalities. The current research in this domain aims to understand the impact of milk protein interactions on the digestibility of dairy products in the human gastro intestinal tract using *in vitro* and *in vivo* systems.

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## Effect of Heat on Food Properties

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### Glossary

**Caramel** Brown material formed by heating carbohydrates in the presence of acid or alkali; also known as burnt sugar. It can be manufactured from various sugars, starches and starch hydrolysates and is used as a flavor and color (E-150) in a wide variety of foods.

**Coagulation** A process involving the denaturation of proteins, loss of their native, soluble, structure, so that they become insoluble; it may be affected by heat, strong acids and alkalis, metals and various other chemical agents. Some proteins are coagulated by specific enzymic action. Denaturation is due to the breaking of hydrogen bonds that maintain the protein in its native structure. As the process continues, there is considerable unfolding of the protein, and interaction between adjacent molecules, forming aggregates which reach such a size that they precipitate.

**Gelatinization** The addition of gelatin or a similar substance to a food to turn it into a gel or Formation of a water-retentive gel by expansion of starch granules when heated in moist conditions.

**Interesterification** Exchange of fatty acids between triacylglycerols in order to modify the properties of the fat; may be achieved by heat treatment or using fungal lipase

**Maillard reaction** Non-enzymic reaction between lysine in proteins and reducing sugars, leading to a brown color. A similar reaction occurs in the glycation of proteins in diabetes mellitus. The first step in the reaction is the formation of a Schiff base (aldimine) between the aldehyde group of the sugar and the  $\epsilon$ -amino group of lysine, followed by isomerisation (Amadori rearrangement). May also occur with other amino acids at the amino terminal of a protein. It takes place on heating or prolonged storage and is one of the deteriorative processes that take place in stored foods. It is accompanied by a loss in nutritional value, since the amino acid that reacts with the sugar is not available.

**Organoleptic** Sensory properties, i.e. those that can be detected by the sense organs. For foods used particularly of the combination of taste, texture and astringency (perceived in the mouth) and aroma (perceived in the nose).

**Pathogenic** a bacterium, virus, or other microorganism have ability to causing disease.

**Retrogradation** Cooked starch has an amorphous structure; the staling of bread and other starchy foods is due to crystallization of the starch, so that crumb loses its softness, a process that can be delayed by addition of emulsifiers (crumb softeners) such as polyoxyethylene and fatty acid monoglycerides. Many modified starches are used to slow the process of retrogradation.

### Introduction

Foods are treated thermally in order to enhance palatability, increase shelf life, destroy pathogenic microorganisms and improve sensory quality (Sun, 2012). Heating method generally depends upon food matrix type, microbial load, heating medium and final product characteristics. Various conventional and innovative thermal treatments are currently being employed for processing of foods including pasteurization (Bornhorst et al., 2017b), sterilization (Sevenich et al., 2014), canning, blanching (Malmgren et al., 2017; Datta et al., 2002), induction heating (El-Mashad and Pan, 2017), ohmic heating (Ramaswamy et al., 2014), radio frequency dielectric heating (Ahmed et al., 2010; Awuah et al., 2014), microwave assisted (Guo et al., 2017; Bornhorst et al., 2017a), pressure-assisted (Ahn et al., 2007; Wang et al., 2017), and pH-assisted thermal processing (Palop and Martinez, 2006). There is an increasing concern regarding the effect of thermal processing treatments and their combinations on properties of different foods and their components (Sun, 2012). Heat treatments can induce chemical changes in foods i.e. hydrolytic decomposition (hydrolysis of fats, proteins) (Nawar, 1969), oxidative rancidity (Frankel, 1998), denaturation of proteins (decomposition and modification of amino acids) (Anema, 2014), color degradation (Suh et al., 2003; Medina-Meza et al., 2015), enzymatic degradation (Oerlemans et al., 2006), caramelization of carbohydrates, and interactions between proteins and organic molecules. Other chemical changes include homofermentative or heterofermentative reactions, emulsion breakdown and production of ethanol (Clark et al., 2014). These changes can result in either desirable or undesirable effects (e.g. increase in shelf life or nutritional losses respectively) (Ling et al., 2015). Some of the effects of heat treatment on physicochemical and organoleptic characteristics of major food components as well as various food groups are summarized in following sections.

## Effect of Heat on Major Food Components

Foods mainly comprise of four major components including water, carbohydrates, proteins and fats. Starch and sugar are among the most common types of carbohydrates present in plenty of food products. When sugar is exposed to heating, it starts melting and converts to concentrated syrup. With increasing temperature, its color changes from clear to yellow (Chen and O'Mahony, 2016). Further increase in temperature results in browning is a phenomena known as caramelization) together with the production of a unique flavor (Kocadağlı and Gökmen, 2018). When sugar combines with amino acids, another reaction phenomena starts, which is called as Maillard reaction. Maillard reaction produces various by-products which result in color changes, intensive flavor and aroma production (Martins et al., 2000). Both caramelization and Maillard reaction are initiated at the temperature above 150 °C. Starch is a complex carbohydrate, which gelatinizes when mixed with water during heating process. During gelatinization, each starch granule absorbs water and swells, which causes the liquid to thicken. Different starches gelatinize at different temperatures (Gerschenson et al., 2017).

Heating has significant effect on solubility and structural properties of proteins as it mostly denatures or causes unfolding by thermal processing (Malmgren et al., 2017). These conformational changes are associated with the intensity or severity of heating treatment. Sometimes these changes (denaturation or agglomeration) are desirable as in the case of meat and eggs (Tornberg, 2005).

Fats and oils impart flavor and silky mouthfeel to the end product and enhance the nutritional composition (Drake et al., 2010). Fats usually melt during heating at around 37–40 °C (Bhardwaj et al., 2016). However, melting point of fats varies from product to product according to their chemical structure. Fats are composed of fatty acids and vary in number of double bond, number of fatty acid and type of fatty acids. After melting, further heating can cause degradation of fats resulting in the formation of free fatty acids. Heating at elevated temperatures (e.g. in frying) can lead to the smoke point. Hence, oxidization is the end point, which may coincide with smoke point. Although, smoke point of fats is far above normal cooking temperature, in some cooking methods like deep frying, fat is heated to such elevated temperatures (Brühl, 2014).

## Cereal Products

Bread, pasta, cookies, flakes and cakes are the most common cereal products and mainly comprise of starch, protein and minute quantity of fats. These are consumed all over the world in raw form as well as in processed form (Caldwell et al., 2016).

Bread is the most eaten cereal product having diverse types depending upon the mode of processing and ingredients. The first observable effect in bread after the dough enters in oven is the formation of thin elastic outer film that prevents the dough to collapse and helps to retain gas formed due to yeast activity. This film becomes strong as the temperature of dough rises (Mann et al., 2014). As the internal temperature increase gradually the volume of the dough tends to rise to  $\frac{1}{3}$  of its original volume called as oven rise (gas expansion). This is due to the decrease in solubility of gas by increase in internal temperature hence the pressure increases that leads to rise in volume. This process continues till internal temperature reaches 50 °C. As internal temperature of the dough increases up to 50–60 °C, starch gelatinization begins, and starch granules swell by absorbing water (Yamada et al., 2015). Yeast activity increases by increase in temperature up to a certain limit and dough tends to rise more as more gas is produced. When temperature reaches at 60 °C, thermal death of yeast occurs, and gas generation stops.

As the internal dough temperature reaches at around 75 °C, gluten starts to coagulate. Moisture is removed from gluten molecules which is taken up by starch granules and aids in starch gelatinization and retrogradation. Activity of enzyme ( $\alpha$ -amylase &  $\beta$ -amylase) is greatly affected by the temperature and with every 10 °C rise in temperature, it becomes double. Above certain specific temperature, enzymes disrupt and stop their activity (Zhang et al., 2017).

Caramelization in bread starts at around 170 °C and produces colored substances known as caramels. This process is only confined to crust because internal dough temperature does not reach as high as needed for caramelization to take place (Purlis and Salvadori, 2009). Similarly, Maillard reaction also starts at around 170 °C and produces colored substances called melanoidins. These components also impart color and flavor to the final product (Helou et al., 2016) in Table 1.

## Dairy Products

Thermal treatments in dairy industry is of paramount importance because it has various benefits that are needed to ensure the safety of consumers, to maintain specific characteristics of product and to increase the stability of product. Along with all its benefits, it imparts various desirable as well as undesirable changes in nutritional, chemical, and organoleptic properties of final product.

Quality of milk fats is affected due to the formation of ketones and lactones through hydrolyzation and interesterification (Kim et al., 2017). Milk proteins are affected in a way that most of the enzymes become inactivated due to unfolding and casein micelles are aggregated that leads to coagulation (Rynne et al., 2004). Denaturation of some serum proteins occurs and becomes insoluble hence decrease the consistency of milk.  $\kappa$ -casein forms a complex with  $\beta$ -lactoglobulin. Denaturation of whey proteins (immunoglobulins and agglutinins) starts at 72 °C that eventually affects creaming properties as well as antimicrobial nature of milk (Schong and Famelart, 2017). Some of the enzymes such as alkaline proteinase, plasmin and plasminogen are heat resistant (Kelly et al., 2012; Sutariya et al., 2017). Decrease in redox potential happens due to formation of free sulfhydryl groups liberated during ultra-high temperature UHT (Hoffmann and van Mil, 1997).

**Table 1** Compositional changes in meat during heating

Meat components	Temp. (°C)	References
Fat melting, protein unfolding	30–40 °C	Yu et al. (2017), Liu and Lanier (2016)
Carbohydrates gelatinization	40–45 °C	Bombrun et al. (2015)
Water losses starts	46 °C	Bombrun et al. (2015)
Sarcoplasmic protein, Myosin and actinin denaturation	40–50 °C	Tornberg (2005), Yu et al. (2017), Liu et al. (2016)
Collagen and myoglobin denaturation, fat liquefaction	50–60 °C	Christensen et al. (2012), Shabbir et al. (2015)
Myofibrillar protein aggregation, connective tissue proteins shrinkage	65 °C	Li et al. (2010), Tornberg (2005), Christensen et al. (2011)
Titin denaturation	60–75 °C	Pospiech et al. (2002)
Actin denaturation, Connective tissue proteins gelatinization	70–80 °C	Scussat et al. (2017)
Tropomyosin and troponin denaturation, Perimysial and endomysial gelatinization	80–85 °C	Shabbir et al., 2015
Myofibrillar shrinkage	50–90 °C	Han et al. (2014)
Sarcoplasmic protein aggregation	90 °C	Yu et al. (2017)

Gases are important especially O<sub>2</sub> due to its role in oxidation reaction and it must be eliminated or decreased up to the safe limit. During heating, O<sub>2</sub> along with CO<sub>2</sub> are removed hence the risk of oxidation as well as the growth of bacteria becomes very low. Partial degradation of lactose occurs and is converted into organic acids and lactulose through Maillard reaction (Stapelfeldt et al., 1997). Maillard reaction decreases the availability of lysine hence decreasing the nutritional value of milk (Meltretter et al., 2007). Heat treatment results in an increase in the amount of inorganic phosphate due to hydrolyzation of phosphoric esters and phospholipids. There is also a decrease in pH observed due to creation of organic acid by lactose degradation and colloidal phosphates hence the titratable acidity increases (Lajnaf et al., 2018; Ma and Barbano, 2003).

Raw milk has a yellowish creamy white color that is a blend of individual effect of its constituents. Any change in these constituents may result in change in color (Chugh et al., 2014). Carotenes, caseinates and fat globules imparts yellow color and play a role in scattering light. Mild heat treatment increase whiteness due to increase in colloidal phosphates and decrease in Ca ions. But high heat treatment imparts brown color due to production of Maillard products (Kelly et al., 2012). Moreover, flavor of milk is due to lactose, minerals and fat and decrease in the amount of these contents results in flavor changes during heat processing. Sometimes, UHT produces cooked or heated flavor due to lactoglobulin denaturation and the liberation of volatile sulfur compounds (Al-Attabi et al., 2014). Viscosity also decrease by mild heat treatment but high heat treatment results in evaporation, hence increases solids and viscosity (Sutariya et al., 2017).

## Meat Products

Meat and meat products are of foremost importance as it nutritionally contains essential functional ingredients as well as valuable components such as iron, zinc, calcium, vitamins, proteins, carbohydrates, fat in conjugation with other bioactive compounds. There are different heat processing techniques or cooking methods that can be adopted to achieve desired product quality. These techniques include dry heat cooking (roasting, barbecuing, frying, broiling, and grilling) using electric or gas ovens (Suryanto and Armunanto, 2015), moist heat cooking (boiling, steaming, simmering, and poaching), their combinations (stewing and braising) and some other treatments like microwave heating, and sous vide. Heat stability of meat components need to be measured before commercially declaring safe, wholesome and nutritious meat and meat product (Pearson and Tauber, 1984).

During heat processing or cooking of meat the temperature of meat surface can rise as high as 300 °C compared to interior (around 80 °C). Major changes in meat structure start at the temperature of 30–40 °C which include softening and melting of meat fats and protein unfolding. At the temperature of 45–50 °C, gelatinization starts while actin and myosin (sarcoplasmic proteins) begin to denature with shrinkage of myofibrils. Myosin and actin denaturation enhances the toughness of meat structure (Yu et al., 2017). Changes in water contents start at 46 °C where it decreases in myofibrils and increases in intermyofibrillar space producing gel like structure (Tornberg, 2005). At the temperature of 50–60 °C denaturation of collagen and myoglobin starts and sarcomere length decreases (Scussat et al., 2017). At this point, the meat fats start liquefaction called rendering. Optimum physicochemical characteristics like tenderness, juiciness, flavor etc. can be achieved at this temperature. Afterwards collagens start to collapse, which squeezes out pink colored juice into muscle fibers and meat turns drier at 60 °C. However, at 66 °C, another meat protein called actin starts to contract and coagulate, which toughens the meat (Christensen et al., 2011). Above 70 °C moisture begins to evaporate, and inactivation of microorganism occurs. At 80–90 °C tough collagen starts to gelatinize, actomyosin and myosin undergo transitional changes, dehydrated muscle fibers start to detach from bones making the meat more tender and easy to shred (Bombrun et al., 2015). On the other hand, Maillard reactions render the meat surface crunchy and brown (Yu et al., 2017).

Raw meat has bright pink color which gradually converts into brown color as temperature increases. Changes in meat color depend upon denaturation or oxidation of muscle proteins (myoglobin) during heating at around 60–67 °C (Christensen et al.,



2012). At that point, myoglobin pigment starts shrinking or precipitating (Shabbir et al., 2015). During oven heating process, some gases like carbon monoxide or nitrogen can turn meat into pink color by reacting with myoglobin. When protein is denatured, it can convert the translucent meat into opaque appearance. This phenomenon is the result of change in protein configuration by coagulation process which blocks the passage of light through meat. Dry heating methods produce browning surface of the meat as compared to moist heating methods.

Heating induces odor and flavoring components in meat due to major alteration of fat, amino acids and carbohydrates as well as lipid degradation components. Millard reactions plays a significant role in the production of meat flavor. The dry heating process (frying and roasting) can increase the level of Millard reactions and produces the quality flavor as compared to moist heat methods e.g. boiling (Shabbir et al., 2015). On the other hand, juiciness of the meat is greatly affected by heating method. Elevated temperature decreases the juiciness regardless of the connective tissues level. More temperature drives more water removal resulting crusty and drier meat surface (less juiciness) (Boles, 2010). The overall summary about the effect of heating on meat is given in Table 1.

## Miscellaneous

Traditionally, legumes and beans are cooked through common heating and boiling techniques until they turn soft. During heating starch gelatinization and proteins denaturation produces brown color in addition to softening of tissues (Clark et al., 2014). However, research demonstrated that heating can also reduce the anti-nutritional components and toxic factors in legume seeds such as tannins and phytic acid (Rehman and Shah, 2005). Additionally, it increases the protein and starch digestibility, dietary fiber while reduces the stachyose, ash, potassium, magnesium and raffinose (Wang et al., 2010).

In fruits and vegetables heating process converts the chlorophyll into pheophytin, which is a green or brown color pigment while carotenoids are heat stable (Sumonsiri and Barringer, 2014). Other changes include textural or rheological changes (softening of tissues), and some nutritional quality changes. Heat treatments can inactivate some enzymes such as pectin methyl esterase, polygalacturonase lipxygenase, and polyphenol oxidase in fruits and vegetables (Ahmed and Shivhare, 2005).

## Conclusion

Thermal treatment produces several physical and chemical changes in foods. These changes result in both beneficial and harmful effects on nutritional, functional and organoleptic properties of foods and their components. Currently, a number of thermal techniques are being employed for food processing. Optimization of thermal processing techniques with respect to the type of foods is essential to ensure the availability of foods with better nutritional and sensory quality.

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# Effect of Emerging Processing Technologies on Maillard Reactions

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## Introduction

Browning of foods occurs by enzymatic and non-enzymatic mechanisms. It is difficult to distinguish between the two mechanisms unless, for example, enzymes are inactivated by heat. Non-enzymatic browning occurs during heating and is subdivided into three reaction categories. The first is a Maillard reaction, which occurs between amino compounds (e.g. in proteins, peptides, amino acids) and carbonyl groups of reducing sugars (e.g. glucose, lactose, fructose). The second reaction is caramelization, where sugars react with each other under more drastic conditions. The third reaction category is ascorbic acid oxidation, which is the nearest to enzymatic browning involving ascorbic acid oxidase. Depending on the process used during food preparation, a wide variety of modifications may occur in a food matrix facilitating browning by one or more of the above mechanisms. Some researchers investigated the use of some emerging technologies (e.g. pulsed electric fields, high-pressure processing, etc.) in order to reduce food browning during transformation, transportation, and/or storage. In this chapter, the positive and negative effects of emerging technologies on Maillard reactions will be highlighted with the aim of gaining insights on the suitability of these technologies to be effectively used in sensitive food products.

## Maillard Reaction in Foods

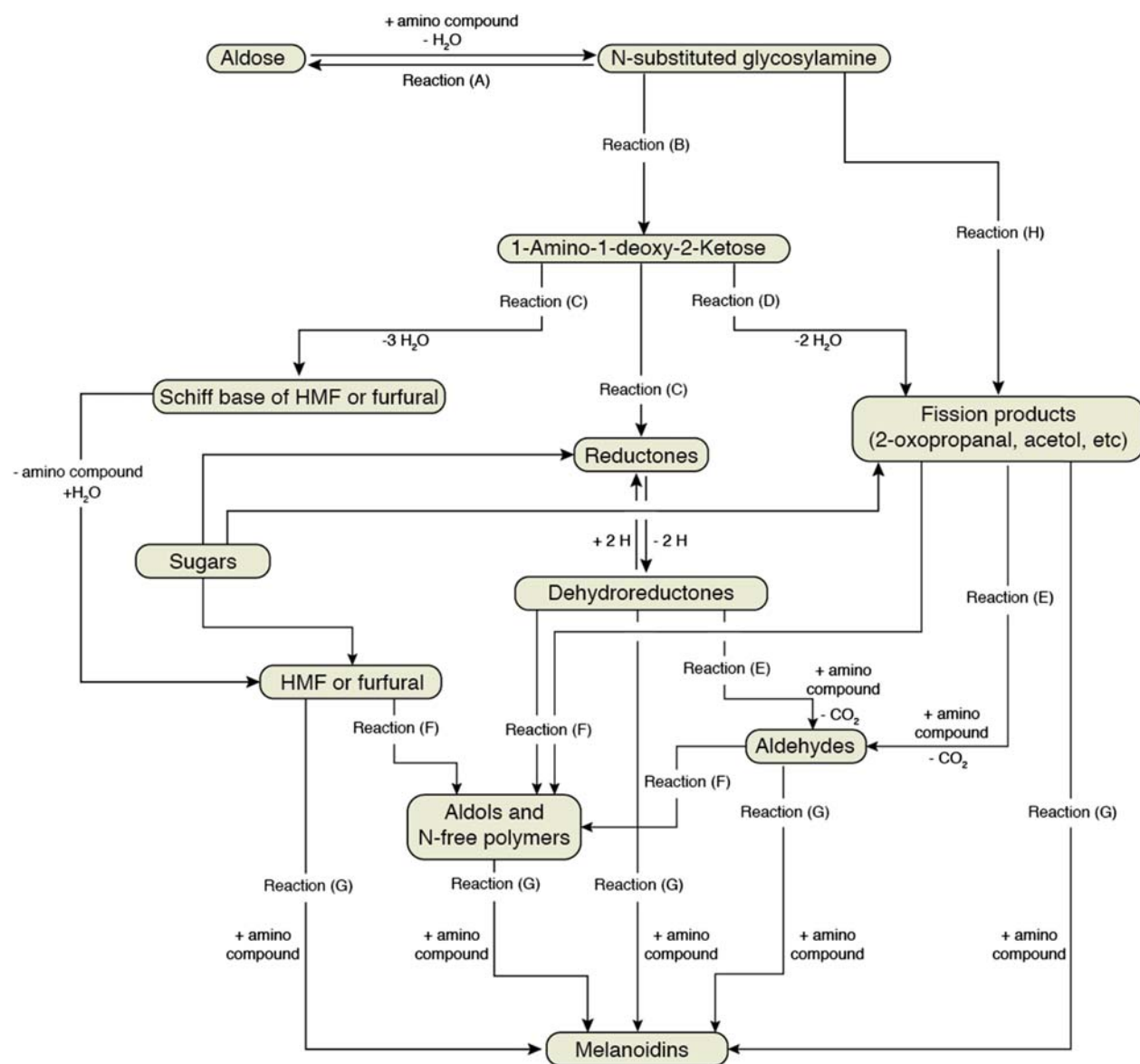
Maillard reactions were first described in 1912 by Louis-Camille Maillard (1878–1936); a French scientist qualified in medicine from the University of Nancy in 1903 (Feather and Waller, 1983; Nursten, 2005). Maillard reactions are very complex. For instance, as detected by HPLC and TLC, a condensation reaction between glucose and ammonia could lead to the formation of more than 15 compounds. The reaction between glucose and glycine gives more than 24 compounds, and the reaction between xylose and glycine gives about 100 compounds (O'Reilly, 1982). Typically, Maillard reactions result in the formation of a Schiff base followed by rearrangement to Amadori or Heyns products (Hodge, 1953; Hellwig and Henle, 2014). Several reviews have been published in the literature detailing Maillard reactions (Thorpe and Baynes, 2003; Peng et al., 2011; Hellwig and Henle, 2014; Lund and Ray, 2017). Hodge (1953) subdivided Maillard reaction into three stages as follows:

1. *Initial stage*: the product remains without any color and without ultraviolet (UV) absorption. Two reactions occur at this stage: sugar-amine condensation (reaction A) and Amadori rearrangement (reaction B).
2. *Intermediate stage*: the product's color may change to yellow with UV absorption. This stage will involve sugar degradation (reaction C) and fragmentation (reaction D), as well as degradation of amino acids (reaction E).
3. *Final stage*: the product becomes highly colored, and reactions such as condensation of aldols (reaction F), condensation of aldehyde-amine and formation of heterocyclic nitrogen compounds (reaction G) will occur.

The different reactions above-mentioned (reaction A–G) are described in Fig. 1. These three stages are known in literature as Early, Advanced, and Final Maillard reactions, respectively (Mauron, 1981). Melanoidins are the final products of non-enzymatic browning and should be distinguished from melanins, which are the final products of enzymatic browning. The dark-brown products formed in foods by enzymatic and non-enzymatic reactions are very complex to differentiate and their chemical analysis is difficult since they are relatively intractable.

Maillard reactions occur particularly when food products are processed at high temperatures (for example roasting, baking, extruding, etc.), when the processed products are stored for long periods at ambient temperature, or during transportation overseas where the temperature may increase up to 70 °C in the transportation containers (Meltretter et al., 2013, 2014). Depending on the time–temperature profile applied during food processing, as well as on the matrix, distinguished aromas could be formed (Ames, 1990). Many food industries are concerned by the occurrence of Maillard reactions especially when the product flavor is very important such as chocolate and coffee. Some of the changes found in non-enzymatic reactions are summarized in Table 1.

It should be noted that while Maillard reactions generate desired changes (i.e., especially flavors) in some food products such as coffee, bread, roasted meat, and dark beer (Vanderhaegen et al., 2006), these changes are perceived as off-flavors in other food products such as ultra-high-temperature (UHT) processed milk (Jansson et al., 2014a, 2014b). Thus their contribution to the quality of the product is product-dependent. Consequently, controlling Maillard reactions during food processing and storage is of paramount importance in order to maintain high-quality product. For instance, protein hydrolysis may occur in several food products, either naturally or during food processing (e.g., pasteurization, cooking, drying, tenderization ...) contributing to the release of free amino acids thus increasing the availability of amino groups for reactions. Another example is the



**Figure 1** Non-enzymatic browning. Adapted from Hodge (1953).

**Table 1** Changes in foods after non-enzymatic browning

Modification	Initial stage	Intermediate stage	Final stage
Color change or discoloration	—	+	+++
Flavor change or off-flavor	—	+	++
Water production	+	+	+
CO <sub>2</sub> production	Unknown	+	Unknown
pH decrease	Unknown	Unknown	Unknown
Increasing antioxidant activity	+	+	+
Solubility decrease	—	—	+
Loss of activity for vitamin C	+	—	—
Loss of proteins' value	+	+	+
Metals chelation	—	Unknown	+
Toxicity development	—	Unknown	Unknown
Fluorescence emission	—	+	+

Adapted from Nursten (2005), Nursten and MacCarthy (1986).

production of lactose-free UHT milk where lactose is hydrolyzed into glucose and galactose, which causes 10 and 20 times faster reaction rates with amines than lactose, respectively, and thus increases the vulnerability of the product to Maillard reactions (Naranjo et al., 2013).

The functionality of proteins (e.g. properties of emulsifying, foaming, gelation, solubility, texturing, etc.) can also be impacted by Maillard reactions. Other changes in protein properties such as surface charge, hydrophobicity, and tertiary structure, can occur due to Maillard reactions, which allow manipulation of certain functionalities.

### Effect of Emerging Processing Technologies on Maillard Reaction

Some emerging technologies such as pulsed electric fields, ohmic heating, and high-pressure processing have shown their efficiency to be used as alternatives to conventional heating treatments, especially for the inactivation of microorganisms (Misra et al., 2017; Barba et al., 2017). Different results have been reported concerning their impact on Maillard reactions during food processing, and their mechanism of action seems to be complex and not well understood.

#### Pulsed Electric Fields

Many food industries have implemented pulsed electric field (PEF) systems in their processing chains and the technology was successfully tested for various applications. Extracting valuable compounds from plant and microbial matrices, inactivation of microorganisms, enhancing fermentation conditions, and accelerating unit operations of drying and freezing are among the multiple applications of PEF technology (Koubaa et al., 2016a; Lebovka et al., 2007; Roohinejad et al., 2014a, 2014b; Mota et al., 2018; Parniakov et al., 2016). The principle of PEF treatment consists of applying high voltage (usually < 50 kV/cm) pulses for short periods of time ( $\mu$ s to ms) to foodstuff placed between 2 electrodes (high voltage and grounded electrodes) (Koubaa et al., 2016a; Puértolas et al., 2016; Roohinejad et al., 2017). Generally, PEF equipment consists of an electrical generator producing square or exponential decay pulses, and either a batch or continuous treatment chamber (Huang and Wang, 2009). A schematic of a typical PEF treatment installation in the food industry as well as pulse shapes produced by PEF generators are presented Fig. 2.

PEF, as a non-thermal technology, does not increase the temperature of the processed food product and thus reduces the formation of undesired heat-derived or chemical changes. It has been demonstrated that applying PEF treatment, as compared to conventional heating, inhibited the formation of hydroxymethylfurfural (HMF) in some fruit juices (Aguiló-Aguayo et al., 2009). Lower levels of HMF were found in orange juice following PEF treatment compared to conventional pasteurization, along with reduced browning during the 6 week storage period (Cortés et al., 2008). Recently, it has been stated that removing one or more reactive compounds of the Maillard reactions could be the most promising use of PEF (Lund and Ray, 2017). For instance, treating potatoes by PEF increased glucose diffusion, which resulted in lowering the level of one of the components of Maillard reactions (Janositz et al., 2011a). Similarly, the impact of PEF treatment on the quality parameters of white asparagus (*Asparagus officinalis* L.) was investigated (Janositz et al., 2011b). Results from this study showed that PEF-treated samples had lower Maillard reaction substrate (glucose) compared to non-treated control samples.

It should be noted that applying PEF treatment at high intensities might promote the formation of Maillard reaction products, as demonstrated in an asparagine-glucose model system (Guan et al., 2010). The authors applied a PEF intensity of 40 kV/cm for 7.35 ms and noticed that asparagine and glucose levels were reduced by 14% and 66% (used for Maillard reactions), respectively. A similar observation was reported in a study of a glycine-glucose model system (Wang et al., 2011). The authors observed a consumption of 13.09% glycine and 50.76% glucose during the Maillard reaction and concluded that PEF treatment, especially with higher intensity over 30 kV/cm, can significantly promote Maillard reactions. These studies demonstrated that although PEF treatment has numerous advantages over conventional heating for food processing, its application should be controlled in order to achieve the required impact through Maillard reactions.

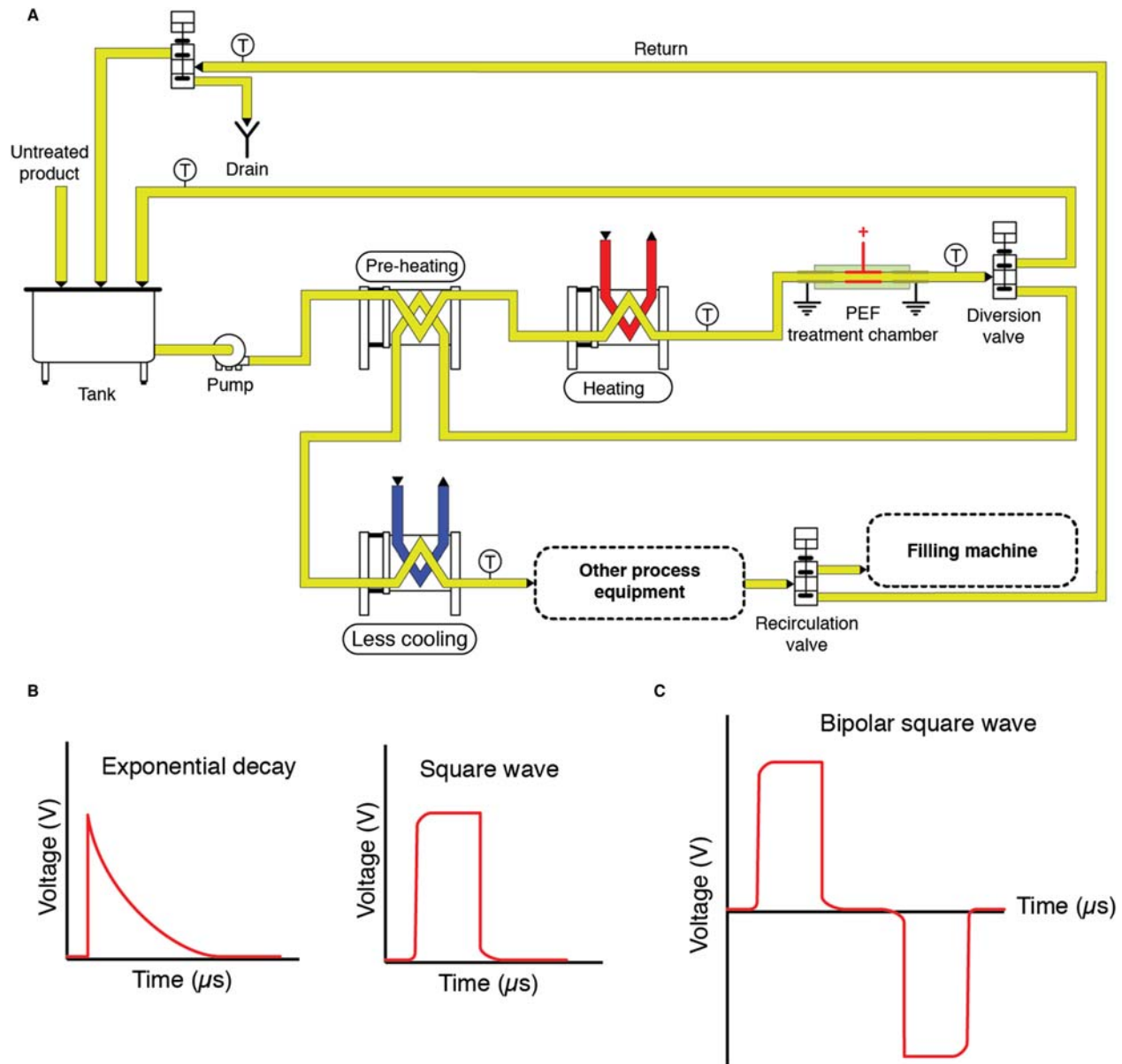
#### Ohmic Heating

Ohmic heating is a process used to heat materials when an electric current passes through them (Fig. 3) (Koubaa et al., 2016b; Hashemi et al., 2017). In ohmic heating, the food sample is not in direct contact with hot surfaces, and the process allows short-time and high-temperature thermal processing, which has the potential to control or minimize Maillard reactions (Lund and Ray, 2017). It has a low maintenance cost and high-energy conversion efficiency (Pereira and Vicente, 2010).

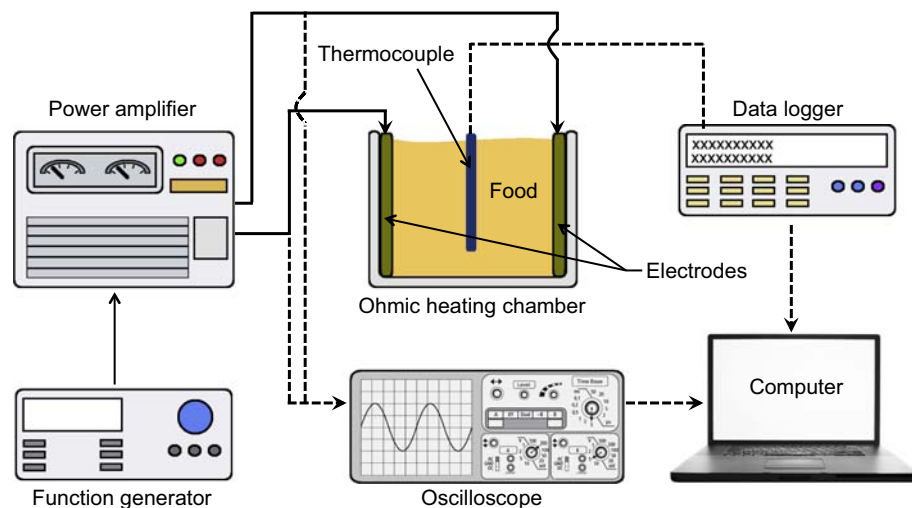
Due to its numerous advantages over conventional heating, ohmic heating is currently used in many applications such as blanching, evaporation, dehydration, fermentation, extraction, sterilization and pasteurization. The absence of heat exchanger surfaces leads to a better control of the thermo-chemical changes in the processed food. For instance, protein deposition on the walls of the plate exchanger during UHT milk processing leads to overheating and therefore chemical modifications, which could be avoided when using ohmic heating (Fillaudeau et al., 2006). This technology also showed its efficiency in limiting non-enzymatic browning by the inactivation of pea peroxidase after short treatment time, compared to sterilization with hot water (Icier et al., 2006).

In a recent study, the impact of ohmic heating in reducing the formation of furan in sterilized vegetable and vegetable/meat baby foods was investigated (Hradecky et al., 2017). The authors reported a reduction of 70%–90% of furan content in ohmic heated samples, compared to conventional sterilization. They suggest that this result is due to the faster heating time in ohmic heating,





**Figure 2** (A) Schematic diagram of industrial installation using PEF technology for the inactivation of microorganisms in food products. (B) Exponential and square waves used in PEF treatments. (C) Bipolar square wave. Reprinted with permission from Barba et al. (2017).



**Figure 3** Schematic representation of Ohmic heating equipment used for food applications. Reprinted with permission from Koubaa et al. (2016b).

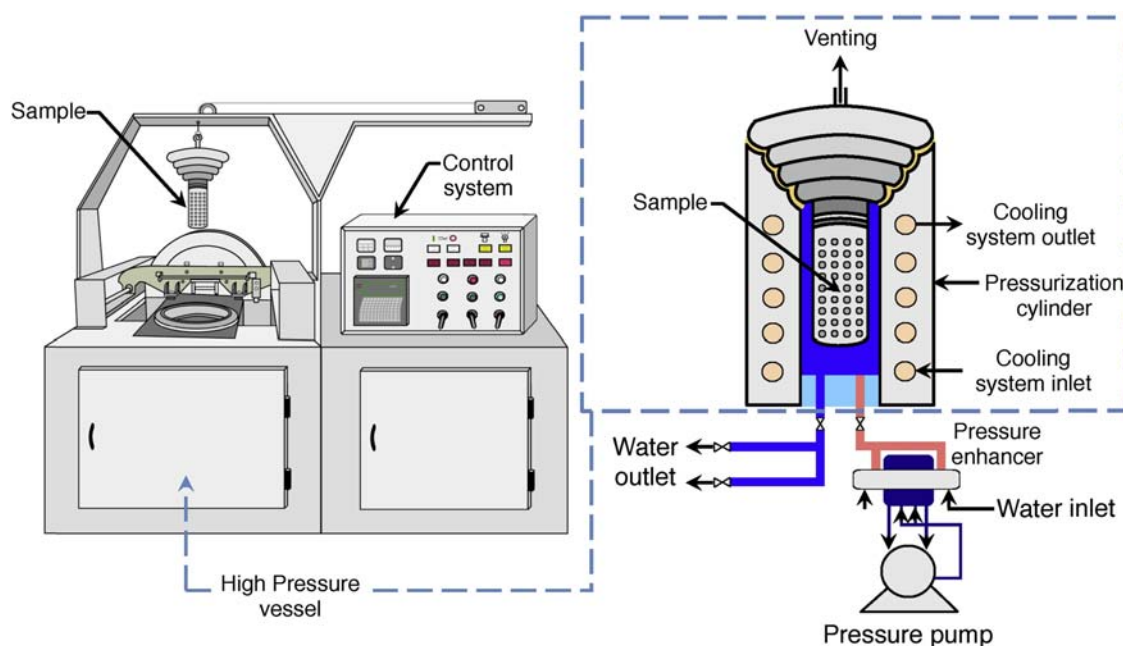
which leads to reduce the degradation of furan precursor. In addition, analysis of compounds formed during Maillard reactions and fatty acid oxidation were significantly higher in conventionally sterilized samples compared to those treated by ohmic heating. These studies show that ohmic heating can lead to reduced Maillard reactions in processed foods.

### High Hydrostatic Pressure

Products treated with high hydrostatic pressure (HHP) were first commercialized in the Japanese market in 1990. Currently, various HHP-treated foods are commercialized and equipment is sold in many countries and used at industrial scale. This success is related to the efficiency of this technology in inactivating spoilage and pathogenic microorganisms, which offers a similar level of safety as afforded by heat sterilization. The principle of high pressure treatment consists of applying pressure to the food product in the range of 400–600 MPa from few seconds to several minutes (Oey et al., 2016). Nonetheless, HHP efficiency was demonstrated in the range of 100–1000 MPa (Oey et al., 2008). This efficiency was further enhanced when investigating the inactivation of bacterial spores by applying high pressure and high temperature ( $\approx 90$ – $120$  °C) (Martínez-Monteagudo et al., 2014; Mathys et al., 2009; Rajan et al., 2006). The process is thus called pressure-assisted thermal sterilization (PATS) or high-pressure sterilization (HPS) and was first developed for the treatment of mashed potato (Sizer et al., 2002) and green beans (Leadley et al., 2008) in order to extend their shelf life. The advantage over heat treatment is obtaining processed food that preserves most of the organoleptic, sensory, and nutritional properties compared to the fresh product (Barba et al., 2012, 2015). A schematic representation of HHP equipment used at laboratory scale is given in Fig. 4.

Recent works investigating the effects of HHP on Maillard reactions show that the related downstream reactions are complex and it would be wrong to affirm that HHP, as a non-thermal technology, provides minimal chemical changes to food products (Martínez-Monteagudo and Saldaña, 2014). Investigation in this field showed that the effect of HHP on Maillard reactions depends on the processing temperature, and it is more pronounced when combining high pressure and high temperature. To understand the mechanisms of action, model systems were used. For instance, Maillard browning was investigated in lysine/arginine-sugar model systems under HHP (Ma et al., 2017). Results from this study demonstrated that high pressure has different effects on lysine-sugar and arginine-sugar models. Intermediate and final stages were retarded in the lysine-sugar model, whereas degradation rate of Amadori compounds was decreased in the lysine-glucose model and increased in the arginine-sugar model. The influence of temperature was also studied. The author showed that Maillard reactions were accelerated along with the emission of fluorescent compounds.

The effects of HHP on oenological characteristics, amino acids and volatile profiles of Hongqu rice wines during pottery storage was investigated (Tian et al., 2016). The authors demonstrated that after 18 months of storage, a faster decrease in total sugars, lower free amino acid content, and higher ketone content were observed in HHP-treated wines. They suggested that these changes could be attributed to the occurrence of Maillard and oxidation reactions. In a similar study, accelerated changes in the volatile composition of sulphur dioxide-free HHP-treated wine was observed during storage (Santos et al., 2015). The authors reported that after 9 months storage, the pressurized wines (500 MPa and 425 MPa for 5 min) contained higher contents of furans, aldehydes, ketones, and acetals compared to untreated samples (wines with 40 ppm of SO<sub>2</sub> and without preservation treatment). They concluded that



**Figure 4** Schematic representation of a high hydrostatic pressure equipment used at laboratory scale. Reprinted with permission from Barba et al. (2016).

this behavior could be related to accelerated Maillard reactions in HHP-treated wines, thus giving similar composition of volatiles when compared to faster aged and/or thermally treated wines. The same research team also previously reported evidence of Maillard reaction acceleration in wine (Santos et al., 2013).

Investigations on reconstituted skim milk treated either with high-pressure thermal processing (HPTP) or conventional thermal processing showed color change and proteolysis in both samples (Devi et al., 2015). Maillard reactions were evidenced by the increased consumption of free amino acids proportionally to the increase in temperature, which indicates higher Maillard reaction rates rather than proteolysis.

## Conclusion

This chapter discussed the impact of emerging technologies (pulsed electric fields, ohmic heating, high hydrostatic pressure) on Maillard reactions in foods. Reported results from different studies showed that using these technologies do not completely inhibit Maillard reactions. However, applications of pulsed electric fields and ohmic heating treatments have shown the potential to control or minimize Maillard reactions in the processed foods, especially due to the mild processing temperature and low treatment time, respectively. Finally, positive and negative impacts of high hydrostatic pressure on Maillard reactions were reported during processing at low and high temperatures, respectively. In order to further explain the mechanisms of action of emerging technologies on Maillard reactions, further investigations should be conducted.

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## Effect of Storage on Fruit Bioactives

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### Glossary

**Bioactive** Producing an effect in living tissue or in a living organism.

**Hyperbaric storage** Of fruit and vegetables, storage above atmospheric pressure.

**Hypobaric storage** Of fruit and vegetables, storage below atmospheric pressure to enhance loss of CO<sub>2</sub> and ethylene, so slowing ripening.

**Phytochemicals** Any naturally occurring plant substance, some of which have been shown in research to protect against disease.

### Introduction

Fruit is a rich source of valuable components like vitamins, minerals, and antioxidants. It is used to enhance/promote healthy living. However, most fruit are perishable commodities and normally available on a seasonal basis. Therefore, fruit is stored to increase its availability throughout the year. Various strategies have been developed to increase the shelf life and to ensure fruit availability throughout the year. However, consumers demand fresh and best quality fruit with all its nutritional and functionally bioactive components. Fruit quality deteriorates rapidly after harvesting due to increased rates of respiration and dehydration. Subsequently, deterioration results in weight loss, color changes, softening, surface pitting, browning, loss of acidity, and microbial spoilage. Therefore, the principle of storage technology is based on reduction of respiratory and enzymatic activities (Thompson, 2011). Other goals are described in Fig. 1. For this purpose, in the 19th century low-temperature storage or refrigeration storage technology was introduced, whereas controlled atmosphere storage and modified atmosphere packaging grabbed attention in the 20th century.

Evaluation of different storage technologies for improving organoleptic characteristics, reducing unnecessary losses, and preserving nutritional quality has become the center of attention now. Traditional fruit storage technologies include postharvest heat treatments, chemical treatments, and pressure-regulating storage. Some advanced storage practices are hypobaric or hyperbaric storage, ionizing or ultraviolet radiation treatment storage, high-pressure electrostatic field storage, ozone storage, magnetic field sterilization, ultrasonic processing technology, plasma treatment, and super low temperature ice technology or ice film storage (Ji et al., 2012). However, these technologies do not provide ideal condition to maintain quality, freshness, and other attributes. These technologies affect potential bioactive components of fruit, which are the focus of this chapter.

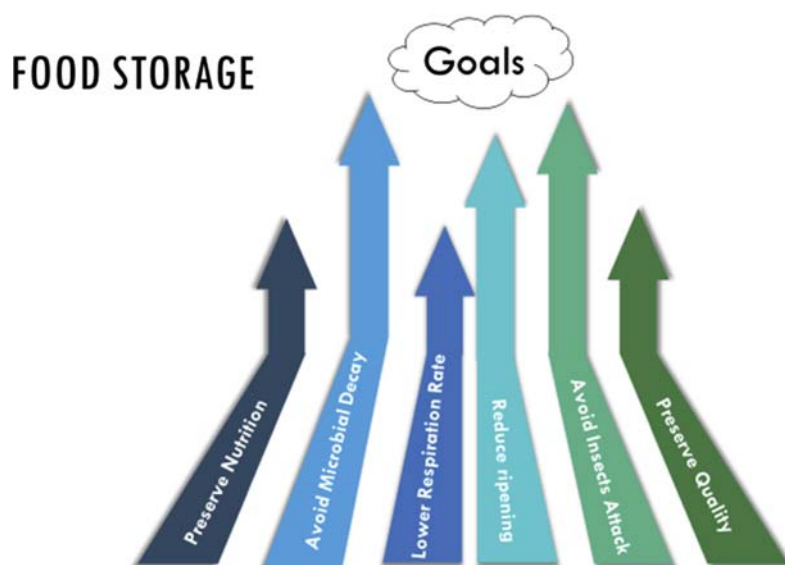


Figure 1 Food storage goals.



## Phytochemicals or Health-Promoting Compounds in Fruit

Fruit as nutritional commodities have an imperial role in human diet because of their countless functions in the prevention of diseases and improving health in conjunction with delightful sensory characteristics. Besides, fruit have various valuable components known as bioactive compounds including terpenoids, phenolic and polyphenolic compounds, alkaloids, sulfur-containing compounds, glucosinolates, carotenoids, anthocyanins, and lycopene. Among these compounds, polyphenols are secondary metabolites of plants and major antioxidants found in fruit. They have exhibited medicinal advantages such as lowering blood pressure and reducing cardiovascular diseases (CVDs), strokes (Russo et al., 2017; Williamson, 2017), and anticarcinogenic potential (Ferreira et al., 2017). They are subdivided into flavonoids (flavones, isoflavones, flavonols, flavanols, flavanones, anthocyanidins, anthocyanins, flavonones, and chalcones), tannins (hydrolyzed and condensed), lignans (sesamol, sesamin, sesamolol, and sesamololol), phenolic acids (hydroxybenzoic and hydroxycinnamic acids), and coumarins (Tokusoglu and Hall, 2011).

Flavonoids are further divided into subcategories including flavanones (hesperetin, hesperidin, naringenin, naringin, narirutin, didymin, eriocitrin, eriodictyol, neoriositrin, neohesperidin, isosakuranetin, pinocembrin, ponsirin, prunin), flavonols (quercetin, kaempferol, myricetin, quercitrin, isoquercitrin, rhamnetin, isorhamnetin, kaempferide, rutin, astragalin, hiperoside), anthocyanidins (cyanidin, malvinidin, delphinidin, pelargonidin, petunidin, peonidin), flavons (luteolin, baicalein, kryptin, diosmin, genkwanin, isorhoifolin, rhoifolin, tectokirisin), anthocyanins (grape extract), flavononols (dihydroflavonols), isoflavones (daidzin, genistein biokenin A, formononetin, glisitein, daidzin, genistin, glisitin) and chalcones (Tokusoglu and Hall, 2011). Flavonoids have clinical importance as antioxidants (Skrovankova et al., 2015) that are effective against many disorders including type 2 diabetes, CVDs and cancer (Martinez-Perez et al., 2014; van Dam et al., 2013). Other numerous advantages of flavonoids include antineoplastic effects, antiproliferative, anti-inflammatory, enzyme detoxification, activation of the immune system, prevention of cell proliferation, and reducing oxidative stress (Asha et al., 2017; Semaan et al., 2017; Zhang et al., 2017).

Other bioactive components of fruit include terpenoids (hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, triterpenes, and tetraterpenes). Carotenoids (tetraterpenes) are fat-soluble compounds recognized as natural coloring components in fruit such as orange, red, yellow, and violet colors. These compounds are associated with much bioactive functionality such as preventing chronic diseases like CVDs (Meléndez-Martínez et al., 2014). They are classified into four main groups: carotenoid hydrocarbons (carotenes), carotenoid alcohols (xanthophylls), carotenoid ketones, and carotenoid acids.  $\beta$ -carotenes are precursors of vitamin A production within the body as well as have potential antioxidant ability. Xanthophyll absorbs light energy, so it is a factor in keeping human eyes healthy and functioning properly (Thomas and Harrison, 2016). Other bioactive functions of carotenoids include preventing atherosclerosis (Amengual et al., 2017), anticarcinogenic by scavenging of peroxy radicals, inhibiting cell proliferation, and antiinflammatory and immunity-boosting properties. Color is the key quality parameter, which can indicate the freshness of fruit. In fruit, carotenoids can be used as quality parameters because of their color-imparting characteristic. In tomatoes, lycopene (carotene) is a major pigmented component, which is nutritionally valuable as an antioxidant, particularly in reducing the risk of CVDs (Cheng et al., 2017; Müller et al., 2016). Table 1 shows a summary of bioactive components of different fruit.

**Table 1** Bioactive components of fruit

Bioactive components	Fruit	References
Anthocyanidins including cyanidin and delphinidin	Bananas	Padam et al. (2014) and Singh et al. (2016)
Flavanone including naringenin, eriodictyol and hesperetin	Citrus fruit (lemons, grapefruit, limes and oranges)	del Río et al. (2017), Hwang et al. (2012), and Selvamuthukumar et al. (2017)
Epicatechin. Epigallocatechin and catechin	Rosacea family fruit (Apples, plums, pears, peaches and apricots)	Brahem et al. (2017) and Raudone et al. (2017)
Flavonoids	Blue red and purple berries	Jimenez-Garcia et al. (2018)
Flavanol including myricetin and quercetin	Cranberries and blueberries	Flores and del Castillo (2015)
Flavonoids catechin and epicatechin	Black grapes and blackberries	Nikitina et al. (2000) and Xi et al. (2016)
Cyanidins (dimers) and anthocyanidins	Cherries, raspberries and red grapes, plum, apples	Peña-Sanhueza et al. (2017)
Xanthophyll	orange and yellow fruit	Al-Yafeai et al. (2017)
Flavones including luteolin	Red grape, oranges	Kozłowska and Szostak-Wegierek (2014)
Naringenin	Grapefruit, oranges	Seyedrezazadeh et al. (2015)
Flavan-3-ols (catechin)	Peaches, berries, red grape, bananas	Ageorges et al. (2014)
Gallocatechin	Berries, plums, pomegranate, persimmons	Zanotti et al. (2015)
Cyanidin	Berries, plums	Hubert et al. (2014)
Delphinidin, malvidin, peonidin	Berries, red grapes	Veberic et al. (2015)
Lycopene (carotene)	Tomatoes	Cheng et al. (2017) and Müller et al. (2016)
Carotenoids	Pumpkins, mango and papaya	Meléndez-Martínez et al. (2014)



## Effect of Different Storage Conditions on Fruit

During storage, fruit respire reducing its quality, and can possibly affect the quality of bioactive components in fruit. It may reduce health-promoting components, their concentrations, and antioxidative abilities. However, small changes in the antioxidant contents of the fruit have been reported during storage conditions because of alterations in ripening patterns (Kim et al., 2009; Park et al., 2006). On the other hand, significant increase in total phenolics (Gonçalves et al., 2004) and ascorbic acid concentrations (Khan et al., 2009) have been observed during storage. These advantages of premium quality can be enhanced by applying proper postharvest storage practices. Table 2 shows the effect of different bioactive compounds in different fruit.

### Cold Storage

Fruit deterioration starts with harvest and is enhanced at room temperature. However, use of low temperature may reduce the respiration and prevent microbial spoilage (Lamikanra, 2002). Therefore, it is necessary to cool down fruit as soon as possible to get premium quality fruit for the consumer. Fruit storage at low temperature has been the main postharvest technology to maintain fruit quality (Kader, 2002). Other important benefits of cold storage are reduction of ethylene production, delay in the adverse changes in parameters (color, acidity, and texture) related to fruit ripening and quality.

However, sometimes fruit exhibiting tissue injury or chilling injury show various physiological disorders (metabolic imbalance leading to undesirable chemical substance production in the tissues and eventual cell death) related to the lower temperature limits, which vary according to the type of fruit. Each commodity, kept in a specific storage temperature range (according to their metabolism), carefully monitored environment, and maintained uniformity in storage conditions, assures the retention of quality. Otherwise, storage conditions may affect the bioactive components leading to faster metabolism or cell death. Several types of cooling systems have been used worldwide such as room cooling, cryogenic cooling, hydro cooling, contact icing, forced air cooling, and vacuum cooling (Valero and Serrano, 2010). Among bioactive components present in fruit, phenolics and carotenoids are

**Table 2** Effects of postharvest storage on bioactive components of fruit

Storage	Fruit	Key findings	References
Cold storage	Tomato	Lycopene decrease at 5 °C	Javanmardi and Kubota (2006)
	Apple	Increases in TP, TA decreased at 0 °C after 120 days Nonsignificant flavonoids and TA losses, TP decrease after 3 months at 0°C	Leja et al. (2003) Ju et al. (1996)
	Pomegranate	TAA increases at 5°C, significant increase in AA Decrease in TP at 2°C Increases in TP, decline in TP after 4 months, TAA increase, significant decrease in AA after 3 months TP decrease at 5°C, TAA declined gradually	Miguel et al. (2004) Sayyari et al. (2011) Arendse et al. (2014)
			Fawole and Opara (2013)
CA	Apple	Nonsignificant flavonoids losses Nonsignificant TA losses	Awad and de Jager (2003) Leja et al. (2003)
	Pomegranate	Degradation of TP significantly affected by pressure TAA and TPC increased significantly, polyphenols degraded	Bermejo-Prada and Otero (2016) Matityahu et al. (2016)
		Delayed synthesis of anthocyanins and other phenolics TA decreased with storage duration	Defilippi et al. (2006) Caleb et al. (2013)
		TP, TA and TAA increased slightly and then decreased after 120 days	Selcuk and Erkan (2015)
MAP		Increases in TA, increase in TAA, reduced levels of TP Lycopene and total carotenoid retention	Tavasoli Talarposhti et al. (2016) Singh and Rao (2005)
	Papaya	Retention of TAA	Villalobos et al. (2014)
	Fig	64% retention of TA, 80% AA retention, TP and flavonoid increase,	Rai et al. (2011)
	Jamun		
Hyperbaric storage	Strawberry juice	TP and monomeric TA remained stable at 7th day. A significant decrease after 10 days	Bermejo-Prada and Otero (2016)
	Watermelon juice	Decrease TP	Fidalgo et al. (2014)
	Tomato	Retention of TAA, significant increase in lycopene, nonsignificant effect on TP and AA	Liplap et al. (2013)
		Lycopene increase	Inestroza-Lizardo et al. (2018)
Hypobaric storage	Peach	Retention of AA	Wang et al. (2015)
		Retention of AA	Chen et al. (2010)
	Pear	Retention of TP	Li et al. (2017)
	Chinese bayberry fruit	Higher TP	Chen et al. (2013)

AA, Ascorbic acid; TA, Total anthocyanin; TAA, Total antioxidant activity; TP, Total phenols; TPC, Total phenolic content.

generally stable, whereas ascorbic acid decreases during cold storage (Jaganath et al., 2008). Similarly,  $\beta$ -carotene, total carotenoids, pro-vitamin A, and total phenolics of fruit remain unaffected by storage. However, reduction of lutein contents has been reported during cold storage (Ayala-Zavala et al., 2004; Kidmose et al., 2004; Koca and Karadeniz, 2008). Decrease in anthocyanin contents of strawberry has been observed at 0 and 5°C, whereas it gradually increases at 10°C (Ayala-Zavala et al., 2004).

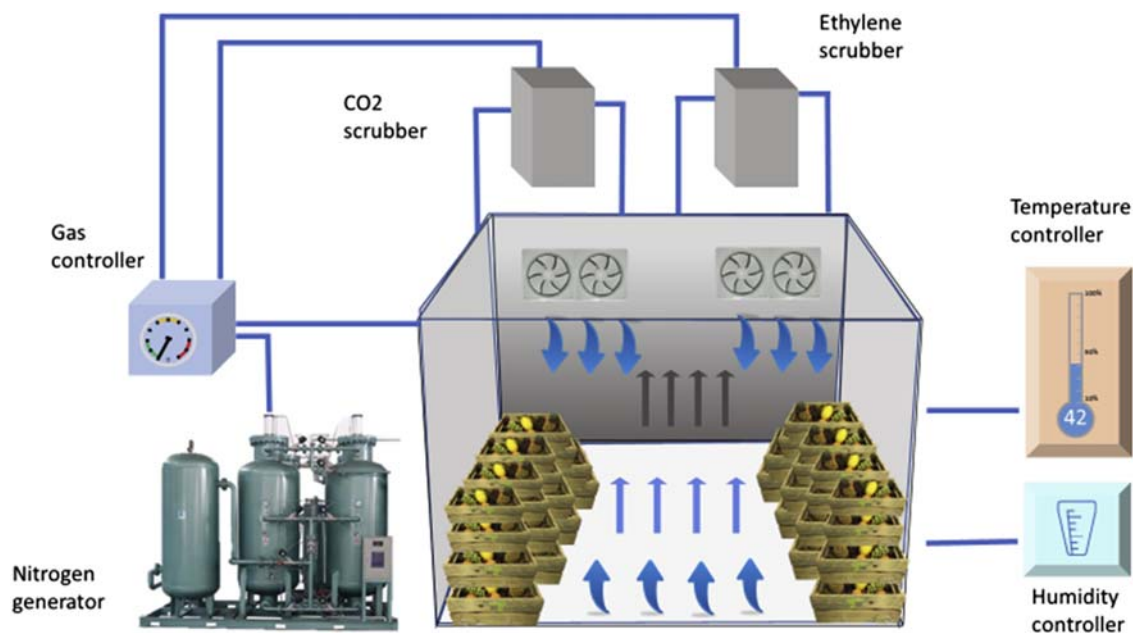
### Controlled Atmosphere Storage

For extending a fruit's shelf life and maintaining its quality characteristics, controlled atmosphere (CA) is a good choice to adopt. Normally, CA is adopted for gaining some advantages such as reducing enzymatic reactions, chilling injuries, respiration rates, substrate oxidation, breakdown of chlorophyll, ripening, rate of production of ethylene, degradation rate of soluble pectin and ultimately maximizing the storage period of fruit. CA storage rooms have been advanced from bricks or concrete-insulated refrigerated storage to metal-faced insulated panels in modern days. In this process, developed equipment are used to regulate and maintain the higher level of CO<sub>2</sub> and lower level of O<sub>2</sub>. Good temperature and humidity maintenance, airtight fitness, appropriate construction and insulation to prevent leakage are important points to be considered before constructing CA storage room (Fig. 2).

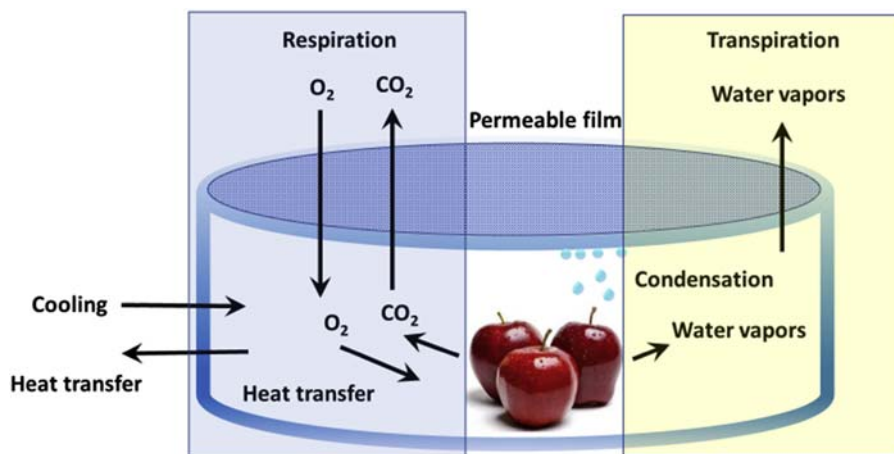
Although CA is a valuable tool for establishing a controlled setup for improving shelf life of fruit, sometimes it is associated with the onset of several physiochemical disorders because of mismanagement or long-term storage period, which affects the overall quality of the fruit. Generally, CA reduces the metabolism of the product, which indirectly affects its eating quality or flavor because of the production of some chemical byproducts (alcohols and aldehydes) during anaerobic conditions. Too-low levels of O<sub>2</sub> and anaerobic fermentation can damage fruit skin or textural properties (darker color, sharp edges). On the other hand, high levels of CO<sub>2</sub> can cause surface-scald browning and pitting without skin softening in addition to increase in acidity of fruit. Various studies on bioactive components of different fruit demonstrated that the total phenolic contents have been increased during CA storage (Leja et al., 2003; Zheng et al., 2003). Besides, a nonsignificant effect of storage was reported on phenolic contents (MacLean et al., 2006; Schotsmans et al., 2007). Other changes may include the synthesis of terpenoid components, synthesis of the carotenoids lycopene, xanthophyll (Goodenough and Thomas, 1981), and chlorogenic acid (MacLean et al., 2006), as well as decrease in anthocyanins (Leja et al., 2003; Romero et al., 2008) and lycopene (Jeffery et al., 1984), and chlorophyll degradation (Ben-Arie et al., 1992; Goodenough and Thomas, 1981). However, vitamin C and total flavonoid contents remained unchanged in CA storage (Martínez-Sánchez et al., 2006).

### Modified Atmosphere Packaging

The main purpose of this technology is to lessen respiration rate of the fruit, which ultimately reduces the metabolism or senescence process. Modified atmosphere packaging (MAP) was studied in the 19th century for the first time on the shelf life of horticultural products, and results demonstrated that anaerobic conditions strongly influenced the ripening process of fruit as unavailability of O<sub>2</sub> will block ripening. Therefore, in MAP fruit is generally packed in a modified environment, which has higher concentrations of CO<sub>2</sub> and lower concentrations of O<sub>2</sub>. This fixed gas composition (different from air) creates an anaerobic environment, which



**Figure 2** Controlled atmosphere storage. <http://www.agroripe.com/controlled-atmosphere-storage/>.



**Figure 3** Summary of MAP passive atmosphere modification phenomenon. Modified from Belay et al., (2016). <https://doi.org/10.1016/j.fpsl.2016.08.001>.

affects the respiration rate of products and enhances shelf life by lowering the rate of respiration. Other crucial factors that greatly influence composite gases are temperature, relative humidity, packaging material, and respiration rate of the product. One disadvantage of this system is that alteration of gas composition by microbial growth or packaged-film permeability can seriously affect the quality of fruit packed in hermetically sealed semipermeable containers. Theoretical considerations indicate that if the container is impermeable, then internal atmosphere can be altered because of the respiration process of the commodity; it will eventually deplete  $O_2$  level (from 21 to 0%) while increasing  $CO_2$  (from 0.03 to 20%) concentrations. On the other hand, semipermeable packaging has the same effect because of the passive atmosphere modification phenomenon (Al-Ati and Hotchkiss, 2002) (Fig. 3).

MAP gas concentration balance is a crucial factor, because an aerobic environment can enhance metabolic activity, sugar or starch consumption associated with flavor alteration, undesirable textural modifications, and production of water vapors, which degrade the other measures of the package selected for MAP. An anaerobic environment with depleted  $O_2$  level is also dangerous for fruit tissues as it reoxidizes reduced metabolites [pyridine nucleotides, nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide plus hydrogen (NADPH)], produces  $CO_2$ , ethanol, and offensive odors. Maintained  $CO_2$  and  $O_2$  levels are required for generating the proper anaerobic environment to slow down respiration rate and the fruit's enzymatic activities. Inappropriate gas composition drastically degrades the physiochemical and sensory quality of the fruit and promote spoilage by pathogenic microorganisms, which ultimately produce undesirable changes in color, texture, aroma, or flavor (Artés-Hernández et al., 2006). Some recent developments of MAP have been introduced such as high-oxygen MAP, controlled MAP, and intelligent MAP (Zhang et al., 2015). MAP storage has advantage over bioactive components of fruit by retention of phenolics, antioxidants, anthocyanins, and ascorbic acid (maintain vitamin C concentration by limiting ascorbic acid oxidation) (Rojas-Graü et al., 2009), and lowering the postharvest ripening process (Jones, 2007). Furthermore, it enhances the sensory quality of the fruit, i.e., overall appearance, color, texture, aroma, etc (Akbudak and Eris, 2004; Martínez-Romero et al., 2003).

### Hypobaric Storage

Hypobaric or low-pressure storage, also known as subatmospheric pressure storage, consists of a ventilated chamber, which establishes a reduced  $O_2$  controlled environment (less than atmospheric pressure) that lowers the respiration rate of the produce and increases its shelf life. Proper monitoring of  $O_2$  level and low pressure are very important factors needed in well-maintained hypobaric storage (Thompson, 2011).

The positive effects of hypobaric storage on fruit have been reviewed by (Burg, 2014; Davenport et al., 2006; Li et al., 2008). Fruit stored in hypobaric conditions continuously respire and produce  $O_2$ , various volatile byproducts, and ethylene, which are constantly removed by vacuum pump, thus risk of ethylene-related disorders is reduced. Other advantages of hypobaric atmosphere storage conditions include inhibition of growth and sporulation of pathogenic microorganisms (fungi; Burg, 2004; Romanazzi et al., 2001) and control of insect infestation in fruit or disinfection [created hypoxic conditions, which reduce adenosine triphosphate (ATP) production and increase membrane phospholipid hydrolysis (Burg, 2010; Davenport et al., 2006; Jiao et al., 2013)]. In addition, advantages include enhanced antioxidant ability and reduced membrane damage of fruit (Wang et al., 2015), removal of scald-related volatile substances of apples (Wang and Dilley, 2000), delayed softening (Dilley et al., 1982), and reduction in the susceptibility to chilling injury (Chen et al., 2013; Zhao et al., 2006).

The major disadvantage of hypobaric is weight loss (higher desiccation) because lower pressure can increase the transpiration rate, which excludes water from the commodity (Burg, 2004). This problem of insufficient relative humidity can be resolved by spraying water and humidity control for maintaining the quality of fruit within hypobaric storage (Laurin et al., 2006).

### Hyperbaric Storage

Hyperbaric storage, the opposite of hypobaric storage, is storage in which high-pressure exposure is adopted for a short time as an alternative to chemical treatment for preserving food quality (Vigneault et al., 2012). Hyperbaric chambers are equipped with CO<sub>2</sub> removal and automatic O<sub>2</sub> replenishment systems and pressurized from 1 to 7 atm, with an automatically monitored gas concentration system, flow meter, CO<sub>2</sub> gas analyzer, and computer-controlled valves.

Advantages include lowering respiration rate and ripening processes (Goyette et al., 2012), reduction of microorganisms (irreversible damage to cell structure; Hite et al., 1914; Yordanov and Angelova, 2010), unchanged flavoring and nutritional components while retaining vitamin C (Day et al., 1998; Hill, 1997), controlling insect/pest infestation (UTZ et al., 2004). Other advantages include saving significant energy (Fernandes et al., 2015), preserving fruit color (López-Malo et al., 1998), improving texture and visual appearance (Luscher et al., 2005), reduced weight loss, improved lycopene retention, reduced chilling injury (Baba and Ikeda, 2002), enhancing antioxidative enzyme activity, oxygen radical absorbance capacity, and phenolic levels (Zheng et al., 2008). Still more advantages include structural rearrangements in enzymes (activation or inactivation; Ludikhuyze et al., 2001), inhibition of undesirable fermentation reactions, delay in enzymic browning (Day, 1996), and reduced peel spotting or browning by decreasing dopamine level (Maneenuam et al., 2007). Extreme high pressure (above 100 MPa) or increased O<sub>2</sub> concentrations may result in irreversible tissue damage, thus causing substantial injuries (Goyette et al., 2007, 2012), higher levels of free radicals (Fridovich, 1986), mealy flesh, browning of skin (Kidd and West, 1934), and peel injury (Solomos et al., 1997).

### Other Fruit Quality Defects During Storage

After picking or harvesting of fruit, deteriorative reactions have been observed, which include physiochemical defects due to environmental stresses as well as microbial infections due to mishandling or improper postharvesting treatments. Quality changes vary according to the nature of plants or trees from where fruit are collected. Fruit are divided into two main categories (climacteric and nonclimacteric) according to their ripening stage. Climacteric and nonclimacteric fruit have different ripening rates (ethylene, sugar, or acid concentration) and are therefore preserved by different storage conditions. Two such chemical alterations, lipid oxidation and nonenzymatic browning, are major factors of deteriorating the quality of fruit in regard to their color and flavor. Common physiochemical defects including weight loss (Galindo et al., 2004), alteration of color (chlorophyll losses; Wrolstad et al., 2005), texture (softening; Bennett and Labavitch, 2008), aroma (volatile profile changes), flavor, and taste (sugar or acid contents) have been observed (Crisosto et al., 2003; Hernández-Muñoz et al., 2006).

### Quality Management During Storage and Distribution

Fruit are perishable and need extensive care and quality control during storage. Superior quality products are the consumer's first demand, whether processed or not, which means the product must meet all quality parameters like taste, aroma, color, and freshness even after storage. There are some quality management strategies that may be adopted to ensure excellent quality during storage. Some of them are First in First out (FIFO), statistical prediction depending upon the effect of a specific parameter on quality loss, and installment of quality indicators that depend on a time-temperature relationship. Additionally, hygiene and cleanliness of storage area have significant effect on improving shelf stability of fruit during storage. Conclusively, quality management strategy depends on type of product to be stored as each food product needs specific strategy. In FIFO system, food must be stored in such a way that new stock should be stored after the older stock, so the older stock will dispatch prior to new stock, hence product will not deteriorate. Statistical prediction enables us to investigate the effect of specific parameters such as temperature, humidity, and other environmental conditions on the final product's quality, so we can calculate estimated shelf life in case of any change in environmental conditions. This system is very helpful during transportation of fruit, because temperature fluctuations occur that ultimately affect the quality. Hence, change in quality parameters may be predicted that helps in estimating final product quality.

### Conclusions

Fruit has been an important part of human diet since ancient times, but its importance is increasing day by day due to increased understanding about bioactive components present in them that are known to improve human health and help in combating diseases. Keeping its importance in mind, preservation of fruit and vegetables is a big concern due to drastic increase in human population and demand for quality food products. Various techniques of preservation have been employed by humans since the start of civilization. These techniques involve control over temperature and gaseous compositions, and their effect on preservation of fruit has been checked thoroughly. The use of hypobaric and hyperbaric environments in storage areas is still under study and has not yet been commercialized.

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# Enzymatic Production of Antioxidants and Their Applications

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## Introduction

Antioxidants are compounds that can slow or prevent oxidation processes. They are required in low concentration for inhibiting the oxidation of oxidisable substrates such as lipids and lipid-soluble compounds. Antioxidants may be present in foods as endogenous factors or may be added to prevent their lipid components from quality deterioration (Shahidi, 2000). The major antioxidants used in foods are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propyl gallate (PG) (Fig. 1). These are chemically synthesized and there are growing health concerns over their use as food antioxidants. For instance BHA and BHT have been suspected of being responsible for liver damage and cancer (Williams et al., 1999). In 1982, BHA was banned in Japan and in 1997 BHT was removed from the “GRAS” (generally recognized as safe) list of the FDA (Food and Drug Administration) in the United States (Hao et al., 2007). Europe and Japan are yet to approve TBHQ as a food antioxidant (Pinho et al., 2000). In Australia, New Zealand, China, USA, Philippines and Brazil, no more than 200 mg/kg of TBHQ is allowed in food (Perrin and Meyer, 2002).

Concerns about the safety of chemically synthesised antioxidants have motivated the food industry to seek natural alternatives. Natural antioxidants are generally derived from plants. The major ones are polyphenols, ascorbic acid (vitamin C) and tocopherols (vitamin E). Ascorbic acid and some polyphenols are water-soluble and are mostly effective in hydrophilic or water-containing food materials, while tocopherols are fat-soluble antioxidants and are effective in fatty or oily food materials. The effectiveness of natural antioxidants depends on the nature of the food materials, including the level of lipid present.

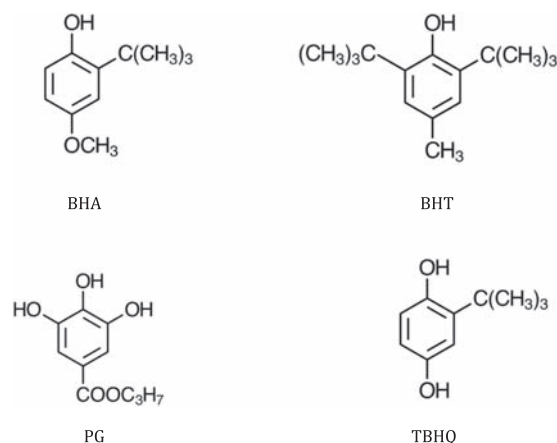
Natural antioxidants can be modified to improve their oxidative stability, solubility properties and bioavailability. For instance, attaching fatty acids to ascorbic acid and some water-soluble polyphenols can make them oil-soluble. Also, specific polyphenols can be attached to tocopherol to improve its antioxidant property. These modifications can be achieved by using chemicals or enzymes. The use of enzymes is preferred because reactions involving enzymes are carried out under mild conditions, with less side reactions compared to chemical methods. Lipases are widely used for these type of reactions. They are the most important enzymes for the formation or transfer of ester bonds, particularly for lipids including triacylglycerols.

Therefore, in this article, lipase-catalysed modification of three major natural antioxidants, vitamin C (ascorbic acid), vitamin E ( $\alpha$ -tocopherol) and polyphenols will be discussed. Specific applications of the modified antioxidants will also be discussed.

## Lipases

Lipases are a highly versatile group of biocatalysts that play an important role in biotechnological and industrial processes, including food, oleochemical and pharmaceutical applications. They are produced from various sources including animals, plants and microorganisms (Olusesan et al., 2009). However, microbial lipases are generally more stable and are cheaper to produce.

Lipases catalyse the hydrolysis of fats into fatty acids and glycerol in aqueous media (Olusesan et al., 2011; Xia et al., 2017). Because of their compatibility with a broad range of solvents, lipases can be used to synthesize or transfer ester bonds in non-aqueous media (Akanbi and Barrow, 2015; Pande and Akoh, 2016). These are known as esterification and transesterification reactions. The high cost of lipases has been recognised as the main constraint to their use industrially. From an economic point of view, these enzymes should be recoverable and reusable for continuous operations. To achieve these, lipases have to be



**Figure 1** Chemical structures of some of the major synthetic antioxidants used in foods.

immobilized so that they can be used in solid form (Akanbi and Barrow, 2017). Immobilized lipases are preferred to free lipases since the immobilized forms tend to be more robust and are more readily recovered and reused at manufacturing scale (Akanbi and Barrow, 2018; Mathesh et al., 2016). *Candida antarctica* lipase B, marketed in an immobilized form as Novozym 435, is a widely used immobilized lipase since it has been shown to catalyse a broad range of reactions.

### Lipase-Produced Antioxidants From Ascorbic Acid

Ascorbic acid is a natural antioxidant, but because it is highly hydrophilic, its use in fats and oils is restricted. This can be overcome by attaching fatty acid to it, thus making it fat soluble. Ascorbyl palmitate is the major fat soluble vitamin C ester available commercially today and it is manufactured through industrial-scale chemical reactions (Humeau et al., 1995). These reactions involve the use of strong acids that can lead to side reactions thus lowering the yield of esters. The use of lipase for producing fatty acid esters of vitamin C is preferred because of the moderate reaction conditions and also because it is considered a natural process. Acyl donors include triacylglycerols, alkyl esters, vinyl esters, fatty alcohols or free fatty acids. Fatty acids can be saturated or unsaturated. Saturated fatty acids are those with only single bonds while unsaturated fatty acids are those with double bonds. The major saturated fatty acids used for the synthesis of vitamin C esters include caprylic (C8:0), decanoic (C10:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acids. Unsaturated fatty acids include oleic (C18:1), linoleic (C18:2), linolenic (C18:3), eicosapentaenoic (C20:5) and docosahexaenoic (C22:6) acids. Since esterification reaction involves the use of solvent, several non-toxic solvents have been identified for the lipase-catalysed production of fatty acid esters of vitamin C. These include 2-methyl-2-propanol (*tert*-butanol), 2-methyl-2-butanol, 1-octanol, acetone and dimethyl sulfoxide (DMSO) (Karmee, 2011; Stamatis et al., 1999). Column chromatography is the principal purification technique for most of these novel compounds. Column chromatography can be used for gram-to kilogram-scale purifications. Different analytical techniques such as capillary chromatography, gas chromatography (GC), nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS) can be used to characterise these esters.

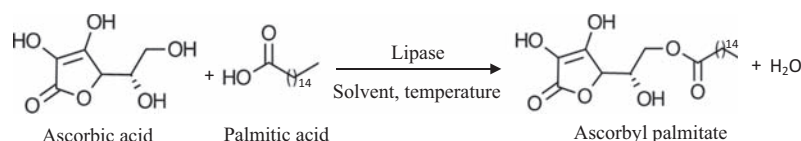
An example of a lipase-produced ascorbyl ester is shown in Scheme 1 where ascorbic acid and palmitic acid, a free fatty acid, are esterified to produce ascorbyl palmitate. As shown, the primary alcohol group from the ascorbic acid is esterified.

### Applications

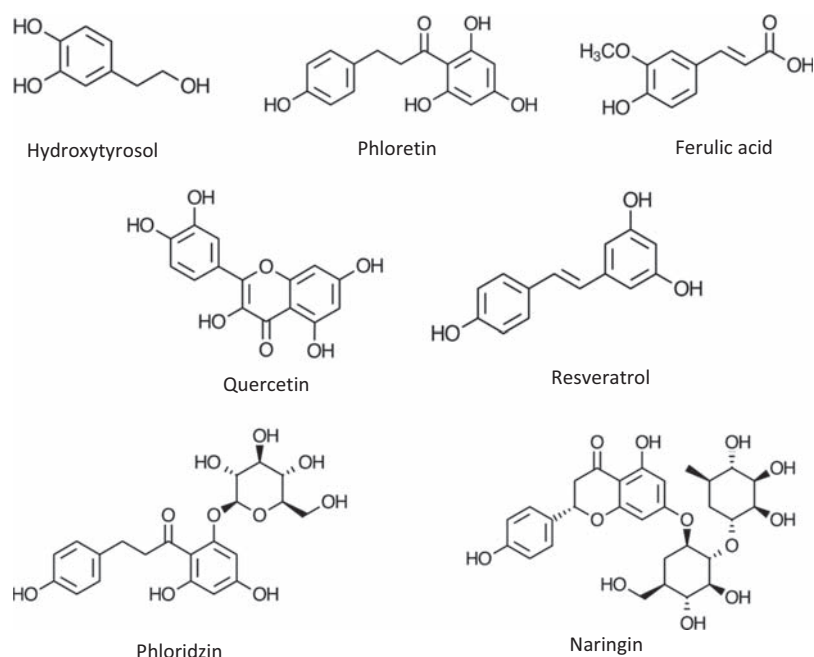
The antioxidant activities of lipase-produced fatty acid esters of vitamin C acid have been previously reported. Rapeseed oil was stabilized using ascorbyl palmitate and oleate produced using Novozym 435 as biocatalyst (Viklund et al., 2003). These authors found that ascorbyl oleate was more effective than ascorbyl palmitate as it could protect rapeseed oil for up to nine weeks when stored at 30 °C. Ascorbyl palmitate was only able to preserve the oil for one week under the same storage conditions. Another study found that both ascorbyl palmitate and oleate produced by *Thermomyces lanuginosus* lipase showed similar antioxidant activity in soybean oil (Reyes-Duarte et al., 2011). Both esters slowed down the formation of peroxides in soybean oil. A study found that ascorbyl linoleate produced using Novozym 435 showed the same antioxidant activity as BHT in soybean oil at a concentration of 200 mg/kg oil (Liu et al., 2011). Ascorbyl decanoate, laurate, myristate, palmitate and stearate were produced using Novozym 435 under ultrasonic irradiation (Jiang et al., 2016). They prolonged the stability of lard in a similar manner as TBHQ. These authors found that these esters were better than TBHQ in stabilizing soybean oil (Jiang et al., 2016).

### Lipase-Produced Antioxidants From Polyphenols

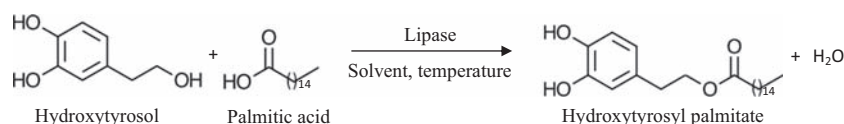
Polyphenolic compounds are the most abundant and widely studied antioxidants from natural sources. They exist in the forms of phenolic acids, stilbenes, flavonoids, lignans, coumarins and tannins (Holland et al., 2017). The chemical structures of some polyphenolic compounds are presented in Fig. 2. All phenolic compounds contain a benzene ring and at least one or more hydroxyl groups, making most of them water-soluble (hydrophilic). The hydrophilic property of these compounds limits their dispersion in fats and oils, thereby reducing their effectiveness in protecting lipids against oxidation (Akanbi and Barrow, 2018). Again, an effective way of making them oil-soluble and with improved antioxidant properties is by attaching fatty acids to them using lipases. Lipases have been used to produce fatty esters of polyphenols, most of which have maintained or showed higher antioxidant activity than their parent polyphenols.



**Scheme 1** Lipase-catalysed synthesis of ascorbyl palmitate.



**Figure 2** Chemical structures of some polyphenolic compounds.



**Scheme 2** Lipase-catalysed synthesis of hydroxytyrosyl palmitate.

**Scheme 2** shows that hydroxytyrosol, an olive oil polyphenol and palmitic acid can be esterified using lipase as catalyst to produce hydroxytyrosyl palmitate.

## Applications

Fatty acid esters of polyphenols have been synthesised using lipases (**Table 1**). Some of these phenolic esters have been used to stabilize fats and oils against oxidation. In our group, we have recently modified the structure of hydroxytyrosol, a major polyphenol in olives, through esterification with a broad range of free fatty acids using Novozym 435. The abilities of these esters to stabilize anchovy oil were investigated (**Akanbi and Barrow, 2018**). We found that hydroxytyrosyl palmitate and eicosapentaenoate effectively stabilized bulk fish oil, fish-oil-in-water emulsions and microencapsulated fish oil, with antioxidant levels higher than those of BHT and  $\alpha$ -tocopherol. These novel antioxidants were also stable for one year when stored at  $-20^{\circ}\text{C}$ . In another study, where lipase was used to synthesize fatty acid esters of tyrosol, the authors found that tyrosyl oleate was the most

**Table 1** Examples of polyphenols and acyl donors reported in the literature

Polyphenols	Acyl donor	Lipase used	References
Hydroxytyrosol	Free fatty acids	Novozym 435	<a href="#">Akanbi and Barrow, 2018</a>
Tyrosol	Free fatty acids	Novozym 435	<a href="#">Pande and Akoh, 2016</a>
Quercetin	Free fatty acid	Novozym 435	<a href="#">Saik et al., 2017</a>
Rutin	Free fatty acid	Novozym 435	<a href="#">Vaisali et al., 2017</a>
Phloridzin	Free fatty acids	Novozym 435	<a href="#">Milisavljevic et al., 2014</a>
Cinnamic acid	Fatty alcohol	Novozym 435	<a href="#">Lue et al., 2005</a>
Ferulic acid	Triacylglycerol	Novozym 435	<a href="#">Safari et al., 2006</a>
Naringin	Vinyl ester	Lipozyme RMIM	<a href="#">Hattori et al., 2016</a>
Resveratrol	Vinyl ester	Novozym 435	<a href="#">Torres et al., 2009</a>

efficient antioxidant for stabilizing an oil-in-water emulsion (Pande and Akoh, 2016). Also, a lipase-produced decanoic acid ester of rutin was found to be a more effective antioxidant than rutin in sardine oil during 20 days of storage (Vaisali et al., 2017). Since lipid oxidation may cause deterioration of other food components such as protein, many of these lipase-produced fatty acid esters of polyphenols can also be used for stabilizing lipid-containing protein rich foods.

### Lipase-Produced Antioxidants From Tocopherols

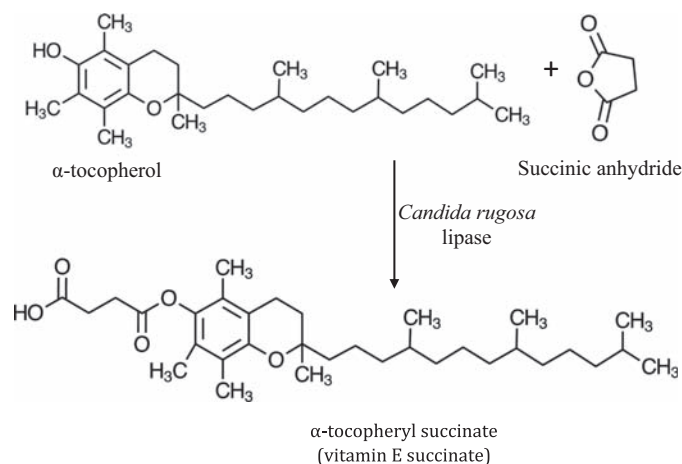
There are four types of tocopherols (alpha,  $\alpha$ ; beta,  $\beta$ ; gamma,  $\gamma$ ; and delta,  $\delta$ ), but the most abundant in nature is  $\alpha$ -tocopherol. Although  $\alpha$ -tocopherol is a natural antioxidant, it is unstable and is readily oxidised in the presence of oxygen, heat and other oxidising agents. Interestingly, esters of  $\alpha$ -tocopherol have been found to be relatively stable to atmospheric oxygen at room temperature. Different microbial lipases have been used to produce vitamin E esters, such as vitamin E acetate, vitamin E succinate and vitamin E ferulate (Ferreira-Dias et al., 2013; Hu et al., 2013). Scheme 3 shows the lipase-catalysed production of vitamin E succinate, where succinic anhydride and  $\alpha$ -tocopherol (vitamin E) are esterified using immobilized lipase from *Candida rugosa* (Hu et al., 2013).

### Applications

Enzymatic conjugation of two natural antioxidants,  $\alpha$ -tocopherol and ferulic acid, produces a potent natural antioxidant, tocopheryl ferulate or vitamin E ferulate. This lipase-produced ester can be used in fats and oils because it is a lipid-soluble antioxidant (Xin et al., 2011). Since ferulic acid is a polyphenol, several polyphenolic esters of vitamin E can be synthesised enzymatically and used as antioxidants in oil and fat-based foods and formulations. Lipases have been used to synthesize other vitamin E esters, such as vitamin E succinate and vitamin E acetate. These esters are more stable in the presence of light and oxygen than their parent vitamin E (Ferreira-Dias et al., 2013). However, the presence of the succinic or acetic groups on the tocopherol block their antioxidant activity, so they cannot be used as antioxidants in fats and oils. However, they have been added to foods to increase their vitamin E content, so that when they are consumed, digestive enzymes like esterase can break the ester bond and release the active tocopherol that then acts as antioxidant *in vivo*. Vitamin E acetate is the most common form of vitamin E used in the human and animal nutrition market (Torres et al., 2008; Ferreira-Dias et al., 2013).

### Conclusion

Enzymes, particularly lipases, are used to modify the oxidative stability and solubility of natural antioxidants, thereby expanding the range of nutritional applications where they can be utilised. The antioxidant activity of some of these lipase-produced esters of natural antioxidants are similar and in some cases better than food-grade chemically synthesised antioxidants such as BHT, BHA and TBHQ. Importantly, the lipase produced antioxidants are considered natural in many regulatory jurisdictions if the starting antioxidant is sourced naturally. In addition, some of these antioxidants, such as vitamin E and vitamin C and their derivatives have positive health benefits in addition to their antioxidant properties, and so can offer a dual benefit in nutritional products such as nutritional supplements and functional foods.



**Scheme 3** Lipase-catalysed synthesis of  $\alpha$ -tocopheryl succinate (vitamin E succinate). Hu, Y., Jiang, X., Wu, S., Jiang, L., Huang, H., 2013. Synthesis of vitamin E succinate by interfacial activated *Candida rugosa* lipase encapsulated in sol-gel materials. Chin. J. Catal. 34, 1608–1616

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# Factors Influencing Red Wine Color From the Grape to the Glass

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## Glossary

**Flavan-3-ol (syn. flavanols, condensed tannins, catechin derivatives, proanthocyanins)** Phenolic compounds consisting of a 15-carbon skeleton with two hydroxylated phenyl rings and a pyran ring with hydroxyl group at C-3 position.

**Interflavan bonds** Covalent bonds formed between flavan-3-ol subunits.

**Nucleophile** Molecule or ion with a free pair of electrons that can be donated to an electrophile to form a covalent bond.

**Oenotannin** A commercially-available tannin-rich product produced by extracting tannins from grapes or oak.

**Tannin (syn. condensed tannin polymer, pigmented polymer)** By definition, tannins are polymeric molecules that bind to protein. In grapes and wine, tannin refers to polymers that consist largely of flavan-3-ol subunits and can incorporate anthocyanins. These tannins also contribute to wine astringency by interacting with salivary proteins. Oak tannins are referred to as hydrolysable tannins and consist largely of gallic acid subunits.

**Veraison** Refers to the onset of grape ripening where sugars begin to accumulate in the berry.

## Nomenclature

BSA Bovine serum albumin

EC Epicatechin

EG Epigallocatechin

ECG Epicatechin gallate

M3G Malvidin-3-glucoside

MCP Methyl cellulose polymer

mDp Mean degree of polymerisation

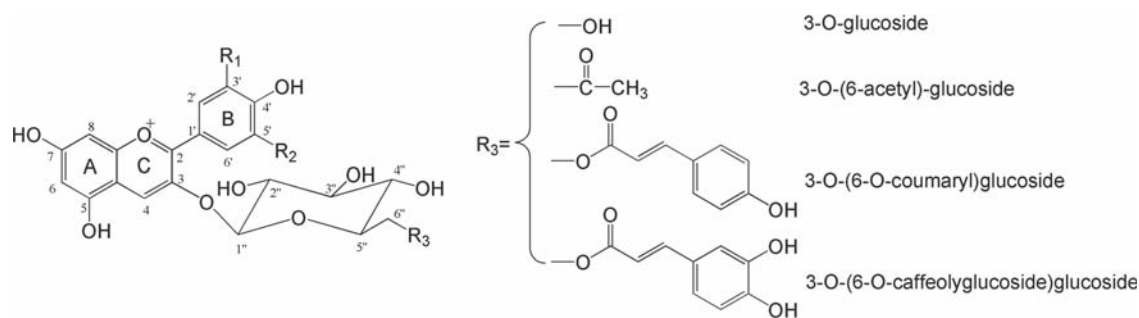
## Introduction

Red wine color is closely related to perceived quality and is influenced by many viticultural and winemaking factors. Wine is a chemically-dynamic system that is acidic (pH 2.8–3.8) and contains high concentrations of oxidizing compounds, particularly phenolics. These factors induce changes to grape and wine pigments from the moment the grapes are crushed to end-product in the glass. Key pigments are extracted from grapes during winemaking and undergo multiple reactions to form purple wine pigments. As wines age, further reactions change the color to a red-brown hue. Such chemical diversity and reactivity makes red wine a fascinating topic of study. This article aims to summarise some of the main influencing factors of red wine color as well as the methods used to measure wine color chemistry.

## Molecules and Mechanisms Responsible for Red Wine Color

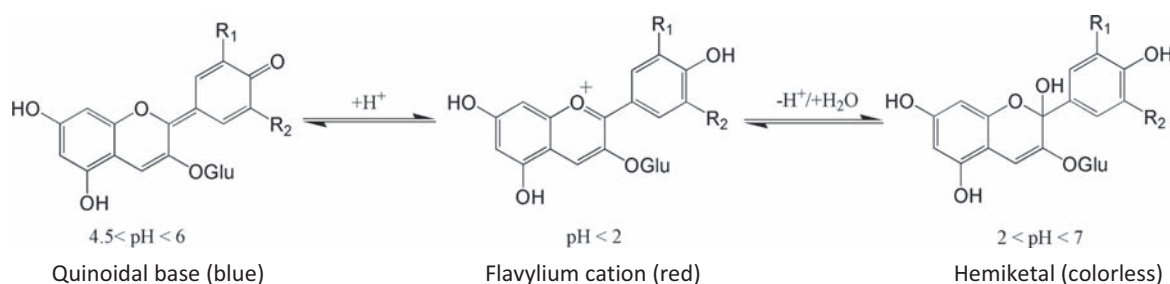
Red wine color is developed from interactions between tannins and anthocyanins extracted from grapes during winemaking. In the principal *Vitis vinifera* varieties used for red winemaking around the world, grape anthocyanins comprise monoglucoside derivatives of several structures with malvidin 3-*O*-glucoside being the most abundant. Other anthocyanins include delphinidin, cyanidin, petunidin, peonidin, and, to a lesser extent, pelargonidin (Fig. 1). Acylation of the glucose moiety at the C-6'' position is also common for grape-derived anthocyanins (Fig. 1), and include acetyl, coumaroyl and cafferoyl moieties (Monagas et al., 2005). The presence and composition of the acylated anthocyanins in grapes varies with variety, for example these types of anthocyanins are not present in Pinot Noir grapes (Cortell et al., 2007a).

The color of anthocyanins depends on the pH of the solution (Fig. 2). In acidic solutions (pH < 2), the anthocyanins exist as red flavylium cations and in higher pH solutions (pH 4.5–6) the blue quinoidal base dominates. Anthocyanins can also undergo hydration reactions which produce a colorless hemiketal form (Cheynier et al., 2006). At pH 3.5, such as in wine, anthocyanins are present in a mixture of colored and colorless forms, with the colorless forms dominating (Monagas et al., 2005). This indicates that while the concentration of free anthocyanins in young red wines might reach levels greater than 1 g/L (Bindon et al., 2014a; McRae et al., 2012), less than half of that concentration contributes to wine color (Monagas et al., 2005). Anthocyanins are



Anthocyanin	R <sub>1</sub>	R <sub>2</sub>
Pelargonidin 3-glucoside	H	H
Cyanidin 3-glucoside	OH	H
Delphinidin 3-glucoside	OH	OH
Peonidin 3-glucoside	OCH <sub>3</sub>	H
Petunidin 3-glucoside	OCH <sub>3</sub>	OH
Malvidin 3-glucoside	OCH <sub>3</sub>	OCH <sub>3</sub>

**Figure 1** Structures of grape anthocyanins. Monagas, M., Bartolome, B., Gomez-Cordoves, C., 2005. Updated knowledge about the presence of phenolic compounds in wine. *Crit. Rev. Food Sci. Nutr.* 45 (2), 85–118.

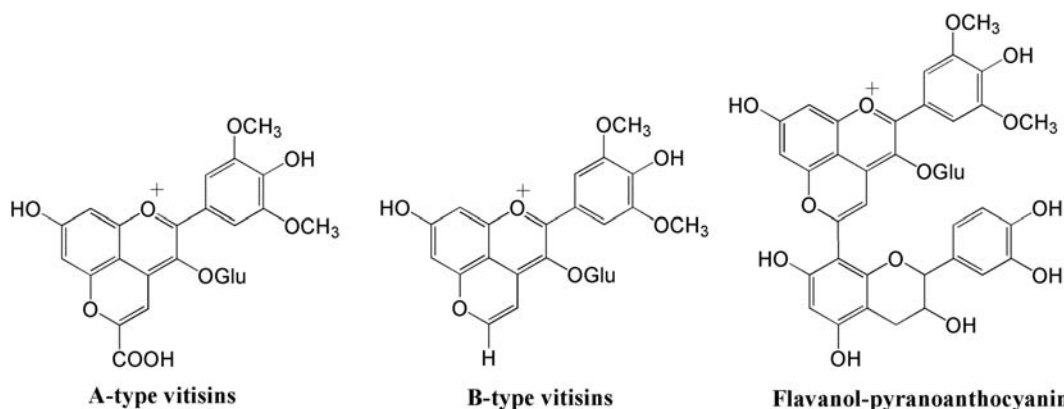


**Figure 2** Anthocyanin forms. Cheynier, V., Duenas-Paton, M., Salas, E., Maury, C., Souquet, J.M., Sarni-Manchado, P., Fulcrand, H., 2006. Structure and properties of wine pigments and tannins. *Am. J. Enol. Vitic.* 57 (3), 298–305.

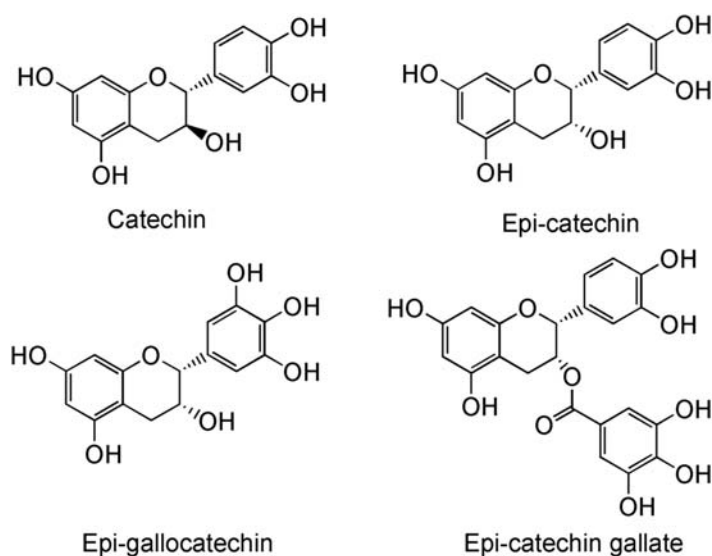
also considered unstable and their concentration in red wine reduces rapidly after winemaking. Substantial reductions in the concentrations of free anthocyanins have been noted within two years after winemaking (Bindon et al., 2014d; McRae et al., 2013). With aging, the concentration of anthocyanins decreases and that of pigmented polymers increases. It is therefore the presence of other compounds, particularly anthocyanin derivatives, that provide the depth of color so strongly associated with quality wine (Somers and Evans, 1974).

The decrease in anthocyanin concentration as wines age relates to the formation of comparatively stable wine pigments derived from anthocyanins such as pyranoanthocyanins and pigmented polymers. These reactions will change the overall wine color from purple to red-brown (Cheynier et al., 2006). Pyranoanthocyanins are formed from the addition of yeast metabolites, such as acetaldehyde and pyruvic acid, to the C-4 position of the anthocyanin aglycone and subsequent reactions lead to the formation of a fourth ring structure (Håkansson et al., 2003; Quaglieri et al., 2017). Pyranoanthocyanins include orange vitisins and blue portisins (Cheynier et al., 2006). Vitisins, including vitisin A and vitisin B (Fig. 3), are the most common group of pyranoanthocyanins in wine and yet concentrations reach only around 5 mg/L in non-Port red wines. This concentration further declines with wine aging (Quaglieri et al., 2017) as free pyranoanthocyanins react with non-colored flavan-3-ols in wine to produce pigmented polymers (Fig. 3).

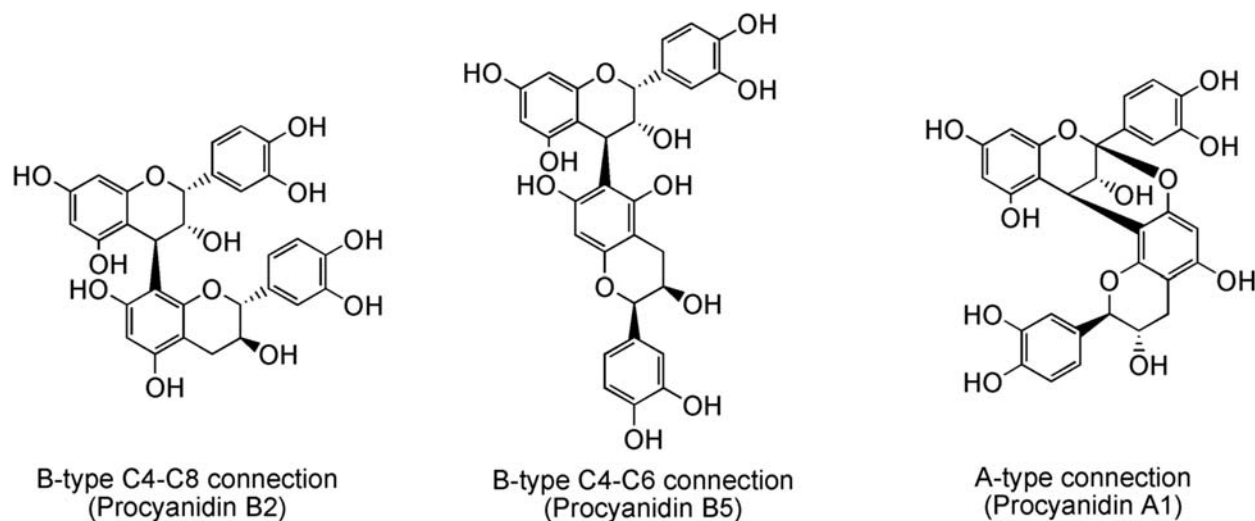
Flavan-3-ols are among the most reactive phenolics in red wine and readily form pigmented polymers via covalent interactions with anthocyanins (Garrido and Borges, 2013; Monagas et al., 2005; Somers, 1971). These pigmented polymers can persist in wines for decades and maintain the color density of red wines (McRae et al., 2012). The predominant flavan-3-ols in wine include catechin, epicatechin (EC), epicatechin gallate (ECG), and epigallocatechin (EG) (Fig. 4) which can be present as monomers, dimers, oligomers and as polymers (known as ‘tannins’) (Monagas et al., 2005). Flavan-3-ols generally form covalent bonds through the C-4 and C-8 positions, although linkages may also form between the C-4 and C-6 positions (Dallas et al., 1996; Wollmann and Hofmann, 2013). These are referred to as B-type linkages (Fig. 5) (Sun and Miller, 2003). Less commonly in grapes



**Figure 3** Examples of pyranoanthocyanin structures. Monagas, M., Bartolome, B., Gomez-Cordoves, C., 2005. Updated knowledge about the presence of phenolic compounds in wine. *Crit. Rev. Food Sci. Nutr.* 45 (2), 85–118.



**Figure 4** Grape flavan-3-ol structures. Monagas, M., Bartolome, B., Gomez-Cordoves, C., 2005. Updated knowledge about the presence of phenolic compounds in wine. *Crit. Rev. Food Sci. Nutr.* 45 (2), 85–118.



**Figure 5** Examples of A-type and B-type flavan-3-ol dimers. Monagas, M., Bartolome, B., Gomez-Cordoves, C., 2005. Updated knowledge about the presence of phenolic compounds in wine. *Crit. Rev. Food Sci. Nutr.* 45 (2), 85–118.

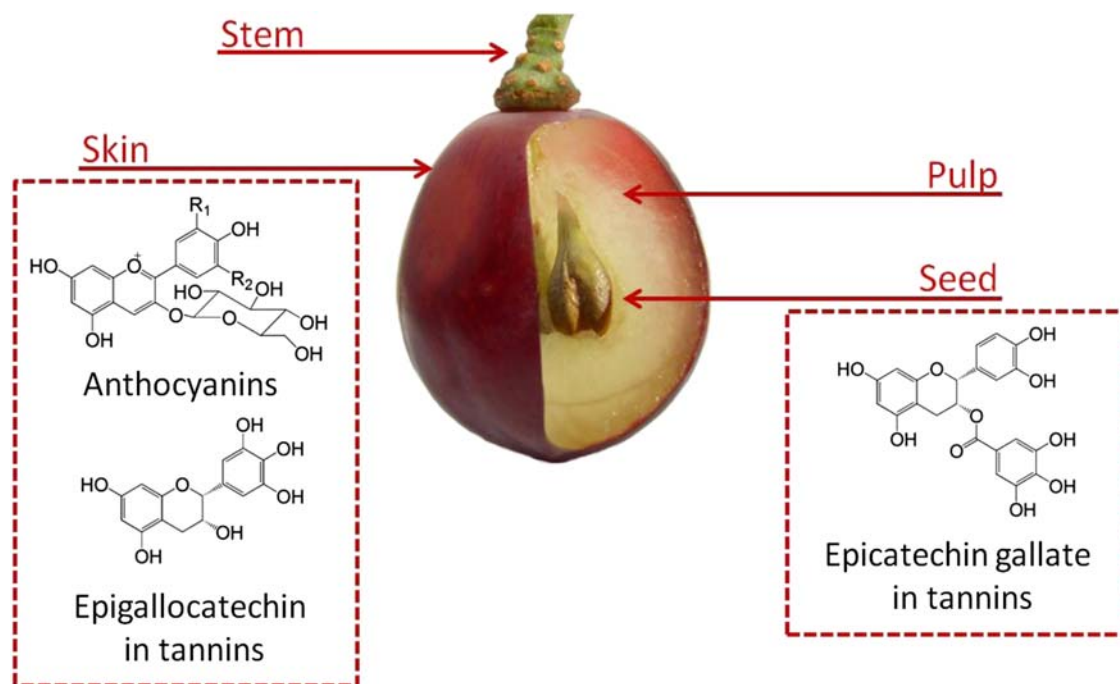
and wine, the C-4 and C-2 positions of one flavanol may interact with the C-6 and C-8 positions of the second flavanol forming A-type linkages (Fig. 5) (Aron and Kennedy, 2007).

Covalent interactions between monomeric flavan-3-ols and anthocyanins form flavanol-anthocyanin (F-A) adducts through either direct or indirect condensation reactions (Cheynier et al., 2006; Monagas et al., 2005). Direct condensation reactions involve the formation of quinones due to oxidation and subsequent polymerisation via B-type linkages producing a red F-A dimer. Indirect condensation reactions involve an ethyl-bridge linkage formed between monomers by interactions with acetaldehyde. This produces a purple F-ethyl-A dimer (Cheynier et al., 2006; Monagas et al., 2005). Both F-A and F-ethyl-A adducts further polymerise over time to produce longer pigmented polymers (Sun et al., 2008). Polymerisation reactions occur more readily between anthocyanins and flavan-3-ol oligomers (3–5 subunits) than with flavan-3-ol monomers, dimers or polymers (25 subunits) (Bindon et al., 2014b; Dallas et al., 1996). Anthocyanin self-association can occur via ethyl- or vinyl-linkages to produce highly colored dimeric or trimeric pigments. These pigments contribute to the color of aged wines and yet are not resistant to bisulfite bleaching (Bindon et al., 2014b; Kilmister et al., 2014). The formation of stable pigmented polymers therefore requires adequate concentrations of both tannins and anthocyanins and in the correct ratios (Bindon et al., 2014b; Kilmister et al., 2014).

Non-covalent interactions between anthocyanins and non-colored phenolic compounds may also influence red wine color via copigmentation. The planar surfaces of anthocyanins interact with the planar surfaces of phenolic moieties via  $\pi$ - $\pi$  interactions to form molecular stacks (Andersen and Jordheim, 2010; Cheynier et al., 2006). Such structures can form from intramolecular interactions between the anthocyanin aglycone and a phenolic acyl group or from intermolecular interactions between anthocyanins and flavan-3-ol oligomers (Escribano-Bailón and Santos-Buelga, 2012). Copigmentation potentially increases color intensity (hyperchromic effect) and purple color (bathochromic shift) (Cheynier et al., 2006; Heras-Roger et al., 2016) although anthocyanin self-association is likely to have a greater contribution to the color of young red wines (Herderich et al., 2006; Lambert et al., 2011).

### Viticultural Factors Influencing Red Wine Color

Many factors influence red wine color before the winemaking begins. The concentration and composition of color-related compounds in red wine depends on the composition of phenolic compounds in grapes and these are influenced by many viticultural factors. Anthocyanin concentrations begin to increase in grape skins after veraison (Pirie and Mullins, 1977) whereas flavan-3-ol monomers in both grape skins and seeds appear to increase prior to veraison and then decline significantly after veraison (Kennedy et al., 2000, 2002). This decrease in flavan-3-ol concentration with ripening does not necessarily relate to tannin formation as tannin concentrations can either increase, decrease or remain unchanged after veraison (Bindon et al., 2014c). Tannin extraction during winemaking depends on anthocyanin concentration (Bindon et al., 2014a; Kilmister et al., 2014) and therefore the concentrations of both species in grapes are linked to wine color.



**Figure 6** Grape structure with significant tannin subunits for skin and seed.

Anthocyanins are generally specific to grape skins while derivatives of flavan-3-ols are distributed throughout the grape including the seeds and pulp (Fig. 6). Tannins in grape skins are longer polymers with an average number of flavan-3-ol subunits (mean degrees of polymerisation, mDp) of around 32 subunits (Kennedy et al., 2001). Epicatechin (EC) subunits dominate grape tannin polymers. In grape skins, the tannins also contain around 20%–50% EG subunits and 5% ECG subunits (Kennedy et al., 2001). Grape seed tannins are much shorter than skin tannins, with mDp around 5 subunits, contain no EG subunits and a higher proportion of ECG subunits (around 24%) (Fournand et al., 2006; Kennedy et al., 2001, 2002).

The concentrations and compositions of grape anthocyanins and tannins in grapes and wines vary greatly and are influenced by genetic origin, grape ripeness, sunlight or UV exposure, plant water status and pathogens. Cabernet Sauvignon, for example contains significantly greater concentrations of tannin than Pinot Noir (Harbertson et al., 2008, 2002). Delaying harvest and increasing grape ripeness can also potentially increase wine color. This may be due to increases in extractable tannin and anthocyanin concentrations as the grape matures, which result in greater pigmented polymer formation, although ripening-related berry shrivel can also underpin this effect (Bindon et al., 2013; Schelezi et al., 2017). Increasing sunlight exposure of grapes, such as by removing leaves in the grapevine canopy, may lead to significantly higher concentrations of both anthocyanins and tannins in grapes (Edwards et al., 2015; Ristic et al., 2010) and shading grapes can reduce the color density of wine (Ristic et al., 2007). This is also related to variations in the concentration of pigmented polymers formed in the wines.

Grapes that receive less water are generally smaller than irrigated grapes which may result in an overall increase in grape skin phenolics, including anthocyanins and tannins, relative to berry volume (Ojeda et al., 2002). Early season water deficit may also enhance the biosynthesis of grape anthocyanins but does not directly impact tannin synthesis (Castellarin et al., 2007). Wine color can be increased by water deficit when both grape anthocyanin and tannin concentrations are enhanced, which in turn results in production of pigmented polymers (Bindon et al., 2014d; Kennedy et al., 2002). The application of elicitors, such as methyl jasmonate or benzothiadiazole, mimic pathogen attack and increase the synthesis of anthocyanins and skin tannins in grapes. This leads to increased grape skin tannin and color, resulting in greater color density of the wine (Ruiz-Garcia et al., 2012). Other viticultural factors can also influence anthocyanin and tannin concentrations in grapes and wine including temperature, nutrient status, crop load, canopy size, grapevine vigor, soil type and elevation (Bergqvist et al., 2001; Cohen and Kennedy, 2010; Cortell et al., 2005, 2007b; Downey et al., 2006; Kennedy et al., 2002).

## Winemaking Factors Influencing Red Wine Color

Reactions of color-related grape compounds begin once grapes are harvested and continue until the wine is consumed. Fermentation of grapes to wine induces the greatest changes in chemical composition. The key factors influencing red wine color during fermentation include oxygen exposure, extent of grape skin contact, variety of yeast for primary fermentation and fermentation additions. Wine pH and oxygen ingress through barrels or bottle closures will also influence color properties during wine aging.

### Oxygen Exposure

Reactions between phenols and oxygen begins when grapes are crushed. Polyphenol oxidase (PPO) enzymes from grapes catalyze the oxidation of caftaric (caffeoyltartaric) acid and cutaric (*p*-coumaroyl-tartaric) acid to highly reactive *o*-quinones. The formation of quinones triggers either oxidation–reduction reactions with anthocyanins or condensation reactions. Anthocyanins with hydroxyl groups on the B-ring, such as cyanidin-, delphinidin-, and petunidin-3-glycosides, undergo oxidation–reduction reactions via coupled oxidation with *o*-quinones of caftaric and cutaric acid to form anthocyanin quinones. Condensation reactions involve nucleophilic attack by *o*-quinones of the C-8 or C-6 position of anthocyanins, particularly malvidin- and peonidin-3-glucoside when in the hemiketal form. This produces colorless adducts that then equilibrate to flavylum forms (Monagas et al., 2005).

Chemical oxidation reactions also occur due to the presence of phenolate ions (Monagas et al., 2005). Such reactions may be catalyzed by iron or copper activation of dissolved oxygen (Quaglieri et al., 2017) and forms products similar to those of enzymatic oxidation (Monagas et al., 2005). The presence of other chemicals as a consequence of oxidation, such as acetaldehyde from ethanol, also promote condensation reactions and the formation of pigmented polymers (Drinkine et al., 2007) and pyranoanthocyanins (Quaglieri et al., 2017).

Oxygen is introduced to wine throughout its lifecycle from grape crushing to bottle aging. Most oxygen is introduced during fermentation due to the practice of pumping wine to break up the grape material, particularly grape skins, that collect at the top of the ferment. Greater oxygen exposure during fermentation can lead to lower concentrations of tannins but an increase in the proportion of more stable pigments (McRae et al., 2015). Oxygen can also be added post-fermentation using micro-oxygenation (MOX) techniques (Schmidtke et al., 2011). Introducing small amounts of oxygen, such as 45 mg/L over three months, can increase the production of stable pigmented polymers and pyranoanthocyanins (Kontoudakis et al., 2011; Quaglieri et al., 2017). The efficacy of MOX is greater in wines of lower pH (Kontoudakis et al., 2011). Further oxygen exposure occurs through barrels during barrel-aging (Quaglieri et al., 2017) and from slow oxygen ingress through bottle closures during long-term storage (Gambuti et al., 2013; Han et al., 2015; Ugliano, 2013). The effect of the long-term oxygen exposure is also greater at lower wine pH (McRae et al., 2013).



## Maceration

The time during which wines are fermented in the presence of grape material, including skins, pulp and seeds, is known as maceration. This process is crucial for the extraction of color-related pigments from grapes. Anthocyanins and grape skin tannins are extracted earlier in the fermentation process due to greater permeability of grape skins (Smith et al., 2015). Seed tannins are extracted later in the ferment as grape seeds are less permeable and require greater hydration for tannin extraction (Casassa et al., 2013; Hernandez-Jimenez et al., 2012; Peyrot des Gachons and Kennedy, 2003). Extending maceration may result in greater color density and higher concentrations of phenolic compounds including grape seed tannins (Aron and Kennedy, 2007; Gomez-Plaza et al., 2001). Addition of enzymes during maceration can lead to higher concentrations of tannins in wine due to the hydrolysis of cell wall pectins which limits the back-binding of extracted tannin, but does not necessarily lead to greater anthocyanin extraction or the improvement of wine color (Bautista-Ortín et al., 2013; Zietsman et al., 2015). More effective maceration techniques for wine color enhancement include thermal treatments such as elevated fermentation temperature, thermovinification, and microwave (Carew et al., 2013b; Sacchi et al., 2005). Thermal treatment can increase the extraction of anthocyanins, tannins and potentially other phenolics, leading to increased polymeric pigment formation and enhanced wine color.

## Yeast Selection

*Saccharomyces cerevisiae* yeast is used to ferment grapes to wine and the selected strain can influence red wine color properties. *S. cerevisiae* AWRI 1631 and RC212 strains can increase tannin extraction from grape skins (Carew et al., 2013a; Holt et al., 2013). *Saccharomyces bayanus* AWRI 1176 and 1375 consistently produced wines with lower tannin concentrations and wine color density (Carew et al., 2013a; Hayasaka et al., 2007; Holt et al., 2013). The higher production of acetaldehyde in these strains can also potentially influence the color development of red wines. Yeast cells can also adsorb anthocyanins, particularly acylated derivatives. The adsorbed anthocyanins settle with yeast at the end of fermentation, reducing their concentration in wine (Morata et al., 2003).

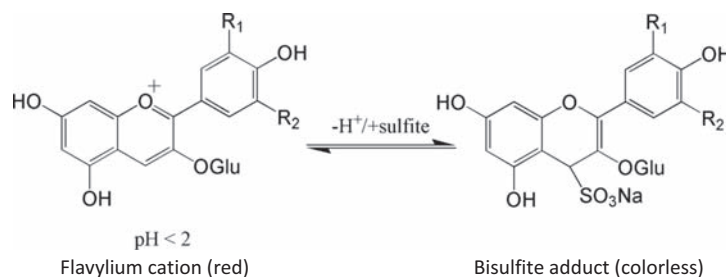
## Fermentation Additions

Many additions are made to wine during fermentation to influence the efficacy of the process as well as the properties of the wine. Some of these additives are known to influence the color parameters of red wines. The most critical of these color-influencing additives is potassium- or sodium-metabisulfite, which is added at the end of fermentation to prevent wine spoilage. Reactions between free anthocyanins and sulphites can reduce the charge state of the anthocyanin forming colorless bisulfite adducts (Fig. 7). Pyranoanthocyanins and pigmented polymers are resistant to this bleaching (Cheynier et al., 2006).

Fining agents, such as with animal-based proteins including casein and gelatin, are sometimes added to wine to remove tannins and lower wine astringency (Maury et al., 2001). This can also potentially reduce anthocyanin concentration and color density (Ghanem et al., 2017; Guerrero et al., 2013).

The addition of copigments prior to fermentation can potentially improve the color properties of wine. Caffeic acid addition, for example, can increase red color produced in wine (Darias-Martín et al., 2002). The addition of enotannins from oak or grape seed can also potentially stabilise color although the efficacy of enotannin addition in improving wine color for many wines may be limited (Li et al., 2017; Versari et al., 2013).

Phenolics extraction from grapes during winemaking is also increased by prefermentation juice runoff or saignée. Removing a portion of juice prior to fermentation increases the ratio of grape solids to juice and thereby improves the efficacy of phenolics extraction. In some cases this can increase the concentration of anthocyanins and tannins in wine (Harbertson et al., 2009; Sacchi et al., 2005) although efficacy of the treatment can vary with vintage and source of fruit (Gawel et al., 2001). The many different winemaking techniques available to winemakers can significantly impact the concentrations of anthocyanins, tannins and copigments in red wines, even in those wines produced from the same package of grapes.



**Figure 7** Bisulfite bleaching of anthocyanins. Cheynier, V., Duenas-Paton, M., Salas, E., Maury, C., Souquet, J.M., Sarni-Manchado, P., Fulcrand, H., 2006. Structure and properties of wine pigments and tannins. *Am. J. Enol. Vitic.* 57 (3), 298–305.



## Analytical Methods for Measuring Red Wine Color

Understanding wine color involves analysis of the diverse range of pigments and copigments in wines as well as the total color metrics. Measuring red wine color therefore uses many different methods to obtain the required information. The color properties of red wines are measured using spectroscopic techniques to give overall wine color properties and concentration estimates for free anthocyanins and pigmented polymers. The concentration and composition of free anthocyanins can be more accurately measured using high performance liquid chromatography (HPLC) analysis with UV detection at 520 nm (Mercurio et al., 2007). Determining the structures of pigmented polymers is best achieved after isolation and depolymerisation reactions combined with HPLC analysis or LC-mass spectrometry (LC-MS) techniques.

Spectroscopic techniques use different wavelengths, wine additions or polymer precipitation to gain information about the overall wine color as well as the type of pigments present. Several techniques are commonly used and each vary in the information provided. CEILAB parameters measure the lightness ( $L^*$ ), angular hue ( $H^*$ ), red-green contribution ( $a^*$ ), yellow-blue ( $b^*$ ), Chroma ( $C^*$ ), and saturation ( $s^*$ ) (Esparza et al., 2009; OIV, 2014). Glories parameters measure absorbance at 420, 520 and 620 nm color intensity (CI), hue (H), brightness (B), and the percent contributions of yellow, red and blue (Glories, 1984). Somers color parameters measure absorbance at 280, 420 and 520 nm before and after treatment with either excess metabisulfite to induce excess  $SO_2$  production, acetaldehyde, or hydrochloric acid. These measurements provide estimations of anthocyanin concentrations, pigmented polymers, hue and wine color density (Mercurio et al., 2007).

Precipitation assays involve interactions between wine pigmented polymers and an added binding polymer such as methyl cellulose polymer (MCP) (Mercurio et al., 2007) or bovine serum albumin (BSA) (Harbertson et al., 2015, 2003). The change in measured absorbance before and after BSA addition and with and without the addition of metabisulfite can also be used to measure pigmented polymers separately from smaller pigments (Harbertson et al., 2003). Changes in the absorbance at 280 nm before and after MCP addition can be used to measure tannin concentration including pigmented polymers. Absorbance is compared to a standard curve of either EC at 280 nm to give tannin concentration as mg/L EC equivalents or malvidin 3-O-glucoside (M3G) at 520 nm to give pigmented polymer concentration as mg/L M3G equivalents.

Anthocyanin monomers are separated and measured using HPLC, monitoring absorbance at 520 nm and/or by MS. Separation is best achieved with a reversed phase C-18 column with a gradient solvent system from aqueous to acidified acetonitrile (Mercurio et al., 2007). Isolating larger quantities of anthocyanins is useful for accurate characterization of structures. This is effectively achieved with multilayer countercurrent chromatography (MLCCC) using quaternary solvent systems such as acetonitrile, water, butanol and butyl methyl ether mixtures (Kneknopoulous et al., 2011). Pigmented polymers and tannins are more effectively isolated from red wines using solid-phase extraction media including Toyopearl (McRae et al., 2010), Sephadex LH-20 (Bindon et al., 2010) or Oasis HLB cartridges (Waters, Australia) (Jeffery et al., 2008).

Compositional analysis of isolated tannins and pigmented polymers is achieved with depolymerisation techniques that enable the determination of the mDp and subunit identification (Cheynier et al., 1997; Kennedy and Jones, 2001). Interflavan bonds are cleaved at elevated temperatures and in acidic solutions to release free monomeric subunits. The addition of nucleophiles will then stabilise the released monomers against further polymerisation. Nucleophiles include toluene-thiol or benzyl-thiol for thiolytic reactions (Cheynier et al., 1997; Poncet-Legrand et al., 2010; Prieur et al., 1994) or phloroglucinol for phloroglucinolysis reactions (Kennedy and Jones, 2001). Polymer extension units and terminal units can be differentiated by the ratio of monomers with and without a nucleophile adduct, respectively, giving the average number of subunits per polymer (mDp). Subunit composition is then achieved with HPLC separation and analysis (Koerner et al., 2009). This method can provide information about the composition of flavan-3-ol subunits in the pigmented polymer, however anthocyanins cannot be detected (McRae et al., 2013) and bonds created as a result of tannin oxidation cannot be cleaved (Poncet-Legrand et al., 2010). Thus the amount of tannin converted in the reaction (mass conversion or % yield) must also be considered for accurate interpretation of results (Poncet-Legrand et al., 2010).

## Conclusions

Research into wine pigments and factors influencing wine color is ongoing. This article highlights some of the key research outcomes in this fascinating area of study and there are likely to be more developments emerging in coming years. These will continue to improve our understanding of wine color and enable the effective production of quality red wines.

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# Fermentation of Grains

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## Introduction

Fermentation, by far, is the oldest, most valued and economical food processing and preservation method practised by mankind dated back to old civilizations of more than 4000 years (Steinkraus, 2004). Thus, fermented foods and beverages are amongst the first types of processed foods produced and consumed by human beings worldwide (El Sheikh and Montet, 2016). Diverse types of fermented foods and beverages are available due to differences in fermentation substrates and microorganisms, fermentation conditions, environmental conditions, ethnic and social preferences, religion, and cultural practices (Tamang, 2010). Based on the substrates used, fermented foods can be classified as grains, vegetables, fruits, meat, and milk-based fermented foods. Fermented grain is the major type of fermented foods and is not only considered as staple food in developing countries but it also considered as healthy food in the developed world. Fermentation of grains is done through both traditional and modern techniques leading to the production of numerous fermented grain products that are distributed, traded and consumed by humans worldwide. Traditionally, the main goal of grains fermentation was to preserve them for a long time and prevent deterioration by spoilage microorganism. However, recently the fermentation of grains had expanded beyond preservation to the enhancement of nutritional, sensorial, and health-promoting potential of grains. This monograph will give an overview of the fermentation of grains, the major fermented grain products, the types of microorganisms involved in the fermentation of grains, and the impact of fermentation on quality of the final products.

## Fermentation of Grains

Grains used for food could be classified as cereals (such as wheat, rice, maize, sorghum, millet, barely, rye, oat, teff, and triticale), pseudocereals (such as amaranth, buckwheat, and quinoa), legumes (such as coffee beans, cocoa beans, soybeans, broad beans, lima beans, kidney beans, mung beans, chickpea, pigeon pea, grass pea, field pea, cowpea, green gram, black gram, lupines, and lentil) and oil seeds (peanuts, canola, cottonseeds, sunflower, safflower, linseeds, rapeseeds, sesame seeds, and mustard) (Graybosch, 2016). In addition to protein and energy, whole grains are rich source of bioactive compounds such as phenolics, flavonoids, sterols, dietary fibre, essential fatty and amino acids, vitamins, macro- and micro-minerals,  $\beta$ -glucans, carotenoids, probiotics, and prebiotics and they are thus considered as important functional foods (Bultosa, 2016). However, whole food grains may contain several antinutritional factors such as phytic acids, tannins, non-starch polysaccharides, cyanogenic glycosides, saponins, and protease and amylase inhibitors that strongly reduce the bioavailability, digestibility, and metabolisms of essential nutrients in these grains (Madsen and Brinch-Pedersen, 2016). Several processes such as fermentation, dehulling, milling, soaking, germination, cooking, and irradiation had been used to overcome such limitations of food grains. Fermentation of grains is exceptionally the most effective method for the reduction of antinutritional factors and toxic compounds of these products (Wood, 2016). In addition, this process is also believed to improve grains storability, safety, digestibility, palatability, availability of essential minerals, enrichment of the fermented products with vitamins and bioactive compounds, as well as improving the health-enhancing potential of grain products (Nout et al., 2007; Wood, 2016). Fermentation increases the diversity of food products and consequently, huge numbers of fermented grains products are now produced and consumed worldwide. Many types of fermentations (alkaline, acetic acid, lactic acid, and alcoholic or a combination thereof) and methods (natural, backslopping, starter cultures, solid state, and submerged fermentations) are used in the production of fermented grain products. In addition, diverse groups of bacteria, yeasts, and moulds are used for fermentation and they are responsible for the production and diversity of the fermented grain products. Fermented grain foods are classified based on the grains classification itself as; cereal and pseudocereals, legumes and oilseeds, and cereal-legumes fermented foods. Some fermented grain foods and beverages are presented in Table 1, and the major types of these products are discussed in the following sections.

## Fermentation of Cereals and Pseudocereal Grains

Cereals are extensively grown world-wide where they considered as important part of diet by peoples all over the world. Although they are not popular like cereals, pseudocereals received great attention in recent years as raw materials for preparation of gluten-free foods. Cereals and pseudocereals contain a high starch proportion and they thus often milled to flours and used for the production of bread and bakery products (Fletcher, 2016). Prior to consumption, a large volume of cereals and pseudocereals grains are processed through fermentation and consequently diverse numbers of fermented foods and beverages are produced from these grains. The fermentation of cereals has a long history of more than 7000 years when Chinese prepared alcoholic beverages from Chu, a mould fermented rice or wheat products, and around 3500 years latter bread and beer making was also developed by Egyptians (Steinkraus, 2004). Since then, thousands of cereal- and pseudocereal-based fermented foods and beverages have

**Table 1** Cereal, legumes, and cereal-legumes based fermented foods and beverages (Adebo et al., 2017; Blandino et al., 2003; Coda et al., 2017; Kohajdová, 2015; Nagai and Tamang, 2010; Nout, 2009; Nout et al., 2007; Tamang, 2010; Taylor, 2016; Vogelman et al., 2009)

Grains group and names	Fermented foods (regions)	Nature of uses	Functional microorganisms in the fermentation
<b>Cereals and pseudocereals</b>			
Wheat ( <i>Triticum aestivum</i> & <i>T. turgidum</i> subsp. <i>Durum</i> )	Babroo (India)	Fried discs used in festivals	Unknown
	Bahtura (India)	Bread	LAB, Yeasts
	Bhatooru (India)	Baked bread, staple foods	LAB
	Bouza (Egypt)	Thick and acidic alcoholic beverage	Unknown
	Boza (Albania, Turkey)	Thick, sweet and slightly sour alcoholic beverage	LAB
	Chilra (India)	Thin, crisp pancake	LAB, Yeasts
	Craft beer (Italy)	Un-filtered beer	Yeasts
	Gulgule (India)	Deep fried sweets used in festivals	Unknown
	Jalebies (India)	Pretzel-like syrup confection	Yeasts, LAB
	Kishk (Egypt)	Dried balls dispersed in water for beverage preparation	LAB, Yeasts
	Kurdi (India)	Solid, fried crisp, salty noodles	Unknown
	Mantou (China)	Steamed cake	Yeasts
	Marchu (India)	Bread	Unknown
	Minchin (China)	Condiment	Moulds
	Nan (India, Pakistan)	Leavened bread	LAB, Yeasts
	Seera (India, Pakistan)	Sweet dish	
	Shamsy bread (Egypt)	Spongy bread	Yeasts
	Siddu (India)	Steamed oval-shaped bread	Unknown
	Sourdough (Worldwide)	Mildly acidic leavened bread	LAB, Yeasts
	Taotjo (East Indies)	Condiment	Yeasts
	Takju (Korea)	Alcoholic turbid drink	LAB, Yeasts
	Tarhana (Turkey)	Dried powder seasoning for soups	LAB
	Aliha (Ghana, Togo, Benin)	Mild acidic non-alcoholic drink	LAB
	Aceeda (Sudan)	Porridge	LAB
	Bogobe (Botswana)	Soft porridge	Unknown
	Burukutu (Nigeria, benin, Ghana)	Creamy liquid alcoholic drink	LAB, Yeasts
	Hulu-mur (Sudan)	Sour and sweet taste non-alcoholic drink	LAB
	Hussuwa (Sudan)	Cooked dough	LAB, Yeasts
	Jiu (China)	Alcoholic beverage	Fungi, Bacteria, Yeasts
	Kisra (Sudan)	Thin pancake bread	LAB, Yeasts
	Merissa (Sudan)	Alcoholic drink	Yeasts
	Nasha (Sudan)	Thick non-alcoholic drink	LAB, Yeasts
	Ogi (Nigeria, West Africa)	Mildly acidic porridge	LAB, Yeasts
	Otika (Nigeria)	Alcoholic beverage	Unknown
	Pito (Nigeria, Ghana)	Dark brown alcoholic beverage	LAB, Yeasts
	Sorghum beer (South Africa)	Acidic and weakly alcoholic drink	LAB, Yeasts
	Talla (Ethiopia)	Alcoholic beverage	Unknown
	Ting (South Africa)	Sour porridge	LAB
	Uji (Kenya, Uganda, Tanzania)	Acid and sour porridge	LAB
Sorghum ( <i>Sorghum bicolor</i> )			



Maize ( <i>Zea mays</i> L.)	Atole (Mexico)	Porridge	LAB
	Banku (Ghana)	Solid maize dough	LAB, Yeasts
	Busaa (Nigeria and Ghana)	Alcoholic beverage	LAB, Yeasts, fungi
	Chicha (Peru)	Spongy solid	Yeast, Bacteria, Fungi
	Chikokivana (Zimbabwe)	Alcoholic beverage	Yeasts
	Ilambazi lokubilisa (Zimbabwe)	Porridge	LAB, Yeasts, Moulds
	Jamin-bang (Brazil)	Cake-like bread	Yeast, bacteria
	Kaanga-kopuwai (New Zealand)	Soft slimy bread	Yeast, bacteria
	Kachasu (Zimbabwe)	Alcoholic beverage	Yeasts
	Kaffir beer (South Africa)	Alcoholic beverage	LAB, Yeasts
	Kenkey (Ghana)	Acidic, solid and steamed dumpling	LAB, Yeasts
	Koko (Ghana)	Porridge	LAB, Acetic acid bacteria, Yeasts
	Maheu (South Africa)	Sour non-alcoholic beverage	LAB
	Mahewu (South Africa)	Sour non-alcoholic beverage	LAB
	Mawe (Benin, Togo)	Sour non-alcoholic beverages and porridges	LAB, Yeasts
	Munkoyo (Africa)	Liquid drink	Unknown
	Mutwiwa (Zimbabwe)	Porridge	LAB, Yeasts, Moulds
	Ogi (Nigeria)	Mildly acidic porridge	LAB, Yeasts, Fungi
	Poto poto (Congo)	Slurry for gruel making	LAB, Yeasts
	Pozol (Mexico)	Mildly acidic porridge	Yeast, Bacteria, moulds
	Rabdi (India)	Semisolid mash	LAB, Fungi, Bacilli
	Seketeh (Nigeria)	Alcoholic beverage	LAB, Yeasts, Fungi
	Tesguino (Mexico)	Alcoholic beverage	Bacteria, Yeasts, Moulds
	Tobwa (Zimbabwe)	Non-alcoholic beverage	LAB
	Uji (Kenya, Uganda, Tanzania)	Acid and sour porridge	LAB
	Ambali (India)	Acidic pancake	LAB
	Bagni (Caucasus)	Liquid drink	Unknown
	Ben-Saalga (Burkina Faso)	Gruel, weaning food	LAB, Yeasts
	Braga (Romania)	Liquid drink	Unknown
	Busa (Syria, Egypt, Turkistan)	Liquid drink	LAB, Yeasts
	Dalaki (Nigeria)	Thick porridge	Unknown
	Darassum (Mangolia)	Liquid drink	Unknown
	Doro (Zimbabwe)	Thick alcoholic drink	Bacteria, Yeast
	Jaanr (India)	Alcoholic paste	Fungi
	Jnard (India, Nepal)	Alcoholic beverage (opaque beer)	Fungi, LAB, Yeasts
	Kwunu-Zaki (Nigeria)	Paste used for breakfast	LAB, Yeasts
	Mangisi (Zimbabwe)	Sweet and sour non-alcoholic beverage	Unknown
	Sura (India)	fermented drink	Unknown
	Tumba (India)	Liquid drink	<i>Endomycopsis fibuligera</i>
Millet ( <i>Pennisetum americanum</i> & <i>Eleusine coracana</i> )			

(Continued)

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Grains group and names	Fermented foods (regions)	Nature of uses	Functional microorganisms in the fermentation
Rice ( <i>Oryza sativa</i> )	Anarshe (India)	Sweetened snack foods	LAB
	Ang-kak (China)	Colorant of dry red powder	Moulds
	Aska (India)	Fermented flour	Unknown
	Bhattejnaar (India)	Sweet-sour alcoholic paste	Yeast, Moulds
	Brem (Indonesia)	Cake	Moulds
	Brembali (Indonesia)	Dark brown alcoholic beer	Yeast, Moulds
	Chongju (Korea)	Clear alcoholic wine	Yeast
	Khanomjeen (Thailand)	Noodle	LAB
	Khaomak (Thailand)	Semisolid, sweet alcoholic beverage	Yeasts, Moulds
	Kichudok (Korea)	Steamed cake	Yeasts
	Lao-Chao (China)	Soft, juicy paste	Yeasts, Moulds
	Me (Vietnam)	Acidic sour condiment	LAB
	Mifen (China)	Fermented noodles for breakfast	LAB
	Mirin (Japan)	Clear liquid seasoning	Moulds
	Puto (Philippines)	Steamed cake	LAB, Yeasts
	Sake (Japan)	Clear alcoholic wine	Yeast
	Shaosinghjiu (China)	Clear alcoholic wine	Yeast
	Sierra rice (Ecuador)	Brown yellow dry rice	Yeasts, Moulds, and Bacilli
	Takju (Korea)	Turbid alcoholic beer	LAB, Yeast
	Tapai pulut (Malaysia)	Semisolid, sweet alcoholic beverage	Moulds, Yeasts
	Tape ketan (Indonesia)	Sweet-sour alcoholic paste	Moulds, Yeasts
	Tapuy (Philippines)	Sour-sweet paste and liquid	LAB, Moulds, Yeasts
	Torani (India)	Seasoning liquid	Yeasts
Barley ( <i>Hordeum vulgare</i> )	Beer (Worldwide)	Alcoholic drink	Bacteria, Yeasts, Fungi
	Chhang/Lugri (India)	Beverage	Unknown
	Pinni/Bagpinni (India)	Solid uncooked dough	Unknown
	Rabadi (India)	Fermented barley dough	LAB, Moulds, <i>Bacillus</i> spp.
	Sourdough (Worldwide)	Leavened dough for bread making	Yeast, LAB
	Thuktal (India)	Steamed food	Unknown
Oat ( <i>Avena sativa</i> )	Beer (Germany)	A berry flavored beer	Yeasts, LAB
	Baijiu (China)	Alcoholic drink	Yeasts, LAB
	Functional beverage (Bulgaria)	Probiotic drink	LAB
	Yosa	Snack	LAB and Bifidobacteria
Rye ( <i>Secale cereale</i> )	Bread (Denmark)	Sandwich bread	LAB
	Kvass (Lithuania)	Non-alcoholic or mild alcoholic beverage	Yeasts, LAB
	Perkarnaya (Russia)	Acidic and aerated bread	Yeasts, LAB
	Pumpernickel (Switzerland and Germany)	Acidic and aerated bread	Yeasts, LAB
	San Francisco bread (USA)	Mildly acidic leavened bread	Yeasts, LAB
	Sourdough (America, Europe, Australia)	Mildly acidic leavened bread	Yeasts, LAB

Teff ( <i>Eragrostis tef</i> )	Bread (Africa) Injera (Ethiopia) katikalla (Ethiopia) Sourdough (Europe) Tela (Ethiopia)	Sourdough and non-sourdough bread Acidic sour, leavened pancake-like flat bread Alcoholic beverages Dough for making bread Alcoholic beverages	Yeasts, LAB LAB, Yeast Unknown LAB, Yeasts Unknown
Buckwheat ( <i>Fagopyrum esculentum</i> )	Aktori (India) Beer (Slovenia) Bread and sourdough (Europe) Mangjangkori (India)	A cake or pancake –like fermented food Gluten free fermented beverage Leavened bread from fermented dough Thick, brown color pancake-like food	Unknown Yeasts LAB, Yeasts Unknown
Amaranth ( <i>Amaranthus caudatus</i> )	Chicha (Peru) Ogi (Africa) Sourdough and bread (Germany)	Traditional beer Porridge Gluten free sourdough bread	LAB, Yeast, Moulds LAB, Yeast, Moulds LAB and Yeasts
Quinoa ( <i>Chenopodium quinoa</i> )	Beer (Europe) Chichi (Latin America) Sourdough and bread (Europe, Latin America)	Gluten free sourdough bread Traditional drink of the Andes Gluten free sourdough bread	Yeast Unknown Yeasts, LAB
<b>Legumes and oil seeds</b>			
Soybean ( <i>Glycine max</i> )	Aakhone (India) Bekang (India) Chee-fan (China) Chiang (China) Chinese yeast Chungkukjang (Korea) Dauchi (China, Taiwan) Doenjang (Korea) Furu or Sufu (China) Hawaijar (India) Ketjap (Indonesia) Kinema (India, Nepal) Meitauza (china) Meju (Korea) Natto (Japan) Pepok (Myanmar) Perayaan (India) Sieng (Cambodia, Laos) Shoyu (Japan) Tempe (Indonesia) Tofu si (China, Japan) Tua nao (Thailand) Tungrymbai (India)	Alkaline and sticky paste used as side dish Alkaline and sticky paste used as side dish Cheese-like food Alkaline paste used as soup Solid and eaten as fresh Alkaline and sticky used as condiment and soup Alkaline paste used as condiment and soup Alkaline paste used as soup Mildly acidic and used as savory Alkaline and sticky paste used as side dish Seasoning syrup Sticky alkaline curry soup Liquid drink Sticky and alkaline paste used as seasoning agent Alkaline and sticky, ammonia odor, side dish Alkaline and sticky, side dish Alkaline and sticky used as side dish Alkaline and sticky used as side dish Alkaline, liquid used as seasoning Alkaline and fried cake Alkaline, liquid used as seasoning Alkaline and dry paste used for soup and curry Alkaline and sticky paste used for soup Cooked fermented beans with ghee and yogurt Flat, glossy, and brown colour product Salty paste Sourdough	<i>Bacillus</i> spp. <i>Bacillus</i> spp. Moulds Moulds Moulds, Yeasts <i>Bacillus</i> spp. <i>Bacillus</i> spp., Moulds Moulds Moulds <i>Bacillus</i> spp. Moulds LAB, <i>Bacillus</i> spp. Mould Mould <i>Bacillus natto</i> <i>Bacillus</i> spp. <i>Bacillus</i> spp. <i>Bacillus</i> spp. Moulds Moulds <i>Bacillus</i> spp. <i>Bacillus</i> spp. <i>Bacillus</i> spp. Unknown Bacilli, LAB Unknown LAB, Yeast Moulds Mould
Common beans ( <i>Phaseolus vulgaris</i> )	Madras (India) Ugba (Nigeria)		
Faba beans ( <i>Vicia faba</i> )	Doubanjiang (China) Sourdough (Italy)		
Peanuts ( <i>Arachis hypogaea</i> )	Oncom (Indonesia) Dage (Indonesia)	Deep fried or roasted cake Tempe-like product	

(Continued)

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Grains group and names	Fermented foods (regions)	Nature of uses	Functional microorganisms in the fermentation
Cotton seeds ( <i>Gossypium hirsutum</i> )	Beverage (Brazil)	Non-alcoholic beverage	LAB, Bacilli
Black gram ( <i>Vigna mungo</i> )	Owoh (Nigeria), Amriti (India)	Condiments, soup	Bacilli, LAB
		Snacks	Unknown
	Bedvin roti (India)	Snacks used for breakfast	Unknown
	Bhallae (India)	Fried patties snack	LAB, Bacilli, Yeast
	Borhe (India)	Fried disc having hole in the centre	Unknown
	Dosa (India)	Snacks used for breakfast	LAB, Bacilli, Yeast
	Maseura (India)	Brittle and friable ball or cone-shaped mixture	Bacilli, LAB
	Mashbari (India)	Staple food	LAB, Bacilli, Yeast
	Masyaura (India)	Side dish	LAB, Moulds, Yeast
	Papadam (India)	Breakfast or snack food	Yeast
	Sepubari (India)	Marriage festival special dish	LAB, Bacilli, Yeast
	Teliye mah (India)	Semi-solid foods	Unknown
	Vadai (India)	Paste used as side dish	LAB
	Wadi (India)	Condiment	LAB
	Wari (India)	Dried, hollow, brittle, spicy, and friable balls, snack, side dish	LAB, Bacilli, Yeast
Locust bean ( <i>Ceratonia siliqua</i> )	Dawadawa (Africa)	Alkaline, sticky Condiment, soup	Bacilli
	Iru (Nigeria)	Alkaline, sticky Condiment, soup	Bacilli
	Kinda (Sierra Leone)	Alkaline, sticky Condiment, soup	Bacilli
	Soumbala (Borkina Faso)	Alkaline, sticky Condiment, soup	Bacilli
	Uri (West Africa)	Alkaline, sticky Condiment, soup	Bacilli
Chickpea/Bengal gram ( <i>Cicer arietinum</i> )	Dhokla (India)	Steamed, soft cake, snack	LAB, Bacilli, Yeast
	Gergoush (Sudan)	Snack used with morning tea	Unknown
	Khaman (India)	Snack	LAB, Bacilli
	Papad (India)	Thin, usually circular, wafer-like product	Yeast
African yam beans ( <i>Sphenostylis stenocarpa</i> )	Oturu	Greyish brown, a strong ammonia-like odour product	LAB, Bacilli, Yeast
Cereals-legumes based	Hamanatto (Japan)	Soft flavoring agent	Moulds
	Idli (India),	Steamed, soft cake, snack	LAB, Yeast
	Kecap (Indonesia)	Liquid flavoring agent	LAB, Moulds, Yeast
	Koji (Japan)	Used to make soya sauce	Mould
	Miso (Japan)	Alkaline paste used as soup	Moulds
	Soybean sauce (Worldwide)	Alkaline, liquid used as seasoning	Moulds
	Tauco (Indonesia)	Alkaline paste used as soup	Moulds

been produced and consumed worldwide. Given the enormous diversity, it is hard to discuss all fermented foods and beverages made from cereals and pseudocereals within this report. However, the general groups and popular fermented product are discussed.

### **Alcoholic and Non-alcoholic Beverages**

Preparation of alcoholic beverages from cereals and pseudocereals has been practised for thousands of years. Historical evidences suggested wine was produced in Caucasus and Mesopotamia as early as 6000 BC (Tamang, 2010). Depending on the raw material, production methods, type and composition of fermenting microorganisms culture, and fermentation techniques and conditions; a vast diversity of traditional and commercial alcoholic drinks are now produced and consumed all over the world (Blandino et al., 2003; Tamang, 2010). These drinks are used for various purposes such as relieving thirst, refreshment, entertainment, social and religious celebrations and ceremonies, and to improve health and wellbeing (Tamang, 2010). The production of alcoholic and non-alcoholic drinks differ from region to another based on tradition, scale (artisanal or industrial), and the level of technology employed in the process. Beer, by far, is the most produced alcoholic beverage, which was initially prepared from barley, but nowadays all types of cereals and pseudocereals are used as raw materials for its production using both modern and traditional technologies (Blandino et al., 2003; Tamang, 2010). Other alcoholic drinks prepared from one or more of cereals or pseudocereals are traditionally produced and consumed in many countries. Among those, Bhaati jaanr, Brem, Chhang, Huangjiu, Judima, Krachae, Lao-chao, Poko, Sake, Satoh, Takju, Tapé, Tapai pulut, Tapuy, and Yakju, which are rice-based alcoholic drinks prepared mainly in Asian countries (Emerald et al., 2016; Tamang, 2010). Ajon, Amgba, Bouza, Burukutu, Darassun, Dolo, Jiu (Chinese Liquor), Inard, Katata, Kweete, Malwa, Merissa, Omuramba, Pito, and Tchoukoutou are alcoholic drinks prepared from sorghum, millet, and maize or mixtures thereof in African and Asian countries (Emerald et al., 2016; Nout, 2009; Tamang, 2010). Several non-alcoholic drinks are also produced from cereals and pseudocereals in some parts of the world. For example, Abreh, Hulu-mur and Nasha, are traditional Sudanese drinks prepared from sorghum and sorghum malt that are consumed to relieve thirst during the month of Ramadan and they believed to enhance the health and promote milk production in lactating women. Agua-agria, Aliha, Kunun-zaki, Mahewu, Mageu, Masvusvu, Mawe, Motoho oa mabela, Munkoyo, Oshikundu, Togwa or Tobwa, and Uji are some of the non-fermented alcoholic beverages prepared from sorghum, maize, and millet (Blandino et al., 2003; Nout, 2009; Tamang, 2010; Taylor, 2016). Boza is also non-alcoholic refreshing drinks that prepared from wheat, barley, millet, oat, and rye (Blandino et al., 2003; Taylor, 2016) and Kvass is non-alcoholic or very mild alcohol drink beverage traditionally produced from rye and barley malt in eastern European countries (Kohajdová, 2015). Both spontaneous and starter culture fermentation processes occur during fermentation of cereals and pseudocereal-based fermented drinks. During traditional fermentation, starch is initially hydrolyzed by amylolytic yeasts and moulds, followed by alcoholic forming yeast, and finally by the flavour-enhancing lactic acid bacteria (LAB) (Tamang, 2010). Generally, wide arrays of microorganisms are involved in the fermentation of cereals and pseudocereals for production of alcoholic and non-alcoholic beverages. Yeasts, moulds, and LAB represent the main genera of microorganisms responsible for the production of these beverages. The major yeast genera involved in this fermentation are; *Saccharomyces*, *Saccharomycopsis*, *Schizosaccharomyces*, *Endomycopsis*, *Pichia*, *Hansenula*, *Candida*, *Kluyveromyces*, *Debaryomyces*, *Torulopsis*, and *Zygosaccharomyces*. Whereas, the major genera of the LAB are *Pediococcus*, *Lactobacillus*, *Enterococcus*, *Weissella*, and *Leuconostoc* and that of moulds are *Actinomucor*, *Amylomyces*, *Mucor*, and *Rhizopus* (Blandino et al., 2003; Nout, 2009; Tamang, 2010).

### **Sourdough and Bread**

As one of the oldest fermentation processes, sourdough fermentation was developed early in human history relaying on spontaneous fermentation of cereal flours by naturally occurring lactic acid bacteria (LAB) and yeast (Poutanen et al., 2009). During the fermentation of sourdough, yeast and LAB work in harmony to develop specific characteristics of leavening (releasing CO<sub>2</sub> by yeasts) and acidification (production of acids by the LAB) in the dough. Maintaining of the combined activities of yeast and lactic acid bacteria in sourdough are obtained and optimized through the use of fresh microorganisms or backslipping (use of small fermented material as a starter). This refreshment approach leads to the development of sourdough with a constant and reproducible acidification and leavening properties (Corsetti, 2013). Based on the processing technologies and microorganisms applied, sourdoughs are grouped as type I, type II, and type III sourdoughs (Corsetti, 2013; Emerald et al., 2016). Type I sourdough contain cultures of metabolically active microorganism(s) that enriched through three stages of fresh culture capable of producing large quantities of lactic and acetic acids, fermentation and leavening without the addition of commercial yeasts (Corsetti, 2013; Emerald et al., 2016). Type II sourdough is normally prepared by a single fermentation step at 30 °C for 15–20 h and is produced at industrial level using bioreactors and the adapted strains are used to start new fermentations. Type III sourdough is made at an industrial level as a dried form of type II sourdough, and it has constant quality (Corsetti, 2013; Emerald et al., 2016). The chemical composition of the materials used, the applied fermentation conditions, and composition and their interactions with the fermenting microbiota have significant effect on the yield and the quality of sourdough and bakery products (Emerald et al., 2016). Sourdough and breads have apparently been made from almost all cereals, pseudocereals, and mixtures thereof. Wheat and rye flour are the most used grains in the preparation of sourdoughs and leavened breads (such as wheat leavened bread, altamura bread, levain tout point, San Francisco bread, white pan bread, Naan, rye sourdough bread, straight rye bread, crispy flat rye bread) (Corsetti, 2013). Also, sourdough leavened bread have been prepared from amaranth, barley, buckwheat, teff, maize, millet, oat, quinoa, and rice (Vogelmann et al., 2009). Microorganisms involved in sourdough fermentation constitute a diverse species of LAB and yeast. The most dominant LAB isolated from cereal and pseudocereal sourdough includes: *Lactobacillus fermentum*, *L. fructivorans*, *L. pontis*, *L. plantarum*, *L. delbrueckii*, *L. sanfranciscensis*, *L. helveticus*, *L. paralimentarius*, *L. spicheri*, and others

(Corsetti, 2013; Emerald et al., 2016; Vogelmann et al., 2009). The dominant yeasts strains responsible for fermentation of sourdough are *Candida milleri*, *C. holmii*, *Saccharomyces cerevisiae* and *S. exiguus*, *Issatchenkia orientalis*, *Pichia anomala*, *P. saitoi*, *P. membranifaciens*, *Torulaspora delbrueckii*, *Debaryomyces hansenii* and others (Emerald et al., 2016; Vogelmann et al., 2009).

### **Gruel, Porridge, Cake, Dumpling, and Pancake**

Cereals and pseudocereals have been also used for the preparation of various types of fermented gruel, porridge, dumpling, cake, and pancake in many countries in the world (Nout, 2009; Taylor, 2016). Numerous types of staple cereal- and pseudocereal-based fermented foods are available worldwide that vary based on the raw materials and processing traditions and techniques used. Kisra is a fermented flatbread prepared from sorghum and is considered a staple food in Sudan (Blandino et al., 2003). Injera, a leavened pancake that considered as staple Ethiopian food, is prepared from fermented flours of teff, sorghum, millet and wheat (Nout, 2009; Taylor, 2016). Puto is a leavened steamed rice cake and Idli is also a spongy cake produced from a fermented mixed batter of parboiled rice (80%) and black gram (20%) (Blandino et al., 2003; Taylor, 2016). Maize-based fermented products, such as uncooked dough (Mawe) and cooked gruel or dumpling (Kenkey), fermented gruel (Ogi), cooked dough (Pozol), are considered a staple food in Benin, Ghana, Nigeria, and Mexico, respectively (Nout, 2009; Taylor, 2016). Aceeda and Ting are fermented porridges made from sorghum and millet and considered as commonly consumed foods in Sudan and South Africa, respectively (Taylor, 2016). Yosa is an oat bran-based fermented snack produced and consumed in Scandinavian countries (Blandino et al., 2003). Several potentially functional fermented foods and drinks have been produced from cereals and pseudocereals (Kohajdová, 2015). Due to the variation in the composition of raw materials and processing conditions of fermented gruel, porridge, dumpling, cake, and pancake, the diversity of fermenting microorganisms is very high. However, the most frequently isolated microorganisms from these products include, but not limited to, LAB such as *L. fermentum*, *Leuconostoc mesenteroides*, *L. brevis*, *L. plantarum*, *L. delbrueckii*, *L. lactis*, *L. confusus*, *L. reuteri*, *L. harbinensis*, *L. parabuchneri*, *Lactobacillus casei*, *L. coryniformis*, *Pediococcus cerevisiae*, *Pediococcus pentosaceus*, *Enterococcus faecalis* and several species of yeast of the genera *Saccharomyces*, *Geotrichum*, and *Torulopsis* and some *Bifidobacteria* (Blandino et al., 2003; Taylor, 2016).

### **Fermentation of Legumes Grains**

Grain legumes are considered as the main sources of protein in human diets in countries where animal proteins are unaffordable and religiously-prohibited. However, these grains contain several antinutritional factors and allergens that could negatively affect the health and reduce the overall benefits of legume-based foods. Fermentation of legumes is a vital approach to reduce the antinutritional factors and allergens and could thus enhance the nutritional, sensorial, functional, and health properties of these products. Although the fermentation of grain legumes is a traditional practice in Asia and Africa, currently fermented legumes products receive high global interest due to their great health-promoting and disease-preventing potentials (Adebo et al., 2017). Due to the high protein content of legumes and microorganism used, the type of fermentation of legumes mostly belong to alkaline fermentation, in which ammonia is released from proteins by the action of microbial or indigenous proteases at elevated pH. In Asian and African countries, legumes-based fermented products represent the major source of daily intake of good quality proteins, vitamins, minerals and bioactive compounds with great potential health promoting properties. Fermented legumes products are highly appreciated in these regions and are very diverse due to differences in the raw substrates, fermentation conditions, and consumer preferences. The major types of legumes-based fermented foods are discussed in the following paragraphs.

#### **Soybean-Based Fermented Foods**

The production and consumption of soybean-fermented foods is the highest among all legumes, especially in Asia. Natto is a traditional fermented soybean product produced exclusively in Japan, with other similarly processed products such as Chungkukjang (soup) in Korea and Thao noa (paste) in Thailand (O'Toole, 2016). Traditionally, the soybeans is soaked in water overnight, boiled, drained, the beans slightly dried, covered with rice straw, and stored at warm temperature (up 36 °C) for 1–2 days to permit the growth of the fermenting bacteria namely *Bacillus subtilis* (natto) and to form a product with viscous sticky slime, with high umami and slightly bitter and sweet tastes, and ammonia odour notes (Nout et al., 2007; O'Toole, 2016). In other types of Natto (e.g. Yukiwari natto and Hama natto), the soybeans are fermented using koji starter culture (*Aspergillus oryzae*) that is prepared by growing the moulds on steamed polished rice (Nout et al., 2007; O'Toole, 2016). Kinema is another soybean-based fermented food traditional produced in India, Nepal, and Bhutan. It is largely resembles Natto with exception that, in Kinema the soybeans are crushed to split the cotyledons. It is a naturally fermented product with the main fermenting microorganism is *Bacillus subtilis* in addition to enterococci, and yeasts (Nagai and Tamang, 2010; Nout et al., 2007). Others Bacilli fermented, sticky, and non-salty soybean products are produced and consumed by ethnic groups in many Asian countries such as Aakhone, Bekang, Hawaijar, Peruya, and Tungrymbai in India, Pepok in Myanmar, and Sieng in Cambodia and Laos (Nagai and Tamang, 2010). Tempeh or Tempe is a mould-fermented soybean product originated in Indonesia and due to its high nutrition, it is considered as a meat substitute. In traditional processing, soybeans are soaked in water or acidified water at room temperature to allow the indigenous LAB bacteria (*L. casei*, *Streptococcus faecium*, *Staphylococcus epidermidis*, and *Klebsiella pneumoniae*) to grow and ferment the beans, the beans are then dehulled, properly cooked, drained, covered with wheat flour, and inoculated with *usar* or *ragi* (Tempe starters) that composed of many *Rhizopus* species and mainly *R. oligosporus* (Nagai and Tamang, 2010; Nout et al., 2007; O'Toole, 2016). Douchi is a salt-fermented soybean product traditionally produced in China, and based on the type of microorganisms used in the fermentation four types of douche are available; *Mucor*-fermented, *Aspergillus*-fermented, *Rhizopus*-fermented, and



*Bacillus*-fermented are produced (Nagai and Tamang, 2010; Nout et al., 2007). Several products similar to *Aspergillus*-fermented douchi can be found in other countries, e.g. Hammanatto in Japan, Tao-si in the Philippines, and Tao-tio in India (O'Toole, 2016). Tofu (Japan) and Sufu or Furu (China) are fermented cheese-like products prepared from soymilk by coagulation and fermentation with moulds of the genera *Actinomucor*, *Mucor*, and *Rhizopus* or bacteria of genus *Micrococcus* and *Bacillus* (Nagai and Tamang, 2010). In addition, there are many other fermented soybean-based products prepared from soybeans and cereals such as Doenjang (Korea), Meitauza (China), Miso and Shoyu (Japan), Soy sauce (worldwide), and Tauco (Indonesia) among others, and the fermentation of these products was attained by various types of moulds, yeasts, and LAB (Nagai and Tamang, 2010; O'Toole, 2016).

### Non Soybean-Based Fermented Legumes

Although the majority of the legume-based fermented products are prepared from soybeans, many other fermented foods are traditionally prepared from various types of legumes by many ethnic groups in Central Asia and West Africa. For example, Maseura (hollow, brittle and friable ball or cone-shaped mixture), Wari (a dried, hollow, brittle, spicy, and friable ball), and Papad (a thin, usually circular, wafer-like product) are fermented products prepared from black gram alone or mixed with Bengal gram, lentil, red gram, or green gram in Central Asia (India, Nepal and Pakistan). Wide range of microorganisms is involved in the fermentation of these products including; bacteria (*L. fermentum*, *L. salivarius*, *Pediococcus pentosaceus*, *P. acidilactici*, *E. durans*, *B. subtilis*, *Bacillus mycoides*, *Bacillus pumilus*, and *Bacillus laterosporus*), yeasts (*Saccharomyces cerevisiae*, *Pichia burtonii*, *Candida castellii*, and *C. versatilis*), and moulds (*Cladosporium*, *Penicillium*, and *Aspergillus niger*) (Nagai and Tamang, 2010; Nout et al., 2007). Dawadawa in an ethnic fermented food condiment prepared from African locust bean (*Parkia biglobosa*) and widely consumed as protein-rich source in West Africa. The fermented product is known under different names such as Iru in Nigeria, Dawada in Ghana, Kinda in Sierra Leone, and Soumbala in Burkina Faso (Nagai and Tamang, 2010). The main microorganisms involved in the fermentation of these products are *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *B. pumilus*, *Bacillus megaterium*, *Staphylococcus xylosum*, *S. saprophyticus*, *S. hominis*, and *Micrococcus* spp (Nagai and Tamang, 2010; Nout et al., 2007). Ugba is a Nigerian flat, glossy, and brown colour fermented legume product prepared from African oil beans, in which the fermentation is carried out by strains belonging to the genera *Bacillus*, *Staphylococcus*, *Micrococcus*, *Corynebacterium*, *Alcaligenes*, and *Citrobacter* (Obeta, 1983). Otiru is a fermented food with greyish brown colour, and a strong ammonia-like odour and is prepared from the seeds of African yam beans in West Africa. A diverse range of bacteria and yeasts were involved in the fermentation of Otiru such as *Lactobacillus jensenii*, *Bacillus coagulans*, *Aerococcus viridans*, *P. pentosaceus*, *Saccharomyces cerevisiae*, and *Candida vini* (Jeff-Agboola, 2015). Oncom is a traditional Indonesian fermented food produced from peanut and peanut cake, in which the fermentation was mainly carried out by different moulds belonging to the genus *Neurospora* (merah or red oncom) and *Rhizopus* (hitam or black oncom) (Nout et al., 2007). Dhokla and Khaman are fermented spongy-textured products made from Bengal gram alone or mixed with rice and the main LAB strains were *L. mesenteroides* and *E. faecalis* (Nagai and Tamang, 2010).

### Impact of Fermentation on the Overall Quality of Grains-Based Products

Fermentation is an incredible process that significantly improves the diversity, storability, nutritional, sensorial, technological, safety, and health potentials of food grains and grain-based products. As the fermentation of grains is mostly done by ethnic groups in developing countries, it is considered as the source of income and employs many peoples in these countries. In addition, fermentation of grain is the easiest and cheapest methods for preservation of grain-based foods from spoilage microorganisms as fermenting microorganisms such as LAB are believed to produce metabolites such as acids, ethanol, hydrogen peroxide, carbon dioxide, and bacteriocins that inhibited the growth of pathogenic and spoilage microorganisms in foods. Fermentation could degrade and remove the toxic and allergic compounds from grains and thus improve the safety of these products. Fermentation also enhances the protein and starch functional properties of food grains and increases the gas, water and oil retention capacities of fermented foods and thus improves the sensory properties and overall acceptability of final products. For example, fermentation reduces the viscosity of porridges by the action of microbial amylases and consequently improves its palatability without effects on the amounts of nutrients and energy (Taylor, 2016). Nutritionally, fermentation of grains are believed to reduce antinutritional factors (tannin, phytate, and protease inhibitors) and complex carbohydrates, increase essential minerals, amino acids, fatty acids, and vitamin content and their bioavailability as a result of improving the protein and starch digestibility. In addition, fermentation improves the yield and profile of polyphenolic of fermented grains products by the action of microbial metabolic activities through phosphorylation, glycosylation, deglycosylation, methylation and glucuronidation and consequently leads to the formation of new metabolites with specific functional and pharmaceutical properties (Emerald et al., 2016). Therapeutic and health promotion potential of fermented grains are diverse and include, but not limited to, antioxidant, anticancer, antihypertension, anti-inflammation, anticholesterol, antidiabetics, and prevention of cardiovascular diseases, gastrointestinal disorders, hepatic disease, obesity, and allergic reactions (Emerald et al., 2016).

## Conclusions

Fermentation of grains was discovered earlier in the history and grain-based fermented foods and beverages became a major part in the diet of all nations and civilizations. Fermented grains indeed will have a great role to play as functional foods in the future. Large number of different grain-based fermented foods and beverages that ranging from indigenous to commercially produced products are prepared and consumed worldwide due to their beneficial roles in nutrition and health. Fermentation of grains provide many desirable outcome, such as reducing the antinutritional factors, removing toxic and allergic materials, improving the safety, shelf-life, digestibility, texture, taste, flavour, micronutrients, and phenolic profile, and add probiotic microorganisms to the products, and thus it contribute to food security. Despite of the great potential of grain-based fermented foods, the processing methods of most of these foods still largely dependent on the traditional acknowledge at home-level or small factories scales that might results in products with changeable quality, safety and low marketability. Thus, application of modern fermentation technologies, using microorganisms with known metabolic pathways, is needed to get the maximum benefits of grain-based fermented foods. In addition, omics approaches (genomics, transcriptomics, proteomics, and metabolomics) could offer opportunities for developing new grain-based fermented products, diversifying the processes, assuring the quality and safety of the products, and validating the health potentials of fermented foods.

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# Fruit Pigment Changes During Ripening

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## Glossary

Ethylene

Natural plant hormone

Functional food

Food that potentially offers health benefits beyond basic nutrition

Pigment

Plant compounds that are perceived by humans to have color

## Pigments in Climacteric and Non-climacteric Fruits

Plant pigments are important in controlling photosynthesis, growth and development of plants (Sudhakar et al., 2016). Pigments act as visible signals to attract insects, birds and animals for pollination and seed dispersal. Pigments also protect plants from damage caused by UV and visible light (Tanaka et al., 2008). Many pigment-rich fruits are consumed in the human diet. The pigment content of fruits has been studied for many decades not only because of the aesthetic appeal of ripe fruit colors, but also due to the wide variety of compounds that are present and are responsible for the characteristic green, yellow, orange, red, blue, and purple colors. Chlorophylls are the source of green in all fruits and function as the primary pigment to capture yellow and blue lights for photosynthesis to produce energy for plant development and growth. Unlike chlorophylls, carotenoids and anthocyanins are accessory pigments (generally with the absorbance spectrum complementary to chlorophylls) and also secondary metabolites that possess much more diverse structures and functions in plants and offer more potential nutritional and health benefits in the diet (Chen, 2015).

Ripening refers to the physiological and biochemical changes of a fruit to attain desirable color, flavor, aroma, sweetness, texture, and thus eating quality. The process of ripening usually does not occur until a fruit reaches its full maturity. When a fruit reaches its full maturity, its size and weight reach a maximum and its growth rate decreases. Ripening of a fruit may occur on the plant or after harvest, depending on the species (Li, 2012). Respiration is the fundamental process whereby living organisms carry out the production of energy. Based on respiration, fruits can be grouped as climacteric and non-climacteric (Lelièvre et al., 1997; McMurchie et al., 1972; Pech et al., 2012). Ripening in climacteric fruits manifests itself by a climacteric rise in respiration and autocatalytic ethylene production (Abeles et al., 1992; Biale and Young, 1981). The main specificity of climacteric fruits is their capacity to produce ethylene and ethylene stimulates its own biosynthesis (Burg and Burg, 1965; McMurchie et al., 1972). Fruits typically contain a mixture of pigments, including the green chlorophylls; yellow, orange, and red carotenoids; red, blue, and violet anthocyanins; and/or other pigments. Examples of climacteric fruits and their main pigments and typical colors are listed in Table 1.

In non-climacteric fruits respiration shows no change and ethylene production remains at a very low level during ripening (Burg and Burg, 1965; Giovannoni, 2001). Hence, the concept of two systems of ethylene production has emerged (Pech et al., 2012). One is characterized by low levels of ethylene production and is present throughout the ripening of non-climacteric fruit. These low levels of ethylene production occur in climacteric fruit during the period preceding the climacteric rise in respiration. Another system operates at the onset of climacteric rise in respiration and is responsible for auto-catalytic ethylene production (Pech

**Table 1** Climacteric and non-climacteric fruits and their main pigments and typical colors

Fruits	Pigment class	Examples	Typical colors
Climacteric	Chlorophyll	Avocado, guava, kiwifruit, apple	Green
	Carotenoid	Tomato, apricot, banana, breadfruit, mango, papaya, peach, passion fruit, durian, jackfruit	Orange, yellow, pink, red
Non-climacteric	Anthocyanin	Fig, plum	Purple, red
	Chlorophyll	Olive, honeydew	Green
	Carotenoid	Orange, grapefruit, lemon, pineapple, lime, carambola (star fruit)	Orange, yellow, pink, red
	Anthocyanin	Blueberry, grape, strawberry, cherry, cranberry, raspberry, pomegranate	Purple, blue, red

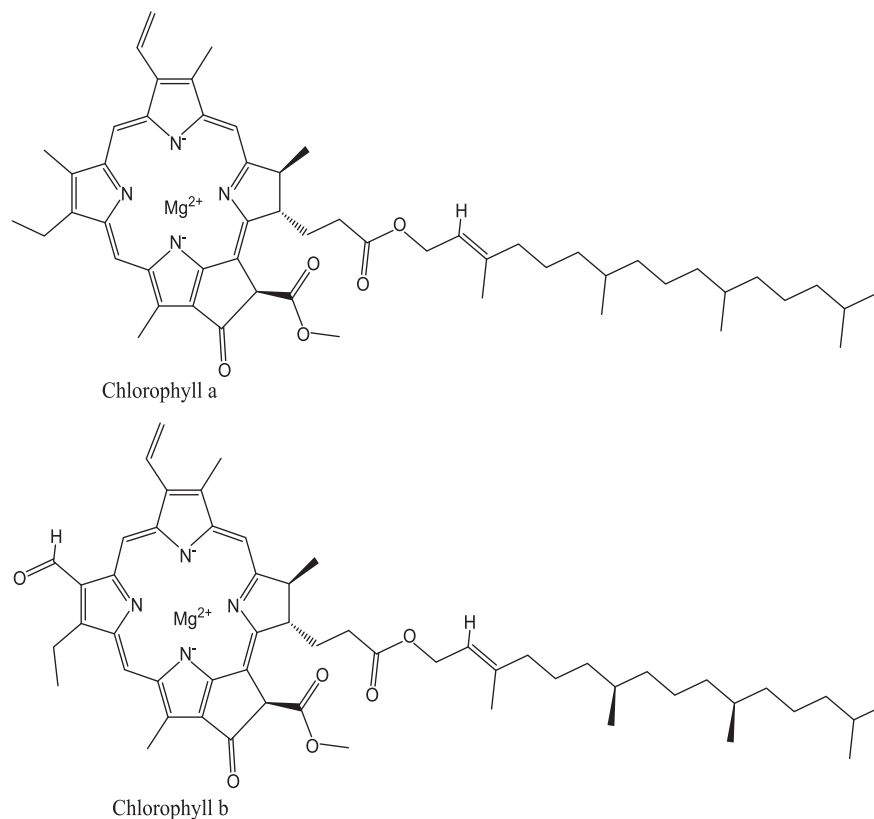
et al., 2012). The respiration rate of non-climacteric fruits responds primarily to temperature (Biale, 1960). Examples of non-climacteric fruits and their main pigments and typical colors are listed in Table 1.

### Chlorophyll Changes During Fruit Ripening

Chlorophylls are the most abundant pigments among biologically produced pigments (Lee and Schwartz, 2005). The green color in all higher plants including fruits is due to chlorophylls. Chlorophylls participate in photosynthesis which is one of the most important life processes by converting energy from light into chemical energy (Chen et al., 2010; Sudhakar et al., 2016). Chlorophylls can absorb visible light in the red region between 600 and 700 nm and the blue region between 400 and 500 nm (Lee and Schwartz, 2005). Chlorophylls are chemically classified within the porphyrin group with a tetrapyrrole macrocycle, linked by methylene bridges and coordinated with a  $Mg^{2+}$  ion. Chlorophyll also contains a modified propionic acid chain in the form of cyclic-ketoester (isocyclic ring) and, on C-17, a chain of propionic acid esterified with the diterpene alcohol phytol. In higher plants there are two types of chlorophyll, a and b. The difference between them is that chlorophyll a has a methyl group on carbon C-7, whereas chlorophyll b has an aldehyde group (Fig. 1) (Artes et al., 2002). Chlorophyll a (blue-green) and b (yellow-green) are present in green plants in the ratio of 3:1 (Lee and Schwartz, 2005).

Chlorophylls of higher plants are located in specialized plastids named chloroplasts. The biosynthesis of chlorophyll occurs in the chloroplast, and all the enzymes involved have already been identified (Beale, 2005; Brzezowski et al., 2015; Tanaka and Tanaka, 2007). In contrast to the biosynthetic pathway, the degradation pathway of chlorophylls is not completely deciphered (Roca et al., 2016). Chlorophyll a is degraded to pheophorbide a, losing the phytol chain and the central magnesium atom (Eskin and Hoehn, 2013; Hörtensteiner and Kräutler, 2011; Kräutler and Hörtensteiner, 2012). The order in which both reactions take place and the enzymes implied in that process are different in senescent leaves than in fruits, and new hydrolases are still to be identified (Kräutler and Hörtensteiner, 2012). An example of enzyme is chlorophyllase which removes the lipophilic phytol anchor of the chlorophyll molecules, which is crucial for binding of the green pigment to the chlorophyll binding proteins and for insertion of the chlorophyll-protein complexes into the thylakoid membranes of chloroplasts (Harpaz-Saad et al., 2007; Kräutler, 2003).

Chlorophyll degradation is central to the degreening process occurring during ripening of most fruits (Hörtensteiner, 2006; Hörtensteiner and Kräutler, 2011). Degradation of chlorophyll causes color change in fruits which in turn unmasks previously synthesized pigments. An example of this process occurs in the degreening of lemons. Degreening process is usually followed by the biosynthesis of other pigments such as carotenoids or anthocyanins (Tucker, 1993). Ethylene has been reported to promote the degradation of chlorophyll during fruit ripening (Burg and Burg, 1965; Hiwasa-Tanase and Ezura, 2014). In most fruits, chlorophyll content increases during green stages and diminishes through maturation, paralleling chloroplast disintegration. There



**Fig. 1** Chemical structures of chlorophyll a and b

are some exceptions of fruits retaining chlorophylls at the ripe stage. For examples, certain apple, pear, fig, plum, limes, avocado, melon, kiwi, and grape cultivars. These fruits are characterized by a low accumulation of pigments other than chlorophylls at the ripe stage (Rodrigo et al., 2012).

### Carotenoids Changes During Fruit Ripening

Carotenoids are lipid-soluble pigments widely found in nature. Carotenoids play an important role in the photosynthetic apparatus (Schieber and Weber, 2016) as they are essential components of the photosynthetic membranes in all photosynthetic organisms (Goodwin, 1986). Carotenoids are much more widespread and not confined to photosynthetic organisms, and structurally and functionally more diverse than chlorophylls (Scheer, 2013). Carotenoids are well known for their diverse yellow, orange, and red colors of most fruits, flowers, and vegetables, and for the characteristic colors of many birds, insects, fish, and crustaceans by intaking carotenoids through the diet (Britton, 1995).

Carotenoids are a large class of pigments of which more than 750 different chemical structures have been reported (Britton et al., 2004; Scheer, 2013). Carotenoids are produced via the general isoprenoid biosynthetic pathway (Da Silva Mendes et al., 2015; Delgado-Vargas et al., 2000; Rosas-Saavedra and Stange, 2016; Tanaka et al., 2008) and normally contain a hydrocarbon backbone of 40 carbon atoms, consisting of 8 isoprene units. The great diversity of carotenoids generally derives from an acyclic  $C_{40}H_{56}$  structure that has a long central light-absorbing conjugated polyene compound constituting the chromophoric system, which absorbs light at 400–500 nm (Alcaíno et al., 2016). Carotenoids are classified into two groups, carotenes and xanthophylls. The carotenes are structurally related to hydrocarbons while the xanthophylls include the corresponding oxidized derivatives (hydroxy, epoxy, and oxy compounds) and are frequently esterified (Eskin and Hoehn, 2013).

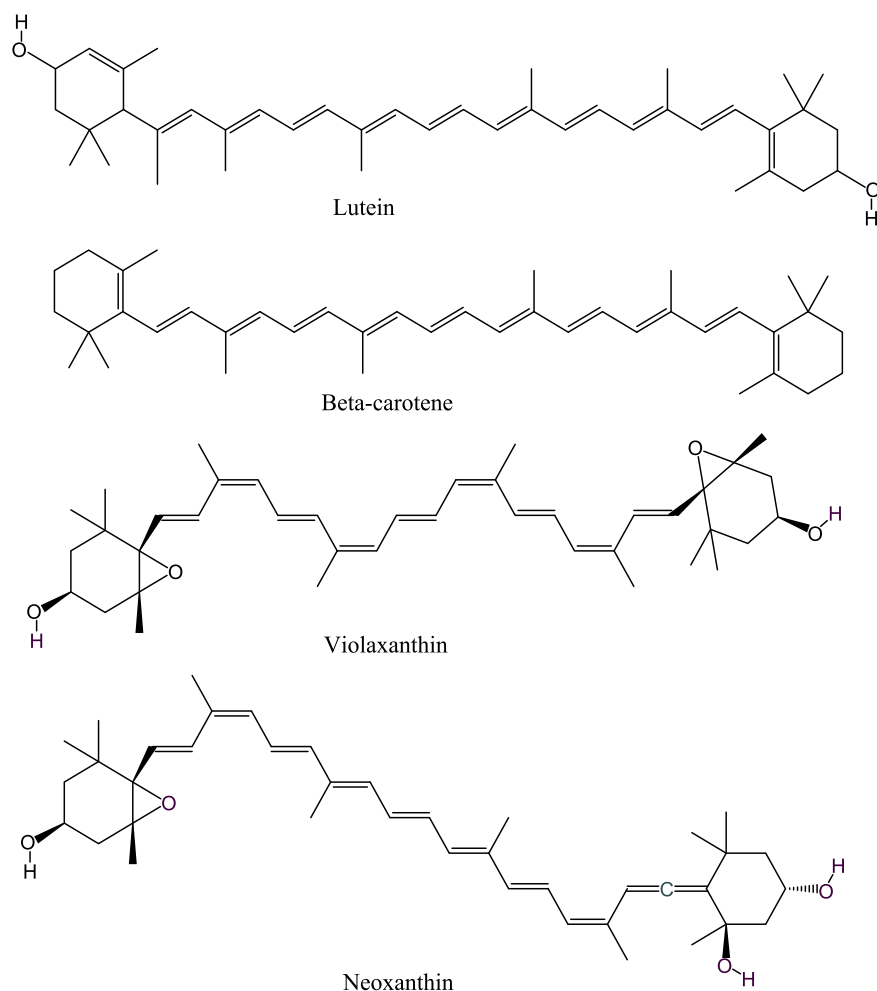
Carotenoids are located in the chloroplasts of plants and often masked by more dominant chlorophyll pigments. Thus, carotenoids are distributed in a wide range of fruits containing chlorophyll pigments with  $\beta$ -carotene being the most common carotenoid, which exhibits a yellow color. Tomatoes (lycopene), carrots ( $\alpha$ - and  $\beta$ -carotenes), pumpkin ( $\beta$ -carotenes), corn (lutein and zeaxanthin), and sweet potatoes ( $\beta$ -carotenes) are typical examples of plant containing carotenoids (Lee and Schwartz, 2005). Among plant organs, fruits display one of the most heterogeneous carotenoids patterns in terms of diversity and abundance (Lado et al., 2016). As in other plant organs, carotenoid content in fruit is directly influenced by the stage of developmental and their environmental conditions. At the green stage, carotenoid composition is the characteristic of chloroplastic tissues, lutein being the most representative, followed by  $\beta$ -carotene, violaxanthin and neoxanthin (Fig. 2), and other minor carotenoids as zeaxanthin and antheraxanthin (Bramley, 2013). Ethylene has been shown to influence the biosynthesis of carotenoids in plants (Marty et al., 2005).

Pigmentation changes during fruit ripening are accompanied by structural changes in the chloroplasts. The granal-integral network, in particular, becomes disorganized, resulting in the formation of chromoplasts (Eskin and Hoehn, 2013). The chromoplasts no longer contain chlorophyll or photosynthetic pigments but become the major site for carotenoid biosynthesis (Camara and Brangeon, 1981). A typical example is in the green-to-red transition of tomato fruit. Carotenoid formation during tomato fruit ripening has been studied extensively and has become the best model system for other chromoplast-containing tissues (Bramley, 2013). During ripening, the concentration of carotenoids in tomato increased between 10- and 14-fold due mainly to the accumulation of lycopene (Fraser et al., 1994). Ikemefuna and Adamson (1984) monitored changes in the chlorophyll and carotenoid pigments of ripening palm fruit and observed a similar degeneration of chloroplasts and formation of chromoplasts. These changes were accompanied by increase in carotenogenesis with the formation of  $\alpha$ - and  $\beta$ -carotenes as the major pigments in the ripe fruit. On the other hand, in fruits which accumulate pigments other than carotenoids, such as grapes, carotenoid content decreases during ripening. A decrease in carotenoid content was observed during ripening of grapes in which lutein content decreased until 66%, whereas  $\beta$ -carotene slowly decreased, having a constant level until the harvest date (Oliveira et al., 2004).

### Anthocyanins Changes During Fruit Ripening

Anthocyanins are water-soluble pigments belonging to the large group of flavonoids, a subclass of the polyphenol family. Anthocyanins give colors in fruits ranging from pink, red, purple to blue (Sudhakar et al., 2016). More than 700 different anthocyanins have been identified (Wallace and Giusti, 2014). Anthocyanins have structures consisting of two aromatic rings linked by three carbons in an oxygenated heterocycle. The basic chromophore of anthocyanins is the 7-hydroxyflavylium ion. Anthocyanin pigments consist of two or three chemical units: an aglycon base or flavylium ring (anthocyanidin), sugars, and acylating groups (Bueno et al., 2012). There are only six different anthocyanidins found in nature that occur frequently and are of dietary importance: cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin (Fig. 3) (Bueno et al., 2012; Kammerer, 2016; Shrikhande and Francis, 1976). The distribution of these anthocyanidins in fruits and vegetables is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%), and malvidin (7%) (Kong et al., 2003). Each aglycon may be glycosylated or acylated by different sugars and aromatic or aliphatic acids. The sugar moiety is typically attached at the 3-position on the C-ring or the 5-position on the A-ring (Bueno et al., 2012). The absorption maxima of anthocyanins in the visible range is between 465 and 550 nm, but they also absorb in the UV range between 270 and 280 nm (Glover and Martin, 2012).

Anthocyanins are mostly accumulated in outer plant parts such as in the vacuoles of epidermal cell layers. As a result, small-sized fruits characterized by a high surface-to-volume ratio are often particularly rich in anthocyanins. This high pigment concentration usually brings about enhanced pigment stability (Kammerer, 2016). In some species anthocyanins are bound to membrane of the



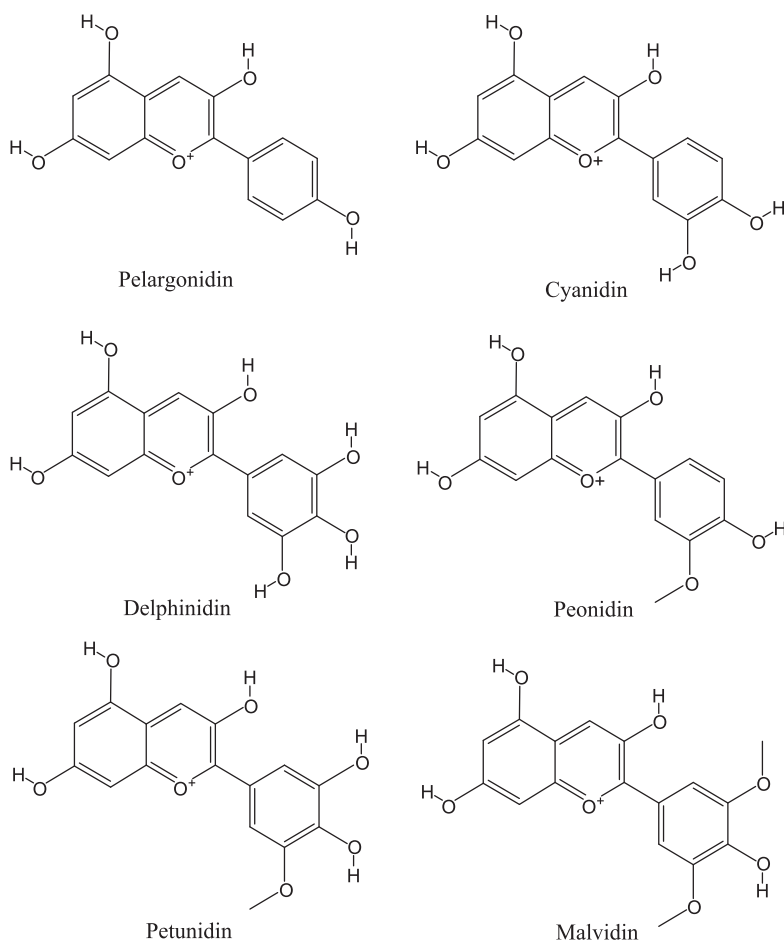
**Fig. 2** Chemical structures of carotenoids.

main cell-vacuole called anthocyanoplasts (Riaz et al., 2016a). Anthocyanins are derived from phenylalanine (phenylpropanoid metabolic pathway) (Riaz et al., 2016b) and synthesized in the cytosol and localized in vacuoles (Rodrigo et al., 2012). The biosynthetic pathway of anthocyanins has been well characterized (Delgado-Vargas et al., 2000; Grotewold, 2006; Schwinn and Davies, 2004; Tanaka et al., 2008). Regulation of the pathway is species-specific and different genes of encoding relevant enzymes of the biosynthetic pathway have been found to be responsible for color variations in different anthocyanin accumulating fruits (Rodrigo et al., 2012). Ethylene has been shown to influence the biosynthesis of light-induced anthocyanin formation in plants (Craker and Wetherbee, 1973).

Anthocyanins are often markers of ripening as many fruits accumulate anthocyanins only during the ripening phase (Macheix et al., 1990). These pigments are formed as the fruit matures and ripens. Examples are for strawberries (Fuleki, 1969), certain varieties of cranberries (Lees and Francis, 1971; Zapsalis and Francis, 1965), apples (Bishop and Klein, 1975; Proctor and Creasy, 1971), black grapes (Liao and Luh, 1970) and blueberries (Suomalainen and Keränen, 1961). Anthocyanins are synthesized at an increasing rate during maturation reaching a maximum in a fully ripe fruit (Rodrigo et al., 2012).

Anthocyanins do not always follow the general pattern of accumulation towards maturity. For example, fruits of some red pear attain their highest anthocyanin levels while immature and red color gradually fades towards harvest (Steyn et al., 2004). Many apple cultivars including varieties that are typically yellow or green at maturity display a peak in anthocyanin levels shortly after fruit set (Lancaster et al., 2000). Anthocyanin-accumulating fruits often display a range of intermediary colors progressing from green to white or pink, then red or blue and finally purple to black with increasing anthocyanin and decreasing chlorophyll levels (Wheelwright and Janson 1985; Willson and Thompson, 1982). Rapid direct color changes from green to black also occur (Willson and Thompson, 1982). In cherry the anthocyanin profile does not change during fruit development but changes in color is due to increasing anthocyanin concentrations. In blueberry a change in anthocyanin profile during fruit development resulting in change in fruit color from red immature fruits to blue and purple in ripe fruits (Macheix et al., 1990).





**Fig. 3** Chemical structures of anthocyanidins.

## Conclusions

Chlorophylls, carotenoids and anthocyanins are important pigments that are highly abundant in fruits. The changes of these pigments during ripening are important knowledge for the fruit industry. In addition, analyzing pigment composition of ripe fruit and fruit products can be used to determine their genuineness. The great biodiversity of plants has generated a concomitant variety of pigment structures from fruits and many to be discovered. Further analysis of different plant species will allow the discovery of novel structures and possibly new biosynthetic and metabolic pathways. Carotenoids and anthocyanins contribute in various ways to quality characteristics of ripe fruit and fruit products. The multiple health benefits of these pigments are of major interest and interest will continue to grow among researchers in the coming years. Further studies on these pigments will contribute to the improvement of agriculture, food, pharmaceutical, and chemical industries involving these pigments.

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# $\alpha$ -Galactosidase and Its Applications in Food Processing

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## Introduction

Enzymes have long been used in various industrial sectors. The heightened need and interest in recent years to utilize renewable materials for biorefinery have attracted further attention to industrial application of enzymes (Kudakasseril Kurian et al., 2013; Loman and Ju, 2016b, 2017). While cellulose and hemicellulose in biomass are often the focuses for biorefinery development, galacto-oligosaccharides and pectic polysaccharides are also abundant in plants, particularly in legumes and vegetables (Sengupta et al., 2015). When consumed as feed or food components, galacto-oligosaccharides and pectic polysaccharides are associated with indigestibility concerns (Kien, 2008).  $\alpha$ -Galactosidase ( $\alpha$ -D-galactoside galactohydrolase, EC 3.2.1.22) is a group of glycoside hydrolase enzymes that catalyze hydrolysis of the terminal  $\alpha$ -galactosyl moieties of both simple and complex oligosaccharides and polysaccharides.

## Classification

Classification of  $\alpha$ -galactosidase has been based on either substrate specificity or amino acid sequence similarity. By substrate specificity,  $\alpha$ -galactosidases can be divided into two groups (Garman, 2007): Group I act on oligosaccharides such as the raffinose family oligosaccharides (RFOs), and Group II act on polysaccharides such as galacto(gluco)mannans. According to the amino acid sequence similarity,  $\alpha$ -galactosidases have been classified into more than 100 glycoside hydrolase (GH) families, notably GH 4, 27, 36, 57, 97 and 110 in the CAZy database (<http://www.cazy.org/>). Most of the eukaryotic  $\alpha$ -galactosidases belong to the GH family 27. GH family 36 includes predominately bacterial  $\alpha$ -galactosidases. Some  $\alpha$ -galactosidases hydrolyze the substrate with retention of the stereochemistry at the substrate anomeric center; others induce its inversion.

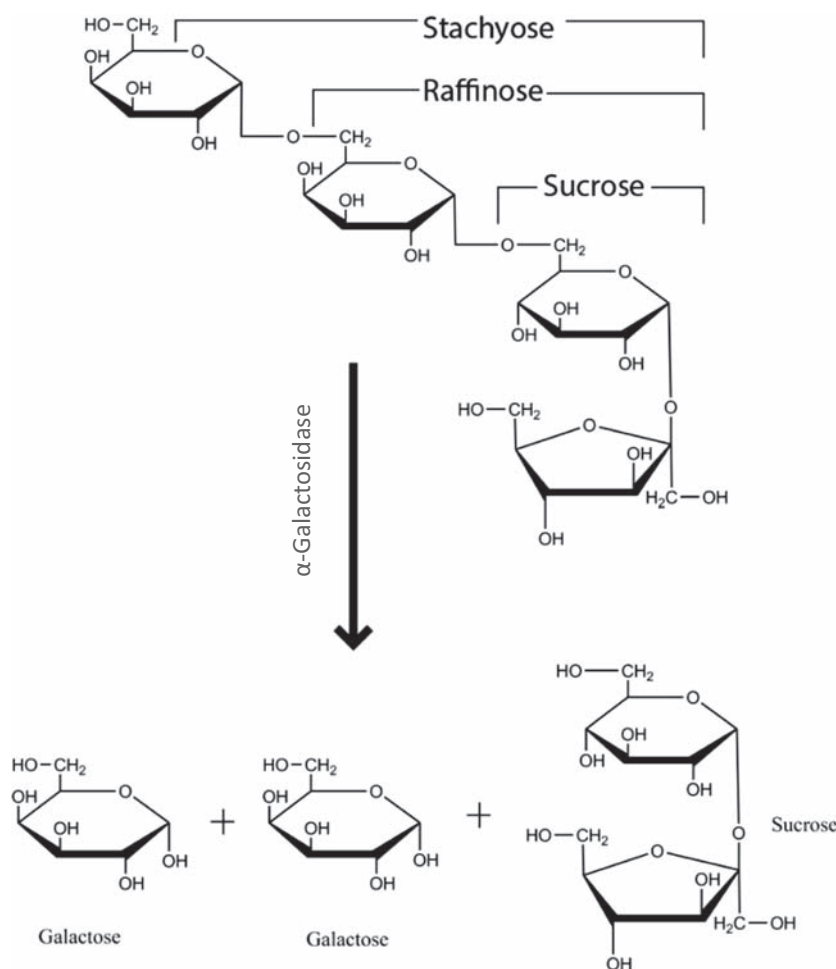
## Substrate

Group I  $\alpha$ -galactosidase catalyzes the hydrolysis of  $\alpha$ -1,6 linkage in low molecular weight galacto-oligosaccharides and galactosides; Group II acts on branched polysaccharides such as galactomannan and galactoglucomannan. Galacto-oligosaccharides are found in legume seeds and different parts of plants. For example, in soybeans galacto-oligosaccharides are the most abundant soluble carbohydrates besides sucrose. In descending order of concentrations, these include stachyose, raffinose and verbascose composed of a terminal sucrose linked with 1 (raffinose), 2 (stachyose) or 3 (verbascose) galactose residues by  $\alpha$ -1,6 linkages. Stachyose is shown in Fig. 1 as an example. These galacto-oligosaccharides can be hydrolyzed to D-galactose and sucrose by  $\alpha$ -galactosidase. Sucrose can be hydrolyzed to glucose and fructose by other enzymes (sucrase, invertase).

## Optimal pH and Temperature

Optimal pH is an important property of enzymes. Generally, fungal (Shankar and Mulimani, 2007) and yeast (Viana et al., 2011)  $\alpha$ -galactosidases are most active and stable in the acidic pH 4.5–5.5. Bacterial  $\alpha$ -galactosidases from *Bacillus* sp. and *Bifidobacterium breve*, on the other hand, have optimal pH at or closer to the neutral range (Gote et al., 2004; Aguilera et al., 2012). The acidic pH optimum is desirable for functioning of the enzymes in the acidic gastric environment. Despite their acidic pH optima, a few fungal  $\alpha$ -galactosidases are stable over a wide range of pH 3–12 (Yanan et al., 2009; Similä et al., 2010; Katrolia et al., 2012). Some bacterial  $\alpha$ -galactosidases are also active in wide pH ranges (Gote et al., 2004). Being active and stable over a wide pH range is a favorable property for industrial applications. For processing of soybean milk, which has a pH of about 7.2, it is desirable to use an  $\alpha$ -galactosidase stable at pH 7–7.5. Cao et al. (2010) used a recombinant *Escherichia coli* with an  $\alpha$ -galactosidase gene from *Streptomyces* sp. S27 (ATCC 41168) to produce the enzyme optimal at pH 7.4 for soybean milk processing.

Most  $\alpha$ -galactosidases have optimal temperatures of 30–40 °C. Thermal stability is desirable for industrial uses, to allow higher reaction rates and process productivities at higher temperatures or to withstand the high temperatures used in, for example, pulp and paper industries.  $\alpha$ -Galactosidases with higher thermal stability have been found from different microorganisms. Most  $\alpha$ -galactosidases from mesophilic fungi and yeast are stable at up to about 50 °C (Simerská et al., 2007; Viana et al., 2010), while a few thermostable enzymes can withstand temperatures above 65 °C (Jin et al., 2001; Gote et al., 2004; Shankar et al., 2009). Some  $\alpha$ -galactosidases from extremophilic bacteria such as *Thermotoga* sp. (Duffaud et al., 1997) and archaeon *Sulfolobus solfataricus* (Brouns et al., 2006) are optimally active at 90 °C and above.



**Figure 1** Hydrolysis of stachyose to galactose and sucrose by  $\alpha$ -galactosidase. Modified from Loman, A. A., Ju, L.-K. 2017. Enzyme-based processing of soybean carbohydrate: Recent developments and future prospects. *Enzyme and Microbial Technology*, 106, pp. 35–47.

## Microbial Production

$\alpha$ -Galactosidase has been isolated from bacteria, fungi, plants and animals (Shankar and Mulimani, 2007). Acidic and alkaline  $\alpha$ -galactosidases differ in their optimal pH for activity. The acidic ones play important roles in seed development and germination.  $\alpha$ -Galactosidase is ubiquitous in legume seeds and vegetables. Filamentous fungi such as many *Aspergillus* (Liu et al., 2007), *Trichoderma* (Savel'ev et al., 1997) and *Penicillium* (Shibuya et al., 1995) species produce generally extracellular  $\alpha$ -galactosidases. Intracellular expression has also been observed such as with *Humicola* sp. (Kotwal et al., 1999).  $\alpha$ -Galactosidase is also widespread in bacteria. Marine proteobacteria and bacterioidetes are reported as good producers of  $\alpha$ -galactosidase that is stable at low temperature and useful for medical and food applications (Bakunina et al., 2014).

For industrial applications of  $\alpha$ -galactosidase, bacteria and fungi are potentially more economic producers (Jin et al., 2001). Plants and animals synthesize this enzyme in lower amounts, necessitating extensive purification for industrial production. Microbial production yield and cost can be more easily optimized with production conditions and use of cheaper substrates. Fungi are considered most suitable because of the extracellular localization of the enzymes produced and availability and stability in a wide range of environmental conditions (Katrolia et al., 2014). Extracellular enzymes are easier to collect and purify. Fungi also give generally higher production yields. Both submerged and solid-state fermentation processes have been used to produce  $\alpha$ -galactosidase (Shankar and Mulimani, 2007; Aleksieva et al., 2010). Agricultural residues have been used as substrate to reduce costs (Gao and Schaffer, 1999; Xiao et al., 2000). Statistical modeling tools like response surface methodology have been used to help optimize process conditions (Liu et al., 2007). Recombinant strains have also been used to increase production (Chen et al., 2000). Compared to fungal sources,  $\alpha$ -galactosidase from yeast has been less explored. For example, intra- and extracellular  $\alpha$ -galactosidases were produced by *Candida guilliermondii* H-404 and *Debaryomyces hansenii* UFFV-1 in media containing melibiose and galactose (Viana et al., 2010, 2011). Several bacteria have been screened for  $\alpha$ -galactosidase production and the culture conditions optimized for different carbon sources, e.g., *S. solfataricus* (Brouns et al., 2006) and *B. breve* (Xiao et al., 2000) on raffinose, and *Bacillus* species on agricultural residues such as wheat bran, rice bran, coconut cake, mustard cake, cotton seed

cake, chana bran and soybean meal (Gote et al., 2004, 2006). Cell immobilization of *Streptomyces griseolalbus* in calcium alginate gel was reported to double  $\alpha$ -galactosidase production yield over the free-cell fermentation (Anisha and Prema, 2008).

## Applications in Food Processing

### Soybean Processing

Soybean protein has a more comprehensive amino acid profile than other vegetable proteins. Soybean-based food has therefore long been considered as promising protein-rich food supplements. The galacto-oligosaccharides in soybeans, however, can cause indigestion problem in monogastric animals and human (Suarez et al., 1999).  $\alpha$ -Galactosidase has been used to reduce the oligosaccharide content in soybean meal, soy protein concentrate, soybean milk and other soybean-based food.

Opazo et al. (2012) produced an enzyme mixture including cellulase and  $\alpha$ -galactosidase by solid-state fermentation of mixed cellulolytic bacteria isolated from different environments. The bacterial mixture included those in the *Streptomyces*, *Cohnella* and *Cellulosimicrobium* genera. They used the enzyme to treat soybean meal and achieved reduction of 83% stachyose, 69% raffinose but only 24% insoluble polysaccharides. Presumably because it contained mainly cellulase and  $\alpha$ -galactosidase, this enzyme was less effective in breaking down pectin which is a major polysaccharide component in soybean meal. More recent studies used the enzymes produced by *Aspergillus niger* and *Trichoderma reesei* to examine the effects of multiple enzyme activities ( $\alpha$ -galactosidase, sucrase, pectinase, xylanase and cellulase) on hydrolysis of soybean meal carbohydrate (Loman and Ju, 2016a,c; Loman et al., 2016).  $\alpha$ -Galactosidase and pectinase were found to be two key enzymes generally insufficiently produced in fungal enzyme mixtures (Coffman et al., 2014; Li et al., 2017) when used for soybean meal processing to enrich protein content and/or remove oligosaccharides and polysaccharides. A new process with partial hydrolysate recycle was developed to retain a large portion of the limiting  $\alpha$ -galactosidase in fungal enzyme mixture so that the carbohydrate monomerization rate could be significantly improved (Loman et al., 2017). Using single-pass retention time of 6 h and recycle rate of 62.5%, the oligosaccharides and polysaccharides were essentially completely hydrolyzed to monosaccharides and the reducing sugar concentration in the harvested hydrolysate reached up to 120 g/L. The continuous-flow recycle process was also shown to potentially increase the volumetric productivity of reducing sugar and protein product by 57% and 300%, respectively, over the corresponding batch process.

Soybean milk is a low-cost substitute for dairy milk and a nutritional supplement for the lactose-intolerant population. It is used in a variety of proteinaceous food and beverage. There are several literature reports on the use of  $\alpha$ -galactosidase from plant, bacterial and fungal sources to remove the anti-nutritional oligosaccharides in soybean milk. For example, Gote et al. (2004) produced a thermostable  $\alpha$ -galactosidase using *Bacillus stearothermophilus* (NCIM 5146). When applying the enzyme at 1 Unit per mL soybean milk at 65 °C for 2 h, they could hydrolyze 100% stachyose and 94% raffinose. In another study, a thermophilic fungus *Humicola* sp. was used to produce an extracellular thermostable  $\alpha$ -galactosidase by solid state fermentation (Kotwal et al., 1999). At the same loading of 1 U per mL soybean milk, this enzyme completely hydrolyzed raffinose and stachyose in 2 h at 50 °C. Cao et al. (2010) used an  $\alpha$ -galactosidase from recombinant *E. coli* together with intestinal protease (trypsin) for degrading oligosaccharides in soybean milk and obtained 25%–30% higher galactose release compared to the use of  $\alpha$ -galactosidase alone.

$\alpha$ -Galactosidase is often immobilized for use in processing soybean products (Anisha and Prema, 2008). Immobilization can improve enzyme reusability and cost-effectiveness (DiCosimo et al., 2013). Natural and synthetic polymers such as calcium alginate, cellulose acetate, chitosan, polyurethane foam, gelatin, and polyacrylamide gel have been used for  $\alpha$ -galactosidase immobilization for soybean milk processing (Sripuan et al., 2003; Kulkarni et al., 2006; Filho et al., 2008; Ansari and Husain, 2010).  $\alpha$ -Galactosidase immobilized in polyacrylamide gel showed better thermal stability (Sripuan et al., 2003). This allows the use of higher process temperature to achieve faster oligosaccharide degradation and limit microbial contamination. Low molecular weight substrates like stachyose and raffinose could easily diffuse into the porous polyacrylamide gel without restricted accessibility to enzyme, which was sometimes a concern for use of immobilized  $\alpha$ -galactosidase. With this immobilized  $\alpha$ -galactosidase, 66% oligosaccharides in soybean milk were degraded in 12 h with retention of 90% enzyme activity and the enzyme could be repeatedly used for at least 4 times (Sripuan et al., 2003). However, polyacrylamide-based enzyme support is not suitable for food application.  $\kappa$ -Carrageenan, a linear, sulfated polysaccharide extracted from marine red algae, was also used successfully to immobilize  $\alpha$ -galactosidase (Girigowda and Mulimani, 2006). At loading of 0.75 U per mL soybean milk, the  $\kappa$ -carrageenan immobilized enzyme degraded more than 76% oligosaccharides in 8 h at 50 °C. However, free enzyme at the same loading hydrolyzed more than 80% of oligosaccharides in 2 h and 91% in 8 h. Despite the lower reaction rate, the immobilized enzyme could be reused; when evaluated in 4 repeated batches, the oligosaccharide reduction was 77%, 72%, 68% and 61% after each successive 4-h processing. The immobilized enzyme by  $\kappa$ -carrageenan was also used in a 150 mL fluidized bed reactor at the same enzyme loading to treat a continuous flow of soybean milk. At 50 °C, this fluidized-bed process reduced 92% oligosaccharides in the soybean milk fed at a 25 mL/h flowrate.

### Other Food Processing

$\alpha$ -Galactosidase has been used for processing other legume foods. Legumes make up an important part of the diet for many people across the world, providing proteins, fiber and other nutrients with lower fat contents. Like soybean, other legumes also contain indigestible galacto-oligosaccharides that can cause abdominal distress. The degradation of galacto-oligosaccharides in various legumes by  $\alpha$ -galactosidase has been demonstrated to be more effective than the traditional methods like soaking and cooking



(Pugalenthi et al., 2006; Katrolia et al., 2014). Pugalenthi et al. (2006) studied the effect of an  $\alpha$ -galactosidase extracted from guar weed *Cassia sericea* on jack bean (*Canavalia ensiformis*) and five types of sword bean (*Canavalia gladiata*). They found 71%–85% reduction of the combined content of raffinose, stachyose and verbascose. In another study, the  $\alpha$ -galactosidase gene (*RmGal36*) from *Rhizomucor miehei* was cloned and expressed in *E. coli* (Katrolia et al., 2012). The enzyme produced was used to hydrolyze the galacto-oligosaccharides in kidney bean flour. There are several other known food processing applications of  $\alpha$ -galactosidase, mainly in the sugar industry; for example, hydrolysis of raffinose in beet sugar syrup is used to improve the crystallization of sucrose.  $\alpha$ -Galactosidase can also be used to improve the gelling properties of galactomannans used as food thickeners (Aleksieva et al., 2010). The enzymes used in food industry are produced by food-grade microorganisms with the Generally Regarded as Safe (GRAS) status.

### Livestock Feed Supplementation

The diets of poultry and swine include high percentages of galacto-oligosaccharide-containing components such as soybean meal. Such diets can cause gastric problems to the non-ruminant animals lacking sufficient intestinal  $\alpha$ -galactosidase. The problems can increase the rate of feed passage in the intestines and reduce the feed utilization. Pre-treatment of animal feed with  $\alpha$ -galactosidase to remove galacto-oligosaccharides improves digestibility and nutritional uptake and reduces the viscosity of digesta. For example, studies have indicated the  $\alpha$ -galactosidase supplementation improves growth performance, nutrient uptake and ileal digestibility of soybean oligosaccharides in piglets and in poultry (Pan et al., 2002; Ghazi et al., 2003). The more advantageous  $\alpha$ -galactosidases for use as feed additives are those active at pH and temperature similar to those in the intestines of mammals and poultry and resistant to protease enzymes (Pan et al., 2002; Ghazi et al., 2003).

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# Influence of Food Processing Operations on Vitamins

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## Importance of Vitamins

Vitamins are organic compounds which cannot be synthesized in adequate quantities by humans and, therefore, must be supplied by our diet. Moreover, they have different regulatory functions that determine the development, physical efficiency, and health status of our body. To date, 15 vitamins have been recognized in human nutrition. Depending on their solubility, vitamins are either classified as water soluble or fat soluble vitamins (Katouzian and Jafari, 2016). As shown in Table 1, water soluble vitamins include vitamin C and the members of the vitamin B group, whereas fat soluble vitamins comprise vitamins A, D, E, and K; also included are the carotenoids that possess varying degrees of vitamin A activity (Pamela et al., 2005; Ball, 2005; Kim and Driskell, 2009).

## Impact of Food Processing Operations on Vitamins

Technological processing applied in the food industry often includes many thermal and non-thermal processes. These processes can have either negative or positive effects on the food materials. Food-processing operations are primarily focused on inactivation of enzymes, food-borne pathogens, prolongation of shelf-life, improved digestibility and bioavailability of nutrients, including augmented antioxidants. In contrast, these processing operations can also bring some unintentional and undesired consequences, such as loss of certain vitamins (Van Boekel et al., 2010; Nayak et al., 2015). Furthermore, the rate of this reduction in vitamin content is influenced by a number of factors: heat, moisture, oxygen, pH, light, oxidising and reducing agents, presence of metallic ions (e.g. iron, copper), and presence of other vitamins (Ottaway, 1993).

Considering the increasing demand of consumers for nutritious and functional foods, manufacturers in the food industry have to develop preservation technologies to ensure that the nutrients of the foods are lost to a minimum. Clearly, different food processing operations such as pasteurization, sterilization, blanching, cooking, frying, high pressure processing, etc. have undesirable effects on vitamins. In this chapter, we will consider these processes in two classes: conventional food processes; and modern processes particularly the non-thermal ones.

## Influence of Conventional Food Processing Operations on Vitamins

Vitamin degradation during heating is dependent on several factors such as the processing method, food type, oxygen, light, moisture, pH, the chemical matrix, type of vitamins, and active enzymes (Awuah et al., 2007; Leskova et al., 2006). A brief overview of studies considering the effect of conventional food processing on vitamins is discussed in this section. Although there are some investigations on the effects of food processing operations on the retention of vitamins, generally speaking, the literature in this field is scarce. More studies have been focused on vitamin C and vitamin B group. In fact, retention of vitamin C during various food processes is a model and control index which predicts the retentions of other vitamins too.

### Vitamin C

Various conventional food processes have been investigated for their effects on the vitamin C content of different fruits and vegetables as shown in Table 2; almost all these investigations have reported to result in some losses of vitamin C. For example, it has been revealed that conventional food processing operations bring about significant decreases of vitamin C content of various foods, such as pasteurization of tomato (Capanoglu et al., 2008), orange (Gil-Izquierdo et al., 2002), guava (Ordóñez-Santos and Vázquez-Riascos, 2010), and strawberry (Klopotek et al., 2005). Blanching as a mild thermal processing has been determined to reduce vitamin C levels in parsley leaves by 47%–51% (Lisiewska and Kmiecik, 1997), and in broccoli by 40% (Wu et al., 1992). Abushita et al. (2000) also reported that during hot-break pulping of tomato, about 38% of its original ascorbic acid was lost, and further processing to produce tomato paste by vacuum evaporation resulted in losses of more than 16% of the ascorbic acid content (Abushita et al., 2000). Moreover, Al-Duais et al. (2009) reported the losses of ascorbic acid, in *Cyphostemma Digitatum* plant, 59% after sun drying.

Vitamin C is very susceptible to chemical and enzymatic oxidation during the processing, storage, and cooking of foods (Ball, 2006). It is clear that raw tomato contains more vitamin C than processed tomato, and there is a higher loss of this vitamin during the production of tomato concentrates than in tomato juice or whole canned tomatoes (Riso and Porrini, 2001). Results from some other studies have also indicated that vitamin C is destroyed mainly due to oxidation reactions and the heat applied in the presence of air during processing (Leoni, 2002). In addition to the effect of oxygen, such high temperature applications themselves cause oxidative stresses. On the other hand, losses of vitamin C are minimal when vegetables are cooked without any water, whereas maximum losses are associated with cooking in a large amount of water (Leskova et al., 2006). For example, Ordóñez-Santos

**Table 1** Classification of the vitamins, their sources and associated anemias

<i>Solubility</i>	<i>Vitamins</i>	<i>Other names/forms</i>	<i>Food sources</i>	<i>Nutritional anemias</i>
Water-soluble	Vitamin C	Ascorbic acid	Tomatoes, strawberries, peppers, broccoli, citrus fruits	Scurvy
	Vitamin B <sub>1</sub>	Thiamine	Pork, beef, yeast, whole grains, legumes	Beriberi, Wernicke-Korsakoff syndrome
	Vitamin B <sub>2</sub>	Riboflavin	Meats, dairy products, enriched cereals, breads, green leafy vegetables	Dermatitis, Cheilosis, Glossitis
	Vitamin B <sub>3</sub>	Niacin Nicotinic acid Nicotinamide	Yeasts, meats, poultry, fish, nuts, and enriched products such as cereals and grains.	Pellagra, Neurological symptoms
	Vitamin B <sub>5</sub>	Pantothenic acid	Liver, yeast, meats, egg yolks, whole grains, potatoes, broccoli, mushrooms, and avocados	Burning foot syndrome
	Vitamin B <sub>6</sub>	Pyridoxine Pyridoxal Pyridoxol Pyridoxamine	Meats, whole-grain products, vegetables, some fruits (e.g. banana), nuts, fortified cereals	Weakness, Sleeplessness, Cheilosis, Glossitis, Stomatitis, Microcytic
	Vitamin B <sub>8</sub>	Biotin	Liver, egg yolks, soybeans, bakers and brewer's yeasts	Dermatitis, Glossitis, Loss of appetite, and nausea
	Vitamin B <sub>9</sub>	Folic acid Folate	Green leafy vegetables, orange juice, dried beans, peas	Macrocytic Megaloblastic Neural tube Defects in the fetus
	Vitamin B <sub>12</sub>	Cobalamin Cyanocobalamin Hydroxocobalamin	Meats, poultry, liver, whole milk, eggs, oysters, fresh shrimp, pork, chicken	Megaloblastic Pernicious Dementia Paranoia Depression
				Night blindness Xerophthalmia Anorexia, Immune deficiencies, Follicular hyperkeratosis
Fat-soluble	Vitamin A	Retinol, Retinal Retinoic acid (A <sub>2</sub> ) $\alpha$ -carotene $\beta$ -carotene $\gamma$ -carotene $\beta$ -cryptoxanthin Echinenon	Meats, egg yolks, fortified food products, dark green leafy vegetables, yellowish-orange fruits and vegetables.	
	Vitamin E	$\alpha$ -Tocopherols $\beta$ -Tocopherols $\gamma$ -Tocopherols $\delta$ -Tocopherols $\alpha$ -Tocotrienol $\beta$ -Tocotrienol $\gamma$ -Tocotrienol $\delta$ -Tocotrienol	Polyunsaturated plant oils, almonds, peanuts	Sensitivity of erythrocytes to peroxide, Abnormal cellular membranes
	Vitamin D	Ergocalciferol (D <sub>2</sub> ) Cholecalciferol (D <sub>3</sub> )	Self synthesis via sun light, fish and fish oils	Rickets Osteomalacia
	Vitamin K	Phylloquinone (K <sub>1</sub> ) Menaquinone (K <sub>2</sub> ) Menadione (K <sub>3</sub> )	Green leafy vegetables, plant oils	Hemorrhagic syndrome

(Adapted from Leskova et al., 2006; Harvey and Ferrier, 2011; Kim and Driskell, 2009)

and Vázquez-Riascos (2010) observed that the production of nectar and pulp from fresh guava decreased vitamin C (28.3%–37%). Consistent with these results, they indicated that the reduction of vitamin C is attributed primarily to the dilution effect generated by addition of water in the product. Another study showed that vitamin C losses during blanching were found to be 10% for sweetcorn, 20% for green beans and 30% for broccoli (Klein, 1997). It has been shown that harvest damage, cutting/slicing, particle size, and type of blancher (steam/water, rotary/cabin) are the main factors affecting the losses of vitamin C (Davey et al., 2000).

Babalola et al. (2010) showed that green leafy vegetables were affected by different processing methods. They reported that boiling (91.5%) and squeeze washing (94.9%) caused more loss than blanching (79.23%). Consequently, it is better to inactivate the oxidase enzyme that destroys vitamin C, blanching vegetables in hot water. Moreover, when comparing various cooking procedures for vegetables, Bureau et al. (2015) observed that boiling was the less suitable method, with a high loss of vitamin C, compared to steaming, microwaving or pressure cooking. Another study conducted by Dewanto et al. (2002) showed that after 2, 15, and 30 min of heating at 88 °C, the vitamin C content significantly decreased by 10.2%–29.4%. Similarly, baking at

**Table 2** The influence of conventional food processing operations on vitamin C

<i>Processing</i>	<i>Product</i>	<i>Result</i>	<i>References</i>
Pasteurization (93 °C)	Tomato	50% ↓	Capanoglu et al. (2008)
Extrusion	Acha/Soybean Blends	No changes	Anuonye et al. (2010)
Blanching	Broccoli	40% ↓	Wu et al. (1992)
Boiling	Spinach	60% ↓	Rumm-Kreuter and Demmel (1990)
Steaming		46.5% ↓	
Pressuõre cooking		58% ↓	
Thermal treatment (88 °C for 2, 15, and 30 min)	Tomato	11%–29% ↓	Dewanto et al. (2002)
Sun drying	Cyphostemma	59% ↓	Al-Duais et al. (2009)
Boiling (10 min at 90 °C)	Spinach	40%–60% ↓	Gil et al. (1999)
Pasteurization	Red tomato	81% ↓	Georgé et al. (2011)
Mild pasteurization (75 °C/30 s)	Orange	12% ↑	Gil-Izquierdo et al. (2002)
Standard pasteurization (95°C/30 s)		19% ↑	
Concentration		2% ↓	
Freezing		2% ↓	
Standard pasteurization (95 °C/30 s)		58% ↓	
Convectional drying (50, 60, and 70 °C)	Sour cherry	71%–73% ↓	Horuz et al. (2017)
Hybrid drying (120, 150, and 180W coupled with hot air at 50, 60, and 70 °C)		63%–84% ↓	
Frozen	Broccoli	50%–55% ↓	Murcia et al. (2000)
Canned (30 min at 121 °C)		84% ↓	
Thermal treatment (Diffrent time and Temperatures)	Beet	1%–8% ↓	Jiratanan and Liu (2004)
Microwaved	Green peas	13% ↓	Hunter and Fletcher (2002)
Boiled		39% ↓	
Overcooked		61% ↓	
Blanching	Parsley	47%–51% ↓	Lisiewska and Kmiecik (1997)
Pasteurization	Strawberry	28%–35% ↓	Klopotek et al. (2005)
Pasteurization	Milk	20% ↓	Moltó-Puigmartí et al. (2011)
Blanching	Pepper	12% ↓	Martínez et al. (2005)
Freeze drying		3% ↓	
Frying		25% ↓	
Roasting		20% ↓	
Low pasteurization	Tomato pure'e	27% ↓	Sánchez-Moreno et al. (2006)
High pasteurization		27% ↓	
Freezing		2% ↑	
High pasteurization plus freezing		31% ↓	
Vacuum Drying			Reis et al. (2017)
50 °C	Litchi	32.78% ↓	
60 °C		25% ↓	
70 °C		34.17% ↓	
Blanching	Vegetables	89.1%–97.3% ↓	Mosha et al. (1995)
Cooking		80.1%–97.4% ↓	
Thermal processing	Pepper	75% ↓	Howard et al. (1994)
Heat Treatments	Milk	16.6%–29.7% ↓	Haddad and Loewenstein (1983)
Pounded and cooked	Cassava leaves	76.87% ↓	Achidi et al. (2008)
Ground and cooked		78.61% ↓	
Baking	Tomato	2%–62% ↓	Gahler et al. (2003)
Thermal treatments	Tomato	10.2%–29.4% ↓	Dewanto et al. (2002)
Boiling	Vegetables	51% ↓	Bureau et al. (2015)
Blanching	Sweetcorn,	10% ↓	Klein (1997)
	Green beans	20% ↓	
	Broccoli	30% ↓	
Pasteurization	Guava pulp	28.3% ↓	Ordóñez-Santos and Vázquez-Riascos (2010)
	Guava nectar	37% ↓	
Sundrying	Green Leafy Vegetables	6.5%–66.9% ↓	Babalola et al. (2010)
Blanching		64.5%–79.23% ↓	
Boiling		74.5%–91.5% ↓	
Squeeze-washing		89.6%–89.9% ↓	
Squeeze-washing with salt		90.5% ↓	
Squeeze-washing and boiling		94.9% ↓	
Heat processing (Toasting)	Edible Winged Termite	16% ↓	Kinyuru et al. (2010)

(Continued)

**Table 2** The influence of conventional food processing operations on vitamin C—cont'd

Processing	Product	Result	References
Heating (98 °C, 10 min)	Apricots, Cherries,	1.2–2.5 fold ↑	Leong and Oey (2012)
Freezing (−20 °C)	Nectarines, Peaches, Plums, Carrots	1.6–2.5 fold ↑	
Thermal processing by increasing nanoparticle concentration (70 °C)			
0%	Tomato juice	37.27% ↓	Jafari et al. (2017)
2%		34.41% ↓	
4%		33.66% ↓	

↓: decrease, ↑: increase.

220 °C of tomato, the vitamin C content significantly dropped by 2%–62% (Gahler et al., 2003). In addition, certain pre-treatments such as thawing cause higher vitamin C loss. Therefore, frozen vegetables must not be thawed before cooking.

These investigations show that vitamin C is a heat unstable vitamin. The losses of vitamin C are primarily due to chemical degradation including oxidation of ascorbic acid to dehydroascorbic acid (DHAA), followed by hydrolysis of the latter to 2,3-diketogulonic acid. Afterwards, polymerization to form other nutritionally inactive products (Dewanto et al., 2002; Ordóñez-Santos and Vázquez-Riascos, 2010) occurs. In a more recent study, Jafari et al. (2017) investigated the effects of thermal processing by nanofluids on vitamin C retention of tomato juice. The results showed that increasing nanoparticle concentration from 0 to 2% and 4% could culminate in the slightly better retention of vitamin C due to shorter process times. On the other hand, Leong and Oey et al. (2012) reported 1.2–2.5 fold and 1.6–2.5 fold increases in vitamin C content of some fruits and vegetables following the heating and freezing, respectively. This study indicated that heating and freezing enhanced the stability of vitamin C, resulting in higher levels after processing compared to fresh. This was due to ascorbic acid oxidase inactivation during heating leading to L-AA protection towards enzymatic oxidation (Leong and Oey, 2012).

### Vitamin E

Some selected studies considering the influence of conventional food processing on the retention of vitamin E has been summarized in Table 3. Generally, vitamin E in foods is predominantly represented by  $\alpha$ -tocopherol. Capanoglu et al. (2008) compared values for the starting material in tomato paste and found that 22% of  $\alpha$ -tocopherol was increased during thermal processing while 69%  $\delta$ -tocopherol and 84%  $\gamma$ -tocopherol were also lost. Seybold et al. (2004) reported that homogenization and sterilization of tomatoes during tomato juice production resulted in significant losses in  $\alpha$ -tocopherol. Also, extrusion process variables could affect this vitamin content. For instance, extrusion cooking was determined to decrease vitamin E levels in cereals by 63%–94% (Zielinski et al., 2001), and in buckwheat by 63% (Zieliński et al., 2006). Moreover, Thammapat et al. (2016) observed that the parboiling process significantly decreased the concentrations of  $\alpha$ - and  $\gamma$ -tocopherols as compared to raw rice (18%–25%). Similarly, Stuetz et al. (2017) noted that concentrations of  $\alpha$ -tocopherol were decreased in hazelnuts, almonds and walnuts (20%–54%). The effects of processing on the content of vitamin E in foods have been the subject of limited investigations. In another study, Murcia et al. (1999) observed that boiling, omelette, and microwaving of egg yolk led to a 21.63%, 53.24%, and 43.12% reduction in vitamin E activity, respectively. The possible explanations could be the formation of peroxides as intermediate products in the autooxidation of the fatty acids, destroying the oxidation sensitive vitamins such as vitamin E since it would react with the peroxides (Murcia et al., 1999).

### Vitamin A

Similar to the observations for vitamin C, loss of  $\beta$ -carotene also varies depending on the type of vegetable (the matrix) as well as the processing method applied (Table 3). It has been reported that boiling and frying of Thai vegetables led to a 14% and 24% reduction in vitamin A activity, respectively (Speek et al., 1988). Moreover, according to Achidi et al. (2008), processing and cooking of cassava leaves caused minimal decrease in  $\beta$ -carotene content. Bolin and Stafford (1974) found that sun-drying (30%), shade-drying (10.1%), and dehydrating (9.2%) of apricot resulted in significant losses in  $\beta$ -carotene. Similarly, thermal processing of peppers resulted in a 25% decrease of total provitamin A activity (Howard et al., 1994).

On the other hand, Mosha et al. (1997) reported that the increase in vitamin A activities were in the range of 32.5%–63.1% for blanched vegetables, and 68.6%–228.6% for cooked vegetables. In another study conducted by Sánchez-Moreno et al. (2006), it was revealed no significant differences in vitamin A contents among low pasteurization, high pasteurization, freezing, high pasteurization plus freezing purée samples in comparison with the untreated sample. As seen in the examples, more researchers have studied the effect of different methods of cooking and processing on the levels of both vitamin A and their vitamers in foods. These results show that vitamin A is sensitive to destruction by heat, light and oxygen. Moreover, thermal food processing can result in remarkable losses of vitamin A activity.



**Table 3** The influence of conventional food processing operations on different vitamins (other than Vitamin C)

Processing	Product	Vitamin	Result	References
Pasteurization (93 °C)	Tomato	$\alpha$ -tocopherol	22% ↑	Capanoglu et al. (2008)
		$\beta$ -tocopherol	128% ↑	
		$\delta$ -tocopherol	69% ↓	
		$\gamma$ -tocopherol	84% ↓	
		$\beta$ -carotene	36% ↓	
Extrusion	Buckwheat	Vitamin E	63% ↓	Zieliński et al. (2006)
Extrusion	Whole-grain (wheat, barley, rye, oat)	Vitamin E	63%–94% ↓	
Extrusion	Oats, maize maize + peas	Thiamin (B <sub>1</sub> )	39%–77% ↓	Athar et al. (2006)
		Riboflavin (B <sub>2</sub> )	14%–30% ↓	
		Niacin (B <sub>3</sub> )	25%–40% ↓	
		Pyridoxine (B <sub>6</sub> )	65%–82% ↓	
Extrusion	Acha/soybean blends	Riboflavin (B <sub>2</sub> )	6% ↓	Anuonye et al. (2010)
		Pyridoxine (B <sub>6</sub> )	86.36% ↓	
Thermal treatment (120 °C for 30 s)	Carrot juice	Vitamin A	49% ↓	Chen et al. (1995)
Sun drying	Digitatum plant	Vitamin E	22% ↑	Al-Duais et al. (2009)
High vacuum flame sterilization	Tuna flake	Thiamin (B <sub>1</sub> )	45% ↓	Seet et al. (1983)
		Riboflavin (B <sub>2</sub> )	16% ↓	
		Niacin (B <sub>3</sub> )	14% ↓	
		$\beta$ -carotene	8% ↓	
Pasteurization	Red tomato	Folic acid	23%–39% ↓	Georgé et al. (2011)
Thermal treatment (various time and Temperatures)	Beet			Jiratanan and Liu (2004)
Roasting (140 °C for 25 min or 160/170 °C for 15 min)	Nuts	Thiamine (B <sub>1</sub> )	11%–84% ↓	Stuetz et al. (2017)
		Riboflavin (B <sub>2</sub> )	–6 – 6% ↑	
		Pyridoxine (B <sub>6</sub> )	–4 – 22% ↑	
		$\alpha$ -Tocopherol	20%–54% ↓	
Parboiling	Rice	Vitamin E	18%–25% ↓	Thammapat et al. (2016)
Pasteurization	Orange-carrot Juice	Vitamin A	7.8% ↑	Torregrosa et al. (2005)
Low pasteurization	Tomato pure'e	Vitamin A	6% ↑	Sánchez-Moreno et al. (2006)
High pasteurization			3% ↑	
Freezing			12% ↑	
High pasteurization plus freezing			12% ↑	
Boiling	Egg yolk	Vitamin E	21.63% ↓	Murcia et al. (1999)
Omelette			53.24% ↓	
Microwaving			43.12% ↓	
Blanching			19%–23% ↓	
Cooking	Vegetables	Riboflavin	10%–91% ↑	Mosha et al. (1995)
		Thiamine	67.7%–85.6% ↓	
			3%–87% ↓ or 15%–27% ↑	
			29.3%–88.6% ↓	
Blanching	Vegetables	Vitamin A	32.5%–63.1% ↑	Mosha et al. (1997)
Cooking			68.6%–228.6% ↑	
Cooking	Egg yolk	cholecalciferol (D <sub>3</sub> )	1%–6% ↓	Mattila et al. (1999)
		hydroxycholecalciferol (25-OH-D <sub>3</sub> )	5%–11% ↓	
		ergocalciferol (D <sub>2</sub> )	1%–14% ↓	
		cholecalciferol (D <sub>3</sub> )	2%–23% ↓	
Water blanching	Lima bean	Vitamin B <sub>6</sub>	19%–24% ↓	Raab et al. (1973)
Steam blanching			13%–17% ↓	
Thermal processing	Pepper	Provitamin A	25% ↓	Howard et al. (1994)
Dehydrating	Apricot	$\beta$ -carotene	9.2% ↓	Bolin and Stafford (1974)
Shade drying			10.1% ↓	
Sun drying	Milk		30% ↓	Haddad and Loewenstein (1983)
Heat Treatments		Thiamine	7.9%–11.9% ↓	
		Riboflavin	0.9%–2.7% ↓	
			25% ↓	
Thermal Processing	Soymilk	Riboflavin	29% ↓	Kwok et al. (1998)

(Continued)

**Table 3** The influence of conventional food processing operations on different vitamins (other than Vitamin C)—cont'd

<i>Processing</i>	<i>Product</i>	<i>Vitamin</i>	<i>Result</i>	<i>References</i>
Heat processing (Toasting)	Edible Winged Termite	Pyridoxine (B <sub>6</sub> )	4% ↓	Kinyuru et al. (2010)
		Folic acid	37% ↓	
		Ascorbic acid	16% ↓	
		Niacin (B <sub>3</sub> )	21% ↓	
		Riboflavin (B <sub>2</sub> )	34% ↓	
		Retinol	30% ↓	
Pounded and cooked	Cassava leaves	α-Tocopherol	20% ↓	Achidi et al. (2008)
		Thiamine (B <sub>1</sub> )	35.14% ↓	
		β-Carotene	6.76% ↓	
Ground and cooked	Cassava leaves	Thiamine (B <sub>1</sub> )	38.95% ↓	Achidi et al. (2008)
		β-Carotene	5.29% ↓	
Boiling	Vegetables	Folate	68% ↓	Bureau et al. (2015)
		B-carotene	9% ↓	
		Folate	53% ↓	
Cooking	Boiled broccoli	Folate	36% ↓	Bassett and Sammán (2010)
	Boiled spinach		10% ↓	
	Boiled onion		37% ↓	
	Baked onion		86% ↓	
	Roasted liver		31% ↓	
	Fried hen egg		2% ↓	
	Boiled hen egg		6% ↓	
	Fried yolk		3% ↓	
	Boiled yolk		57% ↓	
	Boiled lentil		5% ↓	
	Boiled soybean		26% ↓	
	Boiled potato		35% ↓	
	Kisra	Thiamine (B <sub>1</sub> )	16% ↑	
		Riboflavin (B <sub>2</sub> )	10% ↓	
Fermentation	Hulu-mur	Thiamine (B <sub>1</sub> )	15% ↓	Mahgoub et al. (1999)
		Riboflavin (B <sub>2</sub> )	88.5% ↓	
		Thiamine (B <sub>1</sub> )	48.4% ↓	
Baking	Hulu-mur	Riboflavin (B <sub>2</sub> )	< 10% ↓	Mahgoub et al. (1999)
		Thiamine	↔	
		Pantothenic acid	< 10% ↓	
Pasteurization	Milk	Biotin	< 10% ↓	Chapman et al. (1957)
		Vitamin B <sub>12</sub>	< 10% ↓	
		Thiamine	30%–50% ↓	
		Riboflavin	< 10% ↓	
		Pantothenic acid	< 10% ↓	
		Biotin	< 10% ↓	
Sterilization		Vitamin B <sub>12</sub>	90%–100% ↓	
		Thiamine	40%–50% ↓	
		Riboflavin	< 10% ↓	
		Pantothenic acid	10%–15% ↓	
		Biotin	< 10% ↓	
		Vitamin B <sub>12</sub>	90%–100% ↓	
Ultra-high temperature processing		Thiamine	30%–40% ↓	
		Riboflavin	↔	
		Pantothenic acid	< 10% ↓	
		Biotin	10%–15% ↓	
		Vitamin B <sub>12</sub>	90% ↓	
		Thiamine	10%–15% ↓	
Evaporation		Riboflavin	< 10% ↓	
		Pantothenic acid	< 10% ↓	
		Biotin	10%–15% ↓	
		Vitamin B <sub>12</sub>	90% ↓	
		Thiamine	10%–15% ↓	
		Riboflavin	< 10% ↓	
Spray drying		Pantothenic acid	< 10% ↓	
		Biotin	10%–15% ↓	
		Vitamin B <sub>12</sub>	35% ↓	

↓: decrease, ↑: increase, ↔: no changes.

### Vitamin B Group

Athar et al. (2006) reported a significant decrease in vitamin B<sub>1</sub> (39%–77%), vitamin B<sub>2</sub> (14%–30%), vitamin B<sub>3</sub> (25%–40%), and vitamin B<sub>6</sub> (65%–82%) in extruded food products. Similarly, Anuonye et al. (2010) indicated a 6% decrease in riboflavin (vitamin B<sub>2</sub>) and 86.36% decrease in pyridoxine (vitamin B<sub>6</sub>) after extrusion of Acha/soy bean blend. In another study, it was revealed that high vacuum flame sterilization of tuna flake led to a 14% reduction in vitamin B<sub>3</sub> (Seet et al., 1983). Jiratanan and Liu (2004) observed that thermal treatments of beet caused a 23%–39% decreases in folic acid. Al-Khalifa and Dawood (1993) indicated that Thiamin (vitamin B<sub>1</sub>) was more sensitive to heat than was riboflavin. Moreover, the study reported that higher losses of thiamin occurred during roasting and deep-frying, while braising and microwave cooking resulted in lower losses. Bassett and Sammán (2010) investigated the retention of folate in foods after using different cooking processing. They reported that the folate retention was in the range 14%–99% according to both type of food and method of processing. Another study conducted by Achidi et al. (2008) concluded that thiamine losses from 35.14% to 38.95% occurred while processing cassava leaves. In a separate study, Kinyuru et al. (2010) observed that the toasting of frozen green vegetables, pyridoxine (B<sub>6</sub>), folic acid, niacin (B<sub>3</sub>), and riboflavin (B<sub>2</sub>) was found to result in approximately 4%, 37%, 21%, and 34% losses, respectively.

Kwok et al. (1998) investigated effect of thermal processing on thiamine and riboflavin content in soy milk. They reported that thiamine was found to be much more heat sensitive than riboflavin. In another study, Mahgoub et al. (1999) noted that fermentation of kisra increased riboflavin (16%) but decreased thiamine (35%), whereas fermentation of hulu-mur reduced the levels of both riboflavin (15%) and thiamine (10%). Moreover, the same researchers observed that riboflavin was not affected by baking of kisra but thiamine level was markedly decreased. Hulu-mur baking caused reduction of both thiamine and riboflavin. These high losses may be attributed to the both long baking times and high baking temperatures. Chapman et al. (1957) reported that pasteurization (except riboflavin), sterilization, ultra-high temperature processing, evaporation (except riboflavin), and spray drying led to a decrease in all B complex vitamins. Furthermore, 7.9%–11.9% loss of thiamine and 0.9%–2.7% loss of riboflavin was observed on heat processing of milk (Haddad and Loewenstein, 1983).

Raab et al. (1973) showed that steam blanching (83%–87%) of lima beans may have somewhat improved retention compared to water blanching (76%–81%). Kōmura et al. (1990) investigated the effects of different cooking methods, including boiling, steaming, parching, frying on thiamine recovery and noted that the loss of thiamine was largest in boiling (70%), followed by parching (35%) and frying (30%). In a separate study, Mosha et al. (1995) investigated that effect of blanching and cooking processing on the retention of riboflavin and thiamine. They found a decrease in thiamine content by blanching (67.7%–85.6%) and cooking (29.3%–88.6%) of vegetables. This is explained by the water-soluble nature of the vitamin being leached out into the water. Moreover, the losses of thiamine content may have resulted from severity of heat treatment. The rate of thiamine degradation is accelerated by increase in temperature and pH (Kinyuru et al., 2010). In contrast, it has been shown that blanching and cooking resulted in a significant increase in riboflavin content in some vegetables (cowpea, peanut and pumpkin greens (10%–91%)), whereas in amaranth and sweet potato leaves (19%–87%), a significant decrease was observed. These increases in riboflavin are supposed to result from an increased tissue breakdown and increased accessibility of the vitamin to the extracting solvent. Kwok et al. (1998) reported that riboflavin is more heat-stable than is thiamin, and its thermostability is independent of heating methods. Some other results have been briefly shown in Table 3.

### The Influence of Modern and Non-thermal Food Processing Operations on Vitamins

Traditional thermal processing led to remarkable losses in nutritional quality as mentioned in the previous section. Considering the increasing consumer fresh-like products demands, food industries have directed their studies to the search for alternative processing technologies including UV light, high intensity light pulses,  $\gamma$ -irradiation, pulsed electric fields, radiofrequency electric fields, Ohmic heating, microwave heating, ultrasonication, high hydrostatic pressure, supercritical carbon dioxide, ozonation, and flash vacuum (Jiménez-Sánchez et al., 2017). In general, it has been revealed that these technologies seem to be less detrimental than the thermal processing. In this section, the effects of modern and non-thermal food processing operations on retention of vitamins will be discussed and a brief overview of the relevant studies have been shown in Table 4.

#### Pulsed Electric Field Processing

Pulsed electric fields (PEF) have been developed during the last decades as an alternative to thermal pasteurization of liquid foods. A number of studies have shown high vitamin C retention after PEF processing compared to the thermal treatment (Table 4). For example, Odriozola-Serrano et al. (2008) reported that vitamin C retention just after treatment in heat treated tomato juice was 79.2%, whereas in PEF-processed juice, there was a 86.5% retention. Moreover, Elez-Martínez and Martín-Belloso (2007) reported that high intensity pulsed electric field (HIPEF) treated orange juice (87.5%–98.2%) and gazpacho (84.3%–97.1%) always showed a vitamin C retention higher than that of the heat-pasteurized products. Similarly, Elez-Martínez et al. (2006) showed that HIPEF-processing resulted in higher ascorbic acid content in orange juice (91.2%) than thermal pasteurization (82.8%).

In the study conducted by Salvia-Trujillo et al. (2011), they observed 99.5%–97.0% vitamin C retention after applying HIPEF treatment to the fruit juices. Sánchez-Vega et al. (2015) reported that HIPEF-treated broccoli juice exhibited greater relative content of  $\beta$ -carotene than the juice treated by heat. Similarly, Odriozola-Serrano et al. (2009) showed that  $\beta$ -carotene has been shown to substantially increase in PEF-processed tomato juices, whereas  $\gamma$ -carotene content is slightly depleted (3%–6%). Odriozola-Serrano et al. (2013) proposed that HIPEF processing could trigger the biosynthesis of carotenoids, resulting in an increment of some of them. On the other hand, Oms-Oliu et al. (2009) indicated that HIPEF processing of watermelon juice led to a 4%–60% reduction

**Table 4** The influence of non-thermal food processing operations on vitamins

Processing	Product	Vitamin	Result	References
High-intensity pulsed electric fields	Fruit juice	Niacin	↔	Salvia-Trujillo et al. (2011)
		Thiamine	↔	
		Riboflavin	↔	
Pulsed electric field	Broccoli juice	Vitamin C	25.4 ↓	Sánchez-Vega et al. (2015)
		β-carotene	30.5% ↑	
Pulsed electric field	Orange juice	Vitamin C	8.8% ↓	Elez-Martínez et al. (2006)
Pulsed electric field	Tomato juice	β-carotene	37% ↑	Odriozola-Serrano et al. (2009)
		γ-carotene	3%–6% ↓	
High intensity pulsed electric field	Orange juice	Vitamin C	10.7%–12.5% ↓	Elez-Martínez and Martín-Belloso (2007)
	Gazpacho		2.9%–15.7% ↓	
High-pressure processing	Orange juice–milk	Vitamin E	7–28% ↑	Barba et al. (2012)
		Vitamin D	↔	
Ultra-high-pressure homogenization	Almond beverage	α-tocopherol	83%–93% ↓	Toro-Funes et al. (2014)
High pressure processing	Orange juice	Vitamin A	38.74% ↑	Sánchez-Moreno et al. (2005)
High pressure processing	Tomato puree	Vitamin A	39% ↑	Sánchez-Moreno et al. (2006)
		Vitamin C	29.6% ↓	
High-pressure processing	Human milk	Vitamin C	1%–4% ↓	Moltó-Puigmarí et al. (2011)
		α-tocopherol	1%–6% ↓	
High-intensity pulsed electric field	Watermelon juice	Vitamin C	4%–60% ↓	Oms-Oliu et al. (2009)
Ultrasound treatment	Gooseberry juice	Vitamin C	24.4%–78.81% ↓	Ordóñez-Santos et al. (2017)
		Provitamin A	25%–96.24% ↑	
Ultra-high hydrostatic pressure	Strawberry coulis	Vitamin C	11% ↓	Sancho et al. (1999)
	Egg yolk		1.34% ↑	
High intensity pulsed electric field	Tomato juice	Vitamin C	13.5% ↓	Odriozola-Serrano et al. (2008)
High-intensity pulsed electric field	Orange-carrot juice	Vitamin A	6%–69% ↑	Torregrosa et al. (2005)
Ultrasound treatment	Tomato juice	Vitamin C	3.1%–39.3% ↓	Adekunte et al. (2010)

↓: decrease, ↑: increase, ↔: no changes.

in vitamin C. According to these researchers, electric field strength, pulse frequency, pulse width, polarity and treatment time significantly affected vitamin C of watermelon juice. In contrast, Salvia-Trujillo et al. (2011) observed that HPEF treatment did not affect the concentration of group B vitamins, but thermally treated beverages showed lower riboflavin (vitamin B<sub>2</sub>) concentration.

### High-pressure Processing

Similar to other non-thermal food processing operations, high-pressure processing (HPP) has a profound effect on the stability of vitamins in foods. For example, Barba et al. (2012) reported an increase of total tocopherol content (7%–28%) in orange juice-milk blend treated with HPP, mainly due to an increase in α-tocopherol content. These increases in α-tocopherol are supposed to result from an increased disruption of the chloroplasts where α-tocopherol is confined. Sánchez-Moreno et al. (2005) showed that HPP led to an increased vitamin A value (38.74%). Moreover, Sánchez-Moreno et al. (2006) showed that HPP of tomato puree showed the highest vitamin A value (39% ↑) among the samples. A possible explanation is this fact that HPP has the potential to enhance the release of vitamins from vegetables (Sánchez-Moreno et al., 2005, 2006). Toro-Funes et al. (2014) reported that ultra-high-pressure homogenization treatment of almond beverages led to a decrease in α-tocopherol content (83%–93%). On the other hand, Escobedo-Avellaneda et al. (2015) reported that higher vitamin C content than untreated controls were observed for high hydrostatic pressure-treated oranges. The researchers suggest that high hydrostatic pressure increases the release of vitamin C probably due to cellular disruption, making them more accessible for quantification. Similarly, Vega-Gálvez et al. (2012) reported that at 400 MPa, vitamin C showed the maximum retention (93%) and vitamin E increased compared with the initial value of the gel.

### Ultrasound Processing

Ultrasound processing is a technology that has been extensively investigated in recent years. Studies on the influence of ultrasound processing suggests that it has beneficial effects on vitamin C level in some juices. For instance, Tiwari et al. (2009) showed that greater vitamin C (74.5%) retention was happened in sonicated orange juice. Moreover, Cheng et al. (2007) revealed that guava juice treated with both sonication and carbonation showed the highest ascorbic acid contents. Jabbar et al. (2015) reported a significant increase in ascorbic acid of carrot juice treated with ultrasound at 20 °C when compared with fresh untreated juice samples. Aguilar et al. (2017) reported that the ascorbic acid was retained in fruit juices after the ultrasound processing. Moreover, Cruz et al. (2008) suggested that the thermosonication treatment was found to be a better blanching process, since it inactivates watercress peroxidase at less severe blanching conditions and therefore retains higher vitamin C contents.

Rawson et al. (2011) found a higher retention of ascorbic acid at low ultrasound treatments. The higher retention level of ascorbic acid due to thermosonication treatment might be attributed to the milder heat processing as compared to heat treatment. Another reason could be that samples treated with thermosonication helped to eliminate the dissolved oxygen; thus it delayed ascorbic acid degradation (Anaya-Esparza et al., 2017; Cheng et al., 2007; Jabbar et al., 2015). In contrast, Ordóñez-Santos et al. (2017) reported that ultrasound processing of gooseberry juice was found to result in 24.4%–78.81% vitamin C and 25%–96.24% provitamin A losses. Adekunle et al. (2010) showed that the concentration of ascorbic acid in tomato juice ranged from 96.9% to 60.7%. Furthermore, Gamboa-Santos et al. (2013) showed that conventional blanching treatments at high temperature gave rise to carrots with retention of vitamin C (37.5%–85%) higher than blanching with US-probe (4%). It is possible that the degradation of ascorbic acid is attributed to oxidation processes generated during ultrasonic treatments. Moreover, hydrogen ions, free radicals, and hydrogen peroxide are created during the sonolysis of water molecules. Therefore, it could be related to oxidation reactions, enhanced by the interactions with them (Adekunle et al., 2010).

## Strategies to Retain Vitamins During Food Processing Operations

Many studies considering the processing effects on vitamins showed that thermal treatments could adversely affect vitamins compared with non-thermal treatments. Therefore, protection of vitamin is an important task for food producers. In order to decrease degradation of vitamins, preparation of foods should be done according to appropriate procedures including avoiding overcooking; shortening cooking time; cooking with a minimum water, etc. Moreover, steaming, stirfrying, and pressure cooking bring about less vitamin losses than boiling or classical pan frying (Ball, 2005). Another promising approach for protecting vitamins can be micro/nano-encapsulation (Katouzian and Jafari, 2016). For example, Desai and Park (2005) reported that the vitamin C was found to be stable after encapsulation. Another study conducted by Abbasi et al. (2014) indicated that vitamin D<sub>3</sub> encapsulated in nanoparticles degraded less in the presence of oxygen. Assadpour et al., (2016a,b, 2017) and Assadpour and Jafari (2017) also reported that nanoencapsulation of folic acid within double emulsions could result in better protection of this vitamin resulting in more bioavailability of folic acid and targeted release in large intestine. Righetto and Netto (2006), furthermore, investigated the effect of the encapsulating materials on the stability of two sources of vitamin C, green West Indian cherry juice and synthetic ascorbic acid. They observed that the vitamin C of green West Indian cherry capsules was more stable than synthetic ascorbic acid. It has been shown that phenolic compounds present in the juice have an important role in vitamin C protection.

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# Microbial Xylanases in Bread Making

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## Glossary

**Arabinoxylan** A hemicellulose found in both the primary and secondary cell wall of plants, including woods and cereal grains, comprising two pentose sugars viz. arabinose and xylose.

**Bake** Food cooking by dry heat without exposure of direct flame, typically in an oven.

**Bread** A food made of flour, water, and yeast mixed together followed by baking.

**Crumb** A small fragment of bread, cake, or other bakery products.

**Dough** A thick, moldable mixture of flour and liquid used for baking into bread.

**Enzyme** A protein catalyzing a specific biochemical reaction.

**Farinograph** A recording dough mixer that measures and records the torque developed by the action of mixer blades on the dough at time of mixing.

**Fermentation** Microbial breakdown of a substance that gives rise to effervescence and heat.

**Gluten** It is composed of two proteins present in wheat. It is also responsible for the elastic texture of dough.

**Knead** Mold/work the moistened flour into dough with hands.

**Loaf** A quantity of bread, which is shaped and baked in one piece and usually sliced before being eaten.

**Microorganism** A microscopic organism especially a bacterium, virus, and fungus, which is not seen by naked eyes.

**Proofing** It is the final rise of shaped bread dough before baking.

**Rheology** The branch of physics, which deals with the deformation and flow of matter, especially the non-Newtonian flow of liquid and the plastic flow of solids.

**Staling** Decrease in the palatability of bread and other bakery products, but microbial contamination is not included in this.

**Starch** A tasteless, odorless, and white substance occurs widely in plant tissues and chiefly obtained from cereals and potatoes. It is a homopolymer of glucose.

**Xylanase** A hydrolytic enzyme that cleaves the  $\beta$ -1,4 backbone of xylan found in plant cell wall.

## Introduction

Bread is a staple and basic food item, made by baking process in an oven. It is popular around the world and is one of the oldest artificial foods, having been of importance since the dawn of agriculture. Bread is considered as one of the most consumed baked product known to humans and also a good source of iron. Bread is served in different ways at any time of meal of the day, as a snack, as an ingredient for preparation of sandwiches, as a coating for fried items to prevent sticking. In market, breads are sold as white bread, brown bread, wheat meal bread, fruit bread, fancy bread, milk bread, and bun or masala bread or by any other name prepared from a mixture of wheat flour, water, salt, yeast or their fermentative medium, having one or more other ingredients such as milk (whole or skimmed or condensed), curd, gluten, whey, honey, sugar, liquid glucose, malt product, edible starches, margarine or refined edible oil of suitable type or butter or their mixture, protein concentrate, lime water, etc., and other desired components (Khetarpaul et al., 2005).

Wheat flour, a mixture of gluten, lipids, starch and nonstarch polysaccharides, and enzymes, is usually used as a raw material for bread making. The complex biochemical and biophysical processes begin after mixing flour, yeast, and water together and characterizing the dough phase. To improve the baking process, some exogenous enzymes are added in the dough to reduce bread preparation time, decrease staling rate, compensate flour variability, and replace chemical additives (Tramper and Poulsen, 2005). In bakery products, starch is the main component, which acts as a water binder, thickener, fat substitute, gelling agent, and emulsion stabilizer (Synowiecki, 2007). Rheological properties of dough are affected by other components of flour such as arabinoxylans and lipids (Goesaert et al., 2005; Georgopoulos et al., 2006; Gandikota and MacRitchie, 2005). Gluten makes dough more elastic, viscous, cohesive, and extensible with improved water absorption capacity (Wieser, 2007). Goesaert et al. (2009) reported that wheat flour has unique ability to make viscoelastic dough having gas holding properties because of its gluten protein. In addition to starch and gluten protein, nonpolysaccharides (arabinoxylans), enzymes, and lipids of wheat flour remarkably improve the bread making process (Stojceska and Butler, 2012; Peressini and Sensidoni, 2009; Goesaert et al., 2005, 2009). Pareyt et al. (2011) reported that lipids are also important components during bread making, as they have different valuable properties during storage and processing of bread. It was reported that 2% lipids were present in wheat flour either in free form or bounded with wheat components (Belitz et al., 2004), and further they were categorized into starch lipids and free or bound nonstarch lipids on the basis of solubility in different solvents with different polarities (van der Borgh et al., 2005).

## Bread Making Process

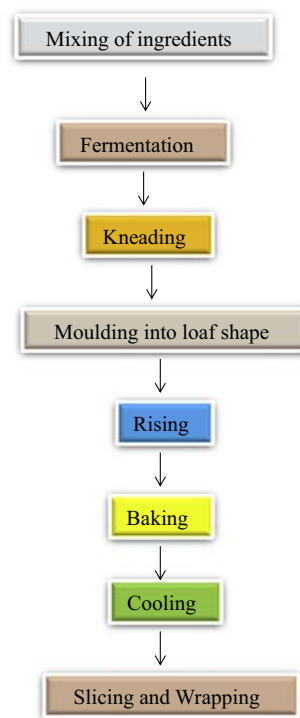
The process of bread preparation can be divided into three main steps that are mixing, fermentation, and baking (Fig. 1). Baking process modifies the taste, aroma, texture, and sensory properties of goods made of raw materials (Abdullah, 2008). Therefore, baking process started a chain of biochemical, chemical, and physical reactions in the product, which includes water evaporation, expansion of volume, denaturation of protein, gelatinization of starch, crust and porous structure formation, and browning reaction (Sablani et al., 2002). Bread is made of elastic, unstable, solid structure made of continuous elastic network of crosslinked molecules of gluten protein and of leached molecules of starch polymer, mainly amylose (Gray and Bemiller, 2003). The properties of final product are affected by mechanical and physical mixing, enzyme catalyzed chemical reactions, and by thermal effects, including baking time and temperature.

Principle stages of bread making process:

1. **Dough formation:** The difference between all kinds of bread making processes is due to the way of dough kneaded. On the basis of method of dough formation, the process is categorized into straight dough method, short dough method, sponge dough method, etc. In this stage, all the ingredients are mixed thoroughly and with the help of mixer the gluten development, air bubble incorporation, and production of dough with desired rheological properties are achieved.
2. **Fermentation and proofing:** During this stage, the yeast acts on fermentable sugars and produces carbon dioxide and ethanol. Ethanol evaporates completely during baking leaving no bad/adverse effect on bread quality, and gluten film entrapped the carbon dioxide gas, which expands during baking process and increases the volume of the bread with porous texture.
3. **Baking process:** This is the final stage of bread making in which because of action of heats the dough is firmed; its structure is stabilized and converted into final baked product (Khetarpaul et al., 2005). The whole process of bread making is summarized in Fig. 1.

## Bread Formulation

So far many different varieties of bread have been developed. In different regions these formulations developed on the basis of seven traditional food habits of the people. Enzymes play an important role in bread preparations by improving its properties. Xylanases have great use in baking industries because they enhance quality of the bread by improving volume of bread, bread crumb structure,



**Figure 1** Whole process of bread making.

**Table 1** Breads categorized on the basis of use

Bread	Features	Area of popularity
Pan bread	These breads are popular in economically developed countries, including Canada, United States, United Kingdom, and European nations.	USA, United Kingdom, Canada
Hearth or sour bread	These breads are produced with or without lactic acid fermentation in an open hearth and are becoming popular in France.	France
Flat bread or chapati/roti	They are unfermented and flat, baked on a flat hot pan. This category is popular in Asian countries.	Asian countries
Rolls and other small fermented breads	These breads generally have higher levels of fat and sugars in formulation. Thus they have sweeter taste and softer bite characteristics.	Europe especially in Germany, Australia, Sweden, Finland, Switzerland, etc.

decrease stickiness of bread, and also significantly increase shelf life and staling of bread when used at optimum levels. Therefore, in baking industries there is an increasing demand toward the application of xylanase for bread preparation (Butt et al., 2007). Xylanases (endo-1,4- $\beta$ -D-xylan xylanohydrolase) are the major group of enzymes, which hydrolyze xylan and release xylose, as well as long- and short-chain xylooligosaccharides (Kheng and Omar, 2005). Xylans are the main constituents of hemicellulose, the world's second resource after cellulose. In cereals, xylans constitute a large amount of L-arabinose, which is known as arabinoxylans. In food industries, major advantageous properties of xylanases are their high stability and optimum activity at acidic pH, and therefore, xylanases are widely applied in food industries (Polizeli et al., 2005; Butt et al., 2008; Harris and Ramalingam, 2010). In bread making, fundamental role of xylanase is the degradation of unextractable arabinoxylan to water-extractable xylan and thus allows the water redistribution in dough with formation of gluten network (Goesaert et al., 2005). This process improves the properties (extensibility, elasticity, and softness) of dry dough. Different breads are categorized on the basis of use in Table 1.

### Microbial Sources of Xylanases

Microorganisms have been regarded as an important source of enzymes, as they can reproduce in higher rates and make industrially important bioactive compounds. The application of enzymes as industrial biocatalysts has been increased exceptionally in recent years, as the enzymes offer more advantages over the application of traditional chemical catalysts. Enzymes exhibit high catalytic activity, are highly specific to substrate, can be produced in large amount at a time, are economically viable, and are not dangerous for our environment because of their highly biodegradable nature (Gote, 2004). In this context, xylanases of microbial sources are preferred biocatalyst for xylan hydrolysis because of their high specificity, negligible substrate loss, mild reaction conditions, potential application on feed, food, and other industries and have no side-product generation (Goswami and Pathak, 2013; Collins et al., 2005). Complete xylanolytic enzyme systems with all of these properties have been found in bacteria, fungi, and actinomycetes (Kulkarni et al., 1999; Sunna and Antranikian, 1997; Elegir et al., 1994). *Aspergillus*, *Trichoderma*, *Streptomyces*, *Clostridia*, *Chytridiomycetes*, *Fibrobacters*, *Phanerochetes*, *Bacillus*, and *Ruminococcus* are some of the important xylanase-producing microorganisms. Filamentous fungi are microorganisms of interest for production of xylanase and other accessory enzymes for xylan degradation, as fungi secrete enzymes into medium and level of enzymes production is higher as compared with yeasts and bacteria. Adsul et al. (2005) reported *Pichia*, *Trichoderma*, *Fusarium*, and *Aspergillus* as great xylanase producers. Qinnghe et al. (2004) reported that white-rot fungi produced extracellular xylanases acting on hemicellulosic substances and produced metabolites useful in different industries such as food, cosmetic, and pharmaceuticals. *Cunninghamella subvermispora* produced xylanase by using polysaccharides of plant cell wall as substrate (de Souza-Cruz et al., 2004). Noteworthy, Subramaniyan and Prema (2002) reported that *Bacillus* sp. produced high level of xylanase at alkaline pH and high temperatures. It was reported that xylanases from various microorganisms, such as *Bacillus firmus*, *Geobacillus thermolevorans*, *Actinomadura* sp. Cpt20, *Streptomyces* sp. S27, and *Saccharopolyspora pathunthaniensis* S582, showed optimum activity at higher (65 to 90°C) temperatures (Verma and Satyanarayana, 2012). Yoon et al. (2004) reported a xylanase from *Thermotoga* sp. having optimum temperature at 100 and 105°C. Mmango-Kaseke et al. (2016) reported xylanase production by *Micrococcus* sp. SAMRC-UFH3 isolated from sawdust under SmF condition. It was found that maximum (2487 U/mL) xylanase production was achieved when birch wood xylan (1% w/v) was used as a substrate at 25°C and pH 10.0. Bala and Singh (2017) reported xylanase production from thermophilic mold *Sporotrichum thermophile* strain BJAMDU5 in SSF at 45°C and pH 5.0 using mixed substrate, wheat straw and cotton cake in 1:1 ratio. They reported that enhanced 3455 U/g DMR (dry moldy residue) xylanase production achieved by the addition of Tween 80. Cerda et al. (2017) reported xylanase production at pilot scale in SSF using coffee husk by a specialized inoculum of bacteria (*Pseudoxanthomonas taiwanensis* and *Sphingobacterium composti*) and yeasts (*Cyberlindnera jadinii* and *Barnettozyma californica*) and obtained enzyme having activity 48  $\pm$  4 U/g DM. A newly isolated bacterium, *Pseudomonas boreopolis* LUQ1, was reported as potent xylanase producer by Lin et al. (2017). They reported that the enzymes have highest enzyme activity (25.61 U/mL) at pH 6.0 and 65°C after 96 h with wheat bran as substrate. Cunha et al. (2018) reported xylanase production under SmF by *Aspergillus foetidus* using soybean residues

**Table 2** Xylanase-producing different microorganisms

Microorganisms	References
<b>Bacteria</b>	
<i>Bacillus cereus</i> BSA-1	Mandal et al., 2012
<i>Bacillus mojavensis</i> A-21	Haddar et al., 2012
<i>B. mojavensis</i> AG137	Sepahy et al., 2011
<i>Bacillus pumilus</i> B20	Geetha and Gunasekaran, 2017
<i>B. pumilus</i> MK001	Kapoor et al., 2008
<i>B. pumilus</i> SV 34S	Mittal et al., 2013
<i>Bacillus subtilis</i> 276NS	Ali et al., 2013
<i>Gracilibacillus</i> sp. TSCPVG	Giridhar and Chandra, 2010
<i>Paenibacillus</i> sp. N1	Pathania et al., 2012
<i>Bacillus</i> sp.	Nkohla et al., 2017
<i>Pseudomonas boreopolis</i> LUQ1	Marco et al., 2017
<i>P. boreopolis</i> LUQ1	Lin et al., 2017
<i>Herbinix hemicellulosilytica</i>	Mechelke et al., 2017
<b>Fungi</b>	
<i>Aspergillus niger</i> CCUG 33991	Shashi et al., 2011
<i>A. niger</i> USMA11	Kheng and Omar, 2005
<i>Humicola brevos</i>	Masui et al., 2012
<i>Penicillium canescens</i>	Bakri et al., 2003
<i>Fusarium oxysporum</i>	Panagiotou et al., 2003
<i>Penicillium echinulatum</i> 9A02S1	Menegol et al., 2017
<i>Aspergillus brasiliensis</i> BLf1	da Silva Menezes et al., 2017
<i>A. niger</i> KP874102.1	Uday et al., 2017
<i>Sporotrichum thermophile</i>	Bala and Singh, 2016

and found maximum xylanase production (13.98 U/mL) at 28°C and pH 7.0 after 168 h. Xylanase production in SSF by *Aspergillus niger* CCUG 33991 in tray bioreactor using low-cost agro-industrial residues was reported by Khanahmadia et al. (2018). They found highest xylanase activity of  $2919 \pm 174$  U/g-IDW (initial dry weight) at 28–35°C and after 48 h using wheat bran with particle size within range of 0.3–0.6 mm. Xylanase producing various microorganisms have been enlisted in Table 2.

## Microbial Xylanases in Bread Making

Bread is one of the traditional and most common food items around the world and closely linked with enzymes. Owing to the demand of a wide range of bread types and increasing mechanization of the baking industry, there is a necessity to modulate structure and viscoelastic properties of dough. To improve bread-making performance, enzymes and chemicals are usually included in bread formulas (Dunnewing et al., 2002). On the other hand, nowadays consumers are aware about the relationship between diet and diseases and from this point of view, for bakers it's a great challenge to make nutritious bread, which is rich in bioactive compounds, high quality in terms of specific properties such as crumb and crust texture, volume, color, taste, and firmness (Paucean et al., 2016). Currently, the treatments with enzymes have been preferred over chemical additives, as enzymes do not have activity in final product (Caballero et al., 2007).  $\alpha$ -Amylases have been used for years in bakery industry. But now bakery industry is changed due to increased demand for natural products, and therefore, enzymes have gained more importance over chemicals because enzymes enhance bread quality by improving dough stability, flexibility, loaf volume, machinability, and crumb structure (Guy and Sarabjit, 2003; Baillet et al., 2003). Gray and Bemiller (2003) reported that xylanases and other enzymes have enhanced the strength of gluten network, directly or indirectly, and thus modified bread qualities. In bread, xylan plays a major role in quality improvement because of its water absorption capacity and its interaction with gluten (Nuyens et al., 2001). Xylanases make dough more forbearing to various processing parameters. Xylanases reduce the sheeting work requirements, reduce final bread volume, and make dough softer (Dervilly et al., 2002; Harbak and Thygesen, 2002). For the last few decades, the application of xylanolytic enzymes is increased due to their effectiveness in bread making. Bakery industry use enzymes that hydrolyze starch and nonstarch carbohydrate as a quality improver (Javier et al., 2007; Polizeli et al., 2005), and hydrolysis of nonstarch polysaccharides by enzymes resulted into enhanced rheological properties (specific volume, crumb firmness, etc.) (Martínez-Anaya and Jimenez, 1997). Water-insoluble hemicelluloses are transformed into water-soluble form by the action of xylanases and bind water in dough and resulted into decreased dough firmness, finer and more uniform crumbs, and increased volume. Xylanase application improves dough-making conditions, i.e., dough becomes machine friendly and does not stick to the machine parts (Rouau, 1993). During the process of gluten–starch segregation, gluten is formed initially as a result of gliadin–glutelin structures. Therefore, for studying the effects of xylanases, they can be added after mixing of other ingredients by simple alteration in glutomatic system. After mixing, reaggregation of gluten protein starts immediately during dilution of dough. Xylanase addition at this stage significantly affects



the formation of gluten. Addition of xylanases before mixing of dough can cause overdose effects, but when xylanases are added later at the time of agglomeration phase, this effect is not observed. Xylanases chiefly act during the reagglomeration of gluten followed by gluten structure break down and affect rheological properties of gluten (Wang et al., 2004). Endoxylanases have abilities for the transformation of water-unextractable arabinoxylan (WU-AX) to water-extractable AX (WE-AX), and they also have ability for the degradation of WE-AX. Endoxylanases of *A. niger* family GT10 and *Bacillus subtilis* family GT11 behave distinctly in bakery applications. Xylanases from *Bacillus subtilis* cleave AX backbone in unsubstituted region, but xylanases from *A. niger* cleave branched region and produce small units when compared with *Bacillus subtilis* xylanase (Biely et al., 1997). *A. niger* xylanase degraded WE-AX, decreased the molecular mass and dough viscosity, and solubilized AX, and the solubilized AX resulted into larger gluten aggregates formation (Romanowska et al., 2003). *Bacillus subtilis* xylanase particularly solubilized WU-AX, increased the viscosity of dough, and also have negative impact on agglomeration of gluten (Courtin and Delcour, 2002) (Table 3).

Xylanases and other enzymes, which hydrolyzed the complex cell wall, are used for improving bread quality and dough-handling properties by reducing staling rate and also to extend the shelf life of bread (Sorensen et al., 2001; Wang et al., 2004). Xylanases as an alternative or in combinations with other emulsifiers can be used for all types of breads (Olse, 1995; Sprössler, 1997), and xylanases also contributed in the replacement of chemical additives such as bromate. The gradual increase in crumb firmness is associated with staling of bread (Gray and Bemiller, 2003; Scanlon and Zghal, 2001), and during storage of bread, xylanases have an antistaling action. Xylanases used in bread making are mostly precise for insoluble AX, and those that are precise for soluble AX show negative effect on quality of bread. Courtin and Delcour (2002) reported that the application of xylanases enhanced the dough softness and tolerance; over spring; and shape, volume, and texture of bread. Baking trials were performed with six types of wheat and different enzymes, and their combinations were reported by Hilhorst et al. (1999). They found that dough containing xylanases and peroxidases showed best result on comparing bread volume, crumb structure, and dough properties. Martínez-Anaya and Jimenez (1997) prepared a bread sample using two laboratory samples and nine commercial enzyme preparations having activity for amylase and/or xylanase, and they found that enzyme addition significantly shortened fermentation with improved volume, aroma, and softer texture of the bread (Martínez-Anaya and Jimenez, 1997). Chemical and high-performance size-exclusion chromatography indicated that xylanase acted on both hot and cold water-extractable arabinoxylans (Hilhorst et al., 2002). Jiang et al. (2005a,b) reported that the addition of xylanase B (120 ppm) from *Thermotoga maritima* improved the specific volume up to 60.3%. It also improved the cell structure of the crumb and enhanced the fresh bread crumb texture. The firmness of the fresh bread was reduced to 42.1%–68.4% as compared with the control. Along with lipase,  $\alpha$ -amylase, and protease, xylanases are considerably effective for making bread with higher specific volume, improved texture profile, and reduced firmness of crumb in microwave oven (Mathewson, 2000; Ozmutlu et al., 2001; Keskin et al., 2004). Laurikainen et al. (1998) added 12 U/g flour of partially purified xylanase of *A. foetidus* at the time of mixing and found 72%–64% reduction in water absorption, increased dough softening, and reduced dough stability. Xylanases of *A. niger* var. *awamori* and *T. maritima* improved specific volume of bread as reported by Maheshwari et al. (2000). The softness of the baked products directly related with its moisture content, and for bread, the ideal level of moisture is in between 35%–40% (Kamaliya and Kamaliya, 2001). Pyler (1988) reported that breads having moisture content less than 30% cannot be refreshed even after heating. Supplementation of xylanase from *Thermomyces lanuginosus* CAU44 in wheat bread preparation increased the final moisture content from 29.4% to 34.5% in fresh bread (Jiang et al., 2005a,b). Shah et al. (2006) found positive influence of partially purified *A. foetidus* MTCC 4898 xylanase on dough. The evaluation of sensory properties showed the favorable effect of enzyme on whole wheat bread qualities. Endoxylanase (500–1000 U/kg flour) from *A. niger* IBT-90 resulted in increased loaf volume in whole meal and wheat–rye bread, which improved on kneading (Romanowska et al., 2003). It also resulted in higher moisture content, better crumb porosity, and extended shelf life without increasing total acidity (Romanowska et al., 2003).

Bread made of composite flour (CF, 60% wheat flour and 40% hull-less barley) showed increased total AX and total and soluble (1,3)-, (1,4)- $\alpha$ -D-glucan but decreased specific loaf volume of bread, but addition of xylanase significantly increased the loaf volume of CF bread and increased soluble AX of wheat flour and CF dough. The combination of xylanase with CF gave bread with increased total and soluble (1,3)-, (1,4)- $\alpha$ -D-glucan content (Trogth et al., 2004). In baking process of unleavened Indian flat bread, which is also known as South Indian parotta, role of different enzymes was studied by Prabhasankar et al. (2004). They compared the effects of xylanase, fungal amylase, protease, and glucose oxidase on microstructure and rheological properties of dough. They reported that the application of xylanase and protease improved the quality score of 92 and 82, respectively, when compared with control (74). A particular endoxylanase of *A. niger* showed effectiveness in improving specific volume of bread without any negative side effect on dough handling (Ahmad et al., 2014).

Novozymes has introduced Panzea, from a new generation of xylanases, claimed to be the first baking xylanase to significantly combine superior volume performance and desired texture and appearance with balanced and dry dough. These enzymes performed very well at very low dosages across a broad range of baked products, baking conditions, and flour types and are not inhibited by inhibitors commonly found in wheat. Bread improver companies and flour mills often have to use a variety of xylanases to find exactly the right combination to achieve the desired baking results. Panzea, produced by *B. licheniformis*, has been documented internally and externally to achieve the desired crumb structure, loaf volume, and dough characteristics at low dosages (Novozymes, 2013).

The comparison of the abilities of xylanases from *Aspergillus oryzae*, *Trichoderma reesei*, and *Humicola insolens* for quality improvement of wheat flour bread was studied by Basinskiene et al. (2006), and they found that *A. oryzae* was more effective among all and increase 8%–13% of specific volume of bread and decrease 15%–24% in crumb firmness, but xylanase from *T. reesei* showed maximum antistaling effect. Pescado-Piedra et al. (2009) reported the effect of enzymes on the rheological properties of dough



**Table 3** Effects of xylanases on nutritional properties of bread

Enzyme	Source	Flour type	Enzyme dose (U/kg flour)	Improvement in quality parameter	References
Xylanase	<i>Trichoderma reesei</i>	Wheat flour	5990	Softening, increased specific volume by almost 20%, reduced staling rate	Laurikainen et al., 1998
Xylanase	Ex <i>Trichoderma</i> (Biocake CX160)	commercial untreated cookie type flour	105,000	Improved handling properties of dough, improved crumb structure, and increased loaf volume increased,	Hilhorst et al., 1999
Xylanase	–	Wheat flour	–	Improve specific volume	Maheshwari et al., 2000
Xylanase	Shearzyme 500L BY NOVO Nordisk A/S (Madrid, Spain)	Wheat flour	14600	Softer crumb, high specific volume, and reduced firming rate	Haros et al., 2002
Xylanase	<i>Aspergillus niger</i> IBT-90	Wheat-rye and whole-meal	500–1000	Improved kneading, increased loaf volume, higher moisture in bread, improved crumb porosity, and extended shelf life.	Romanowsk et al., 2003
Xylanase	<i>A. niger</i> , Biocon, India	Wheat flour	175	Softer dough, delayed crumb formation, and water redistribution	Prabhasankar et al., 2004
Xylanase	<i>Thermomyces lanuginosus</i> CAU44	Wheat flour	26.63–1065.2	Decreased crumb firmness upto 21.4%, reduced firming rate 0.8–0.43 N d <sup>-1</sup> and showed anti-staling effect, improved specific volume (41.4%).	Jiang et al., 2005
Xylanase	<i>Aspergillus foetidus</i> MTCC 4898	Wheat flour	12000	11% decrease in water absorption, 28.5% increase in dough rising, 40.5% increase in final moisture content, 53% increase in volume and 56% in specific volume with better taste, softness and flavour	Shah et al., 2006
Xylanase	Megazyme International Ireland Ltd. <i>Trichoderma longibrachiatum</i>	Super bakers flour	9000	Improved crumb softness, loaf volume, and colour; reduced staling rate, and loered firmness.	Al-Widyan et al., 2008
Xylanase	<i>A. niger</i>	Wheat flour	600	Increased bread volume (610.0 ± 33.96 <sup>a</sup> cc), increased moisture content (36.5 ± 1.48 <sup>a</sup> ), and improved sensory attributes.	Ahmad et al., 2014
Xylanase	Pentomax (Prozyn Ind. e Com. Ltda., São Paulo, SP)	Whole grain-wheat flour	160	Higher specific volume, lowest crumb firmness ( $p \leq 0.05$ )	Jaekel et al., 2012
Xylanase	<i>Penicillium citrinum</i> MTCC9620	Whole-wheat flour	59.9	Increased specific volume (3.99 ± 0.035 mL/g), softer crumb, reduced staling rate, and brighter colour of bread	Ghoshal et al., 2016
Xylanase	<i>Penicillium occitanis</i> Pol6	Whole wheat flour	120	8% decrease in water absorption, 36.8% increase in dough rising, 37.50% increase in final moisture content, and improved volume (17.8%) and specific volume (34.9%)	Driss et al., 2013
Xylanase	Enzymes & Derivates, Neamt, Romania	Wheat/Wheat gram flour	135–162	Improved elasticity and porosity of bread improved the elasticity and porosity	Paucean et al., 2016
Xylanase	<i>P. citrinum</i>	Wheat flour	59.9	Reduced water absorption, softer dough, greater extensibility and less resistance to extension	Ghoshal et al., 2017
Xylanase	<i>Trichoderma stromaticum</i>	Wheat flour and whole grain flour	350	Increase in specific volume of both flour bread, improved stability and elasticity, and mixing tolerance of doug.	Carvalho et al., 2016
Xylanase	<i>Sporotrichum thermophile</i> BJAMDU5	Wheat flour	350	Increased (32.33%) moisture content compared to control (29.13%). Improved amount of metal ions (Na <sup>+</sup> , K <sup>+</sup> , Ca <sup>2+</sup> ), and higher soluble protein (11.23 mg/ml) and amino acid (10.43 mg/mL) amount as compared to control.	Bala and Singh, 2017

and qualities of bread and found increase in water absorption on addition of peroxidase and xylanase. Ahmad et al. (2014) investigated the various treatments of *A. niger* xylanase in bread-making process, during wheat kernels and dough mixing, on dough qualities, i.e., extensibility, dryness, elasticity, stiffness, coherency, bread volume, specific volume, moisture retention, density, and other sensory attributes. They observed that stiffness and dryness of dough were decreased but found increase in elasticity, coherency, extensibility, and volume but decrease in bread density. Xylanase treatment also resulted in improved sensory attributes of bread with higher moisture retention capacity. To analyze the impact of xylanases addition on frozen dough, wet gluten, and quality improvement in bread, a number of studies have been carried out (Steffolani et al., 2012; Cristina et al., 2005; Krishnarau and Hoseiney, 1994). Ren and Ma (2016) investigated the effects of xylanase on quality of frozen dough and steamed bread stored at a freezing temperature for different time intervals. They found that leavening power, specific volume, antistaling rate, and survival rate of yeast in frozen dough and steamed bread can be decreased on prolong frozen storage time. By contrast, addition of xylanase can significantly improve these properties. Supplementation of frozen dough with 80 mg/kg xylanase had low freezable water content ( $28.51 \pm 0.37\%$ ), which was lower than control ( $40.22 \pm 0.43\%$ ). Addition of xylanase significantly lowered the melting point and the melting scope of ice crystals resulted into similar crystal of ice in frozen dough with improvement in frozen dough and steamed bread quality. The influence of supplementation with different doses of xylanase on the properties of loaf bread made of white wheat flour or whole grain wheat flour was studied by Jaekel et al. (2012). They reported that the bread made of white wheat flour and xylanase had higher specific volume as compared with control. Xylanase addition to whole grain wheat flour increased the specific volumes significantly as compared with control. Driss et al. (2013) tested xylanase from *Penicillium occitanis* Pol6 as a quality improver for whole wheat bread and observed a significant increase in dough rising with decrease in water absorption and increase in moisture content (37.50%), specific volume (34.9%), and volume of bread (17.8%), and firmness was decreased more than twofolds. Evaluation of sensory properties showed improved taste, softness, flavor, and overall acceptability. Improved cohesiveness, decrease in gumminess and springiness were also observed. Zheng et al. (2011) studied and compared the effectiveness of two GH10 xylanases, XynA from *Glaciecola mesophila* and EX1 from *Trichoderma pseudokoningii* on bread making. The optimum dosages of both (0.9 U/kg flour XynA and 270 U/kg flour EX1) resulted into improvement if wheat flour dough as well as bread quality with 50% decrease in Brabender units. They also found that XynA more effectively reduced the time to reach maximum consistency as compared with EX1, but both enzymes had similar effects on increasing bread volume (30%).

## Conclusions

Bread is one of the major parts of food item all over the world. Xylanases have great potential to be used in baking industry. Literature have strong evidence, which supported the application of these enzymes in bread making resulted in reduced stickiness, increased bread volume and shelf life, and also increased nutritional value. These enzymes can replace the addition of various chemical additives and emulsifiers in baking industries.

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# Lipases for Biofuel Production

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## Glossary

**Aerobic gram-negative bacterial** These are a group of gram-negative bacteria that are able to grow under an air atmosphere and have an inner cell membrane surrounded by a thin layer of peptidoglycan which is a combination of proteins and sugars with the inner cell membrane unable to retain gram stain dyes typically used to identify bacteria.

**Glycoproteins** These are simply proteins with have sugar molecules attached and are usually found floating in or around the membrane of cells. The sugars may be attached to proteins in the endoplasmic reticulum, and in the Golgi apparatus.

**Isoelectric point** The isoelectric point of a molecule or surface refers to the pH at which a molecule or surface carries no net electrical charge.

**Isoenzymes** These are simply alternative forms of enzymes with different structures that are capable of catalysing the same reaction.

**Moiety** This is used to refer to a part of a molecule for instance the alkyl group of an organic compound responsible for the physical properties of a chemical compound.

**Nonsporogenic** Any fungi that does not produce spores.

**Saprophytic** This refers to the act of getting energy from dead or decaying matter. A saprophyte or saprotroph is therefore an organism which gets its energy from dead and decaying organic matter implying that saprophytes are heterotrophs.

## Nomenclature

List of nomenclature used including all definitions and units.

kDa kilodalton

BCL *Burkholderia cepacia* lipase

CALB (CALA) *Candida Antarctica* lipase B (A)

CRL *Candida rugosa* lipase

GRAS Generally Recognised as Safe status

RML *Rhizomucor miehei* lipase

ROL *Rhizopus oryzae* lipase

TLL *Thermomyces lanuginosus* lipase

## Introduction

Lipases (EC 3.1.1.3) are triacylglycerol ester hydrolases, considered as one of the two major classes of hydrolases, with the second class identified as the so called 'true' esterases (EC 3.1.1.1. carboxyl ester hydrolases). According to Vakhlu and Kour (2005), lipases were previously classified as serine hydrolases due to the presence of serine in the highly conserved domain of their active sites. This serine presents the only common feature shared by all identified lipases sequenced thus far (Vakhlu and Kour, 2005). Lipases are distinguishable from carboxyl esterases via spectral assessment of their substrates, since *p*-nitrophenyl palmitates are cleaved by lipases while *p*-nitrophenyl butyrates are cleaved by esterases. These two major classes of hydrolases can also be distinguished by assessing differences in stabilities in organic solvents, since lipases exhibit higher stabilities compared to esterases (Bornscheuer, 2002). Lipases are recognised as very important industrial inputs as illustrated by applications in the food processing industry (cheese ripening and flavour development) (Saxena et al., 1999), cosmetic industry (aroma production) (Metzger and Bornscheuer, 2006), bioenergy industry (biodiesel production), pollution remediation (oil spill management) (Nakamura and Nasu, 1990) and commercial cleaning industry (alkaline lipase from *Pseudomonas alcaligenes* M-1 used in removing oil stains) (Gerritse et al., 1998).

Lipases are characterised by molar weights ranging from 20 to 75 kDa with their optimal pH and optimal temperature conditions ranging from 6 to 8 and from 30 °C to 40 °C respectively (Giovana da Silva et al., 2012). Typically, the molecular weight of a lipases may be greater than 75 kDa, if the lipase has the capacity to form aggregates in solution (Gill and Parish, 1997). According to Gill and Parish (1997), the majority of lipases secreted by organism function extracellularly and are characterised by acidic glycoproteins containing between 2% and 15% carbohydrates and with the major glycoside residue being mannose. Most importantly, lipases are biocatalysts capable of acting on a range of substrates to enhance a multitude of reactions (Montero et al., 1993; Moura et al., 2017). Lipases are widely available in nature with extracts of lipases readily isolated from plant



**Table 1** Characteristics of some lipases obtained from different sources

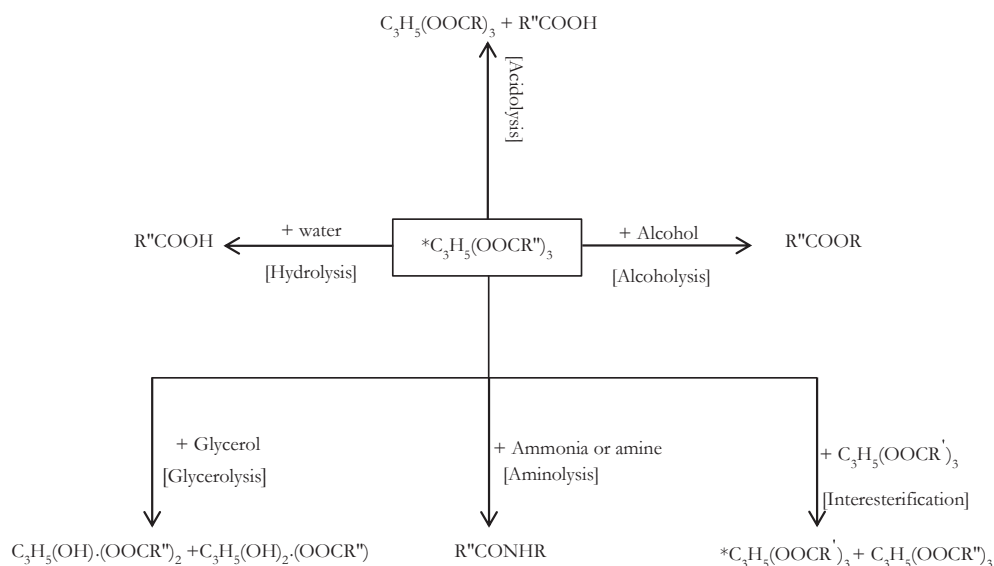
Source organism	Organism source	Short description
<i>Burkholderia cepacia</i>	Bacteria (Thakur, 2012)	BCL lipase is highly tolerant to methanol and displays particularly high conformational stability under conditions employed during transesterification reactions for biodiesel production (Sasso et al., 2016). The lipase source is an aerobic gram-negative bacillus and is one of the most thoroughly studied lipase sources.
<i>Candida Antarctica</i>	Fungi (Thakur, 2012)	The organism produces two lipases namely CALB and CALA, characterised by a similar optimum pH of 7. CALB (CALA) is also characterised by a molecular weight, an isoelectric point and a specific activity of 33 kD (45 kD), 6 (7.5) and 435 (420) respectively (Kirk and Christensen, 2002). Most importantly CALB and CALA differ in terms of their positional specificity toward triglycerides, with a positional specificity of <i>Sn</i> -3 and <i>Sn</i> -2 reported respectively (Kirk and Christensen, 2002). Both lipases are characterised by an almost uniform activity towards straight-chain primary alcohols and carboxylic acids (Kirk and Christensen, 2002).
<i>Candida rugosa</i>	Fungi (Thakur, 2012)	The organism generates lipases that contain at least five isoenzymes of varying proportions and varying stabilities. It is speculated that the existence of these five related but non-identical isoenzymes enhances the adaptability to different substrate sources (Benjamin and Pandey, 1998). The CRL is popularly used in fat-splitting operations in the food industry due to its proven Generally Recognised as Safe (GRAS) status (Gurung et al., 2013) where the organism is nonsporogenic, pseudo-filamentous, unicellular and non-pathogenic in nature (Benjamin and Pandey, 1998).
<i>Rhizomucor miehei</i>	Fungi (Thakur, 2012)	RML is used for many applications in the conversion of fats and oils and in chemical processes. In its free and immobilised forms it has a very high activity and good stability under diverse conditions (Rodrigues and Fernandez-Lafuente, 2010). The lipase also has a high enantiospecificity.
<i>Rhizopus oryzae</i>	Fungi (Thakur, 2012)	The lipase produced by this fungi, ROL, also has GRAS status, which is responsible for its wide acceptance in the food and biotechnology industry (Cantabrana et al., 2015). It is a potent saprophytic and pathogenic fungus which can produce a spectrum of metabolites, such as esters, polymers and bioalcohols (Ghosh and Ray, 2011).
<i>Thermomyces lanuginosus</i> (TLL)	Fungi (Maheshwari et al., 2000)	TLL is used in large amounts in detergents and various synthetic applications. It is characterised by a molar mass of 31.7 kDa and an isoelectric point of 4.4 (Jha et al., 1999). It is a widely distributed thermophilic fungus, thriving at temperatures up to 60 °C but fails at temperatures lower than 20 °C (Singh et al., 2003). It has the capacity to produce high levels of cellulase-free thermostable xylanase, which has wide biotechnological applications (Singh et al., 2003).
<i>Pentaclethra macrophylla</i> Benth	African bean seeds (Enujiugha et al., 2004)	This lipase is characterised by a high thermal stability with lipolytic activity reported at temperatures up to 80 °C (Enujiugha et al., 2004). Optimum temperature and pH conditions have been reported to be 30 °C and 7 respectively. High sensitivity of the enzyme to salts has however been reported with the presence of mercury chloride and sodium chloride significantly inhibiting the catalytic ability (Enujiugha et al., 2004).

and animal sources (Sevil et al., 2012). Table 1 presents some examples of characteristics and sources of common lipases used in the biotechnology industry.

Lipases are also characterised by high bio-catalytic activities when acting on water-insoluble substrates, such as triglycerides composed of long-chain fatty acids, and are typically characterised by a high chemo-, regio- and stereoselectivity, in organic solvents (Barros et al., 2010). Crucially they are generally recognised as environmentally benign with their catalytic effect optimised when mild reaction conditions are imposed (Ferreira-Dias et al., 2013). The effectiveness of lipases in catalysing processes under mild condition also promotes its application in several reactions. Major reactions that can be catalysed using a lipase are presented in Fig. 1.

Fig. 1 shows that lipase can effectively catalyse acidolysis, alcoholysis, aminolysis, interesterification, glycerolysis and hydrolysis reactions, when a triglyceride molecule is the substrate (Pandey et al., 1999).

From Fig. 1, the interesterification reaction involves the exchange of an acyl radical between the parent triglyceride and a 'donor' triglyceride. If however the donor is a free fatty acid, then the reaction is considered as an acidolysis reaction. The exchange of acyl radicals is typically undertaken to improve the properties of the parent triglyceride molecule. On the other hand the displacement of the acyl moiety between the triglyceride and an alcohol (ROH) serves to generate biodiesel. Similar acyl moiety exchanges between the triglyceride and glycerol ( $C_3H_5(OH)_3$ ) is called a glycerolysis reaction. Such glycerolysis reactions serve to generate useful mono- $(C_3H_5(OH)_2(OOCR''))$  and di- $((C_3H_5(OH).(OOCR''))_2)$  glycerides, which are useful products used in the food industry since both products can be used as food stabilisers in air-in-liquid emulsions like cake batter or ice cream.<sup>1</sup> The hydrolysis reaction serves to split the triglyceride molecule into its glycerol backbone and constituent fatty acid ( $R''COOH$ ) fragments. The aminolysis reaction serves to split the parent triglyceride by reacting the triglyceride with ammonia ( $NH_3$ ) or amines ( $R-NH_2$ ) to form a fatty acid amide ( $R''CONHR$ ).



**Figure 1** Lipase catalysed reactions involving triglycerides as the substrate (where  $*C_3H_5(OOCR'')$  represents the parent triglyceride and  $C_3H_5(OOCR')$  represents the 'donor' triglyceride in the interesterification reaction).

According to Ribeiro et al. (2011) most lipase catalysed reactions are initiated by a nucleophilic attack on the carbon present in the ester bond of the susceptible substrate by an hydroxyl group in the serine residue of the active site of the lipase. Nucleophilic attack results in the formation of an acyl–enzyme complex and the release of alcohol from the triglyceride substrate. The regeneration of the lipase is achieved via the hydrolysis of the acyl–enzyme complex. This process may occur via complex or simple mechanisms, depending on several factors, such as temperature, presence of inhibitors and substrate concentration (Artoli, 2008).

The ability of the lipases to catalyse acidolysis, alcoholysis, interesterification, glycerolysis and hydrolysis reactions has been explored extensively in the biofuel industry as the basis for the development of an environmental friendly production process. According to Alonso et al. (2005), the versatility of lipases in enhancing the hydrolysis of ester–carboxylate bonds at the organic–aqueous interphases and esterification–transesterification reactions in water-restricted environments is responsible for its extensive use in the biodiesel (biofuel) industry.

### Lipase Application in Biodiesel Production

The utilisation of lipases in catalysing transesterification (alcoholysis) reactions for biodiesel production processes was first reported by Mittelbach (1990). Mittelbach demonstrated the utilisation of *Pseudomonas fluorescens* lipase to enhance the alcoholysis of sunflower oil using methanol (Mittelbach, 1990). Over the years, more research has been undertaken to explore the activity of lipases in biodiesel production processes when different oil substrates are utilised (Rodrigues et al., 2016; Amini et al., 2017).

This is largely due to the ability of lipases to act on a wide range of oils including low grade oils characterised by a high free fatty acid content, such as dissolved air flotation sludge oil extract (Okoro et al., 2017a), without initiating unwanted side saponification reactions occurring (Rodrigues et al., 2016; Okoro et al., 2017b). Table 2 presents some examples of previous work that have explored lipase utilisation in biodiesel production processes.

### Lipase Immobilisation

Table 2 suggests that high biodiesel yields are feasible when lipases in different forms are used as biocatalysts. However current applications have suggested the preference of the immobilised lipase forms as a means of circumventing several biofuel production limitations identified when lipases in their free forms are utilised (Zhao et al., 2015). Some of these limitations include an escalation of the production costs due to the difficulty of lipase recovery and reuse and the increased possibility of production promoting product contamination with residual enzyme (Ribeiro et al., 2011). Other benefits arising from the application of immobilisation techniques includes an improvement in the thermal stability, pH stability of the lipase and an enhanced tolerance of the lipase to chemical species in the reaction mixture (Zhao et al., 2015; Silva et al., 2013). Such improvements in the properties of the lipase via the application of immobilisation methods will increase the applicability of lipase in continuous biofuel production systems as a result of the enhanced lipase adaptability (Silva et al., 2013).

Lipase immobilisation, in the simplest terms may be considered as the confinement of the lipase in a region or a defined space while simultaneously ensuring that the catalytic activity of the lipase is maintained (Tan et al., 2010). Lipase immobilisation is usually achieved by tethering the lipase to a suitable supporting matrix which must be hydrophilic, inert (to the lipase), biocompatible, resistant to microbial attack, resistant to compression and readily accessible at a low cost (Mohamada et al., 2015). According to Cao

**Table 2** Biodiesel production using lipase as catalyst

Substrate	Alcohol used	Lipase	Form	Temperature (°C)	Reaction time (h)	Performance (%)	References
<i>Ocimum basilicum</i> (sweet basil) seed oil	Methanol	<i>Candida Antarctica</i>	Immobilised on acrylic resin	47	68	94.58 (yield)	<a href="#">Amini et al. (2017)</a>
Jatropha oil	Methanol	<i>Thermomyces lanuginosus</i> (TL) 1,3 specific lipase	Free	35	24	80.7(yield)	<a href="#">Bueso et al. (2015)</a>
Jatropha oil	Ethanol	<i>Chromobacterium viscosum</i>	Immobilised on Celite-545	40	8	92(yield)	<a href="#">Shah et al. (2004)</a>
Waste cooking oil	Methanol	<i>Rhizomucor miehei</i>	Immobilised on textile cloth	45	–	91.08 (yield)	<a href="#">Chena et al. (2009)</a>
Soybean	Methanol	<i>Rhizomucor miehei</i> displaying <i>Pichia pastoris</i>	Used as a whole cell biocatalyst ( <i>Pichia pastoris</i> ).	55	72	83.14(yield)	<a href="#">Huang et al. (2012)</a>
Palm oil	Isobutanol	<i>Candida antarctica B</i>	Immobilised on granular activated carbon	40	45	100(conversion)	<a href="#">Naranjoa et al. (2010)</a>
Waste cooking	Methanol	<i>Thermomyces lanuginosus</i> (Lipozyme TL IM)	Immobilised on hydrotalcite and zeolites	45	105	95 (yield)	<a href="#">Yagiz et al. (2007)</a>
Rape seed oil	Methanol	<i>Saccharomyces cerevisiae</i>	Immobilised on immobilised on Mg–Al hydrotalcite	45	4.5	96 (conversion)	<a href="#">Zeng et al. (2009)</a>
Tallow and Grease	Ethanol	<i>Burkholderia cepacia</i>	Immobilised on a phyllosilicate sol–gel	50	18	94 (yield)	<a href="#">Hsu et al. (2001)</a>
Canola	Methanol	<i>Thermomyces lanuginosus</i>	Immobilised onto hydrophobic microporous styrene–divinylbenzene copolymer	50	24	97 (yield)	<a href="#">Dizge et al. (2009)</a>
Palm oil	Methanol	<i>Aspergillus niger</i>	Used as a whole-cell biocatalyst	40	72	87 (yield)	<a href="#">Xiao et al. (2010)</a>
Soybean oil	Methanol	<i>Rhizopus oryzae</i> ATCC 24563	Used as a whole cell biocatalyst	35	6	97(conversion)	<a href="#">Lin et al. (2011)</a>
Olive oil	Methanol	<i>Candida antarctica lipase B</i>	Immobilisation on an amphiphilic matrix	50	24	90 (yield)	<a href="#">Lee et al. (2010)</a>
Soybean oil	Methanol	<i>Pseudomonas fluorescens</i>	Free	35	90	90(yield)	<a href="#">Kaieda et al. (2001)</a>
Babassu oil	Ethanol	<i>Burkholderia cepacia</i>	Immobilised on SiO <sub>2</sub> –PVA	40–50	48	100(yield)	<a href="#">Da Rós et al. (2010)</a>

(2006), several factors influence the performance of an immobilised lipase such as the pore size of the carrier, the microenvironment of the carrier and the strength of the hydrophobic partition. These factors influence the extent of catalytic activity retention, the lipase stability and the rate of action on a hydrophobic substrate respectively. Thus although the introduction of immobilisation strategies will enhance the ease of the lipase separation for recovery and reuse (Ribeiro et al., 2011), leading to lower operating process cost, the technique and nature of immobilisation must be properly selected based on specific processing targets.

### Immobilisation Methods

There are five major lipase immobilisation methods identified in the literature, namely, covalent, adsorption, cross linking (or aggregation), entrapment and encapsulation methods (Sirisha et al., 2016; Bickerstaff, 2009). These methods have been summarised and illustrated in Table 3 and Fig. 2 respectively.

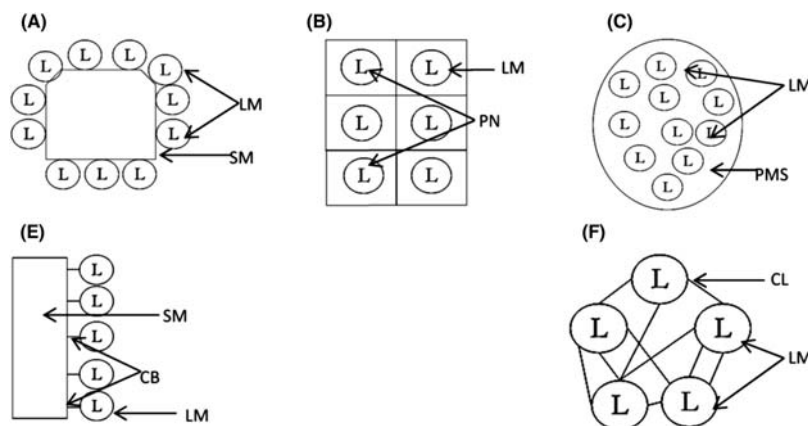
### Future Prospects

It is recognised that the utilisation of lipases in enhancing reactions provides a pathway that is comparatively more environmentally benign compared to reactions that utilise chemical catalysts (acid or alkaline). The high cost of lipase has however thus far limited its use in large scale processes (Poppe et al., 2015). Lipase high cost is exacerbated by lipase deactivation that typically occurs in the presence of alcohols for biodiesel production thus limiting lipase reuse options (Abdelmoez and Mustafa, 2014). Although there have been reports of an improved recoverability of the lipase for reuse via the incorporation of immobilisation techniques, current immobilisation systems are still limited by several factors. Some of these factors include possible losses in catalytic activity of the lipase, high cost of more suitable lipase supports, reduced stability of the immobilised lipase catalyst in oil–water subsystems and increased mass transfer restrictions in the immobilised lipase reaction system (Zhao et al., 2015).

Genetic engineering has therefore been proposed as a tool that can enable improved yields of better performing lipase strains at reduced production cost. This is because lipases can be genetically engineered to have high catalytic activities, to be stable in organic solvents, and enhance product recovery (Abdelmoez and Mustafa, 2014). Examples of successful recombinant DNA technology application include the improvements introduced to *Aspergillus oryzae* and *Pichia pastoris* strains which have been genetically modified to express the properties of several microbial lipases which can be used in biodiesel production (de Simone et al., 2016). The utilisation of whole cells as biocatalyst is also expected to reduce lipase catalysed biofuel production processes since the need for

**Table 3** Major enzyme immobilisation methods

Immobilisation methods	Short discussion
Adsorption bonding [Fig. 2A]	This immobilisation bonding method is recognised as the most straightforward immobilisation method since lipase attachment is achieved in the absence of any chemical transformations. The tethering of the lipase to the support or carrier is achieved using weak intermolecular forces such as van der Waals or dispersion forces (Jegannathan et al., 2008). It is the most widely used immobilisation technique due to its simplicity and low cost (Lukovic et al., 2011). This immobilisation method is however limited by changes in ionic strength during the catalysing process, which may lead to lipase desorption (Rai, 2012). The lipase may also be susceptible to microbial or proteolytic attack.
Entrapment bonding [Fig. 2B]	Entrapment describes the immobilisation of the lipase via the ‘sealing’ of an enzyme within a polymeric network (Brena and Batista-Viera, 2006). The polymeric network enables the transfer of substrate and products across the polymeric network barrier while retaining the lipase within the polymeric network (Mohamada et al., 2015). This immobilisation method is considered as irreversible since the separation of the lipase from the polymeric matrix may require the destruction of the matrix (Mohamada et al., 2015). This immobilisation method is simple and is characterised by no intrinsic changes in lipase properties from chemical modification. This method is however limited by the possibility of enzyme leakage and diffusional constraints of the products and reactants. Furthermore the mass of lipase that can be immobilised within the polymeric network is clearly limited (Mohamada et al., 2015; Brena and Batista-Viera, 2006).
Encapsulation bonding [Fig. 2C]	Encapsulation is the confinement of the enzyme within a porous membrane forming a bilayer (Jegannathan et al., 2008). Encapsulation does not require any interaction between the catalyst and the support, with the size of the pore opening of the support considered crucial as it determines the mass transfer resistance of the reactants and products while also preventing any losses in the encapsulated lipase (Zhao et al., 2006). Several reports have however suggested that such encapsulation may occasionally lead to the inactivation of the lipase molecule (Dwevedi, 2016).
Covalent bonding [Fig. 2D]	This method is based on the formation of covalent bond between the lipase molecule and the support material using side chain amino acids such as histidine with the reactivity of the immobilised lipase influenced by the functional group that is present in the side chain (Sirisha et al., 2016). In cases where the bonds existing between the lipase molecule and the support are due to the presence of strong electrostatic (ionic) forces, such an immobilisation method is referred to as ionic bonding (Tor et al., 1989). The utilisation of such strong (covalent) bonds may however lead to significant changes in the conformational and catalytic properties of the lipase molecule (Dwevedi, 2016).
Cross linking [Fig. 2E]	This method involves the utilisation of bi- or multifunctional reagents to stimulate the aggregation of lipase molecules such that the lipase molecule does not undergo any denaturation (Talekar et al., 2012). This method ensures that the activity or the catalyst productivity is not negatively influenced since the cross linking agent is typically characterised by a molar mass that is large compared to the molar mass of the lipase molecule (Talekar et al., 2012). There is however increased possibility of catalyst loss due to the non-regulation of the aggregation reaction and reduced reactivity due to limitations caused by diffusion (Dwevedi, 2016).



**Figure 2** An illustration of the major immobilisation methods (LM represents lipase molecule; SM represents support matrix; PN represents polymeric network; PMS represents porous support matrix; CB represents covalent bond; CL represents the cross linkage).

lipase recovery and purification is avoided (Soares et al., 2017). Future modifications in existing immobilisation technologies such as the use of biopolymers as mobilisation supports will also enhance lipase recovery and improved product separation while also enhancing the environmental performance of biofuel production processes (Ravindra and Jegannathan, 2015).

Another possible future prospect is the incorporation of intensification approaches, such as the utilisation of ultrasonic and microwave radiation to optimise the activities of immobilised lipases (Subhedar and Gogate, 2017). This is because the application of ultrasonic and microwave radiation provide a low cost option for energising the lipases (Subhedar and Gogate, 2017). Finally, appreciating that the performance and economics of lipase catalysed biofuel production processes are dependent on a proper integration of the aforementioned improvements any future lipase aid biofuel production technology must incorporate an optimisation protocol with the production cost identified as an important objective function (Luna et al., 2016).

## Conclusion

This chapter has highlighted the role of lipases as important biocatalysts in several industries, largely based on their abilities to reduce waste generation and thus lead to favourable environmental outcomes. The importance of lipases to the biofuel industry has been emphasised. Published studies that investigated the applicability of lipases (free and immobilised forms) in the biofuel industry were also highlighted. Enhanced performances of reactions catalysed using immobilised lipases compared to the lipases in free forms, were reinforced and discussions highlighting the benefits of lipase immobilisation, presented. Major immobilisation techniques were also briefly explained. As explained in this chapter, although the utilisation of lipases in catalysing biofuel production processes has many associated advantages, several limitations persist that have thus far hindered large scale applicability. It was suggested that these limitations are largely due to the high cost of lipases, with future perspectives focused on reducing the unit lipase cost. These future perspectives were therefore initially discussed.

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## Lipase/Esterase: Properties and Industrial Applications

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### Glossary

**Acidolysis** A chemical reaction involving the decomposition of a molecule, with the addition of the elements of an acid to the molecule, analogue to hydrolysis in which water plays a similar role to that of acid.

**Carboxylic ester hydrolases** An enzyme catalysing the hydrolysis of an ester bond resulting in the formation of an alcohol and a carboxylic acid in the presence of water.

**Interesterification** Redistribution of the fatty acid moieties present in a triacylglycerol molecule or between two ester molecules.

**Kinetic resolution** A process of differentiating two enantiomers in a racemic mixture with a chiral catalyst or reagent.

**Solid state fermentation** Fermentation process in which microorganisms grow on solid material without the presence of free liquid.

**Structured lipid** Lipids that have been modified from their natural form for specific food and nutraceutical applications.

**Submerged state fermentation** Fermentation process in which microorganisms are grown in free flowing liquid substrate such as corn steep liquor, molasses and nutrient broth

### Nomenclature

API Active pharmaceutical ingredient

CAGR Compound annual growth rate

CALB *Candida antarctica* lipase B

CBE Cocoa butter equivalent

CBS Cocoa butter substitute

DAG Diacylglycerol

DHA Docosahexaenoic acid

EPA Eicosapentaenoic acid

FAE Ferulic acid esterase

FE Feruloyl esterase

FFA Free fatty acid

GLY Glycerol

HCA Hydroxycinnamic acid

HMF Human milk fat

HMFS Human milk fat substitute

IPG 1,2-*O*-Isopropylidene glycerol

LDL Low density lipoprotein

MCFA Medium-chain fatty acid

MCT Medium-chain triacylglycerol

MLCT Medium-and long-chain triacylglycerol

o/w Oil-in-water

PL Phospholipid

PLA<sub>1</sub> Phospholipase A<sub>1</sub>

PLA<sub>2</sub> Phospholipase A<sub>2</sub>

PME Pectin methyl esterase

PUFA Polyunsaturated fatty acid

SFAE Sugar fatty acid ester

SL Structured lipid  
 TAG Triacylglycerol  
 VLCT Very long-chain triacylglycerol  
 w/o Water-in-oil

## Sources and Classification of Lipases and Esterases

### Animal Sources of Lipase and Esterase

Lipases exist in various organs and tissues of human and animal species. Among the various types of lipase, the most widely studied lipase is the pancreatic lipase. Human pancreatic lipase is secreted by the pancreas into the duodenum to hydrolyse the dietary triacylglycerol (TAG) into free fatty acids (FFA), monoacylglycerol (MAG), diacylglycerol (DAG), and glycerol (GLY). Unlike nonhuman pancreatic lipase, human pancreatic lipase does not exhibit phospholipase activity. Apart from pancreatic lipase, the digestion of dietary TAG is also aided by other lipases secreted from gastric, lingual and pharyngeal which accounted for 50% of the dietary fat breakdown (Bauer et al., 2005; Mukherjee, 2003). Besides pancreatic, gastric, lingual, pharyngeal lipase, human milk lipase or the so-called bile salt stimulated lipase which is present in 1% of human milk is also responsible in helping the premature infant to digest and absorb human milk fat. The structure of human milk lipase is unique in such a way that it is considered a glycoprotein having around 10% of carbohydrate attached to the protein moiety (Olivecrona et al., 2003). Further, there are also lipases such as human lipoprotein lipase and hepatic lipase that are involved in the degradation and metabolism of lipoprotein.

Similarly, esterases can also be found in various organs and tissues of human and primate animals (Rhesus monkey, cynomolgus monkey, dog, guinea pig, rabbit, mouse, Nile Tilapia). They are present in the plasma, brain, liver, lung, small intestine, kidney, muscle tissue, saliva and oviducts (Bahar et al., 2012; Li et al., 2007; Valocky et al., 2007; Yue et al., 2009). In humans and animals, esterases are essential in activating drug from pro-drug, inactivating the active therapeutic drug, detoxifying potential toxicants and maintaining the physiological function of the body (Fukami and Yokoi, 2012). Examples of esterases includes cholesterol esterase, cholinesterase, leukocyte esterase, thioesterase, acetylcholinesterase. Among them, cholinesterase and carboxylesterase are the major types of esterases that can be found in the plasma of humans and rodents (Rudakova et al., 2011). Interestingly, arthropod species especially plant bugs such as wild silkworm *Antheraea polyphemus*, *Lygus lineolaris*, *Spodoptera litura*, tobacco hornworm, *Manduca sexta* also contain enzyme esterase (Shankarganesh et al., 2012; Srigiriraju et al., 2009; Zhu and Luttrell, 2012). Among them, the most common insect's esterase is juvenile hormone esterase. This esterase provides bugs with pesticide resistant properties besides maintaining the physiological activity of insects such as digestion, reproduction, and hormone regulation (Kamita and Hammock, 2010).

### Plant Sources of Lipase and Esterase

Plant lipases received very little attention as compared to other sources of lipase. Lipases from plant origin are commonly found in oilseed, cereal, latex-producing plant species, and even marine seaweed (Bele et al., 2014; Keqin and Xinxin, 2008). This includes peanut, linseed, rapeseed, palm, soy castor bean, oat, grain, wheat, rice and so on (Villeneuve, 2003). The most ubiquitous types of plant lipase are oilseed lipases (Barros et al., 2010). They are mainly located on the aleurone (bran) of the lipid bodies and cellular components (embryo). The lipase activity was found to be exceptionally high particularly in germinating oilseeds since they are necessary to hydrolyse the stored TAG into FFA and GLY for the synthesis of sugars, amino acids, and carbon chains in order to meet the energy demand for embryonic growth. A review of different sources, applications, and properties of seed lipases was reported by Barros et al. (2010).

Meanwhile, plant esterases are found in vegetables and fruits such as soybean, rice, sorghum, wheat, papaya, orange peel maize (Hou et al., 2012; Jianke, 2008; Keqin and Xinxin, 2008). Esterases are widely distributed along the cell wall of the plant cell. The most common plant esterases are ferulic acid esterase (FAE) and pectin methyl esterase (PME) (Yeom et al., 2002). They display a diverse role in the biological process and regulation of bioactivity of endogenous products activation. This includes hydrolysis of ester containing xenobiotic molecule, growth, and development, stomatal movement, insecticidal resistance against infection, ripening, cell expansion, reproduction and spoilage.

### Microbial Sources of Lipase and Esterase

Microbial lipases/esterases encompass all classes of microorganisms ranging from fungi, bacteria to actinomycetes (Patil et al., 2011). A review on the up-to-date list of microbial lipases was provided by Hasan et al. (2006). The microbial lipases/esterases can either be isolated from the environment, sourced from culture collection centres or industry manufacturers. There is a prominent interest lately in utilizing microbes for large scale production of lipases which is in line with the high demand of microbial lipases for various industrial applications. Microbial lipase offers multifold advantages ranging from its versatility, availability to its

stability. Additionally, the extracellular nature of microbial lipase in which the lipase is secreted from the microbial cell into the culture medium allows convenient and easier isolation, separation and purification process. The move has prompted several large conglomerates such as Novozymes (A/S Bagsvaerd, Denmark), Amano Enzyme Inc. (Nagoya, Japan), Meito Sangyo (Tokyo, Japan) to manufacture and commercialise microbial lipases for industries.

The diversity of the microbe's Kingdom allows new lipases/esterases with desired functional properties to be isolated from different sources. There is high possibility that microbial lipases/esterases can be found in oil containing substances or oil contaminated areas. Some researchers managed to isolate microorganisms having esterase activity from the surface of cheese, oil contaminated areas and desert (soil, sand, stone and monument) (Essoussi et al., 2010; Heath et al., 2009; Soliman et al., 2007; Xue et al., 2012). Examples of microbial species having esterase activity include *Bacillus licheniformis* S-86 and lactic acid bacteria (Torres et al., 2009).

The presence of active lipases/esterases can be identified using agar plate assay (Hasan et al., 2009). Identification of esterase is conducted using chromophoric substances (*p*-nitrophenyl ester) or tributyrin supplemented agar plate whereas that of lipase is performed using substrate spectra (*p*-nitrophenyl palmitate and *p*-nitrophenyl butyrate). A halo formation on the agar plate is associated with the presence lipases/esterases. Sometimes, fluorescent substances like rhodamine B, Lugol's iodine solution are used to detect and enhance the clear zone. Lately, a metagenomic approach was used to screen for lipase (Reis et al., 2009).

The microbial lipases/esterases isolated are then cultured either in solid state or submerged state fermentation in order to optimise its culture conditions to obtain a high yield of enzyme lipase/esterase prior to large scale production which is usually performed in a bioreactor (Treichel et al., 2010). Several vital parameters that affect the growth of microbes which comprises of 1) nutritional factors such as nitrogen source, carbon source, lipid source, surfactants and metal ions, 2) physical factors like incubation time, pH, temperature, inoculum size, shaking condition, aeration will then be studied. Sometimes, lipid and fatty acids are introduced into the culture media to stimulate the secretion of lipase. Since purification of lipase is an important process in ensuring its high activity, therefore it is common that upon isolation from cells and mycelium, the enzyme lipase is purified to separate from other contaminants using ammonium sulphate followed by ion exchange/affinity/gel filtration column chromatography (Tan et al., 2015). Lately, with the advancement in biotechnology, the performance of lipase can further be enhanced through techniques such as chemical and physical mutagenesis as well as genetic modification (Anobom et al., 2014; Hoesl et al., 2011).

## Properties of Lipase and Esterase

Both esterase and lipase are made-up of  $\alpha/\beta$  hydrolase protein folding consisting of  $\alpha$  helices and  $\beta$  sheets. Lipase shows high activity toward insoluble or heavily aggregated long chain fatty acid esters. As such, lipase activity is largely dependant on the substrate area rather than the concentration of the substrate. Lipases catalyse both forward and reverse reactions. In the presence of water, lipase shows preferences for carrying out hydrolysis, alcoholysis, and aminolysis of ester bonds to breakdown TAG into FFA and partial acylglycerols. Under limited amount/absence of water, lipase-catalysed esterification reaction will occur which involves the formation of either TAG, DAG or MAG from fatty acid and GLY. Meanwhile, esterases display broad substrate specificity catalysing not only the hydrolysis of ester bond of short chain fatty acid ester but also non-ester bonds such as thiols, amides, and carbamates. Compared to lipase, esterase acts on water soluble substrate. Lipase and esterase have high stability and activity at pH above 8 (alkaline) and pH around 6 (acidic), respectively.

The enzymatic reaction takes place at the active site of lipase which comprises of a catalytic triad containing Ser-His-Asp residue. The active site of lipase is covered by mobile lid domain that is in the form of loop and helices which is amphiphilic in nature. The lid is positioned in such a way that hydrophilic side faces the solvent and the hydrophobic site is arranged towards the catalytic pocket. The activity of lipase as such is predominantly controlled by the lid domain. In non-aqueous media, the hydrophobic layer triggers the confirmation of lid to open, allowing the substrate to enter the active site. However, in pure aqueous media, the lipases are inactive as the lids are in closed position and the active sites are inaccessible to the environment substrate. Since the lid domain is involved in the determination of the activity of lipase, several studies were conducted to alter the lid through protein engineering in order to intensify the activity of lipase (Khan et al., 2017). Although most of the lipase contains one or more lids in the form of helices/loops with different sizes, certain lipase from genuine pig was found to have no lid domain. Lipase activity is activated only at lipid water-interface giving rise to its interfacial activation property (Reis et al., 2009). The activity of lipase occurs in emulsion system either in normal phase emulsion (water-in-oil) or invert phase emulsion (oil-in-water). Unlike certain enzyme lipase that may require cofactors to remain active, esterase does not require a cofactor.

Meanwhile, esterases have either no lid or a small lid covering its active site. The active site of esterase is comprised of Ser, His, and either Asp or a Glu residue. Study found that the lid covering the active sites of cholesterol esterase from *Ophiostoma piceae* OPE is made up of one  $\alpha$ -helix and two  $3_{10}$ -helices flanked by two loops. During esterase activation, lid displacement occurs to change the loop  $\alpha 16$ -  $\alpha 17$  from a close to open structure which in turn leads to the exposure of the catalytic site (Gutiérrez-Fernández et al., 2014).

## Selectivity of Lipase and Esterase

Lipase can be classified into three different classes based on its positional and fatty acid specificity. Most of the lipases belong to sn-1,3 specific lipase. These lipases hydrolyse/esterify fatty acid specifically at the either/both sn-1 and sn-3 position. The sn-2 fatty

acids are prevented from binding to the active site of lipase due to steric hindrance effect. Example of this group of lipase includes human pancreatic lipase, *Aspergillus niger*, *Rhizomucor delemar*, *Rhizomucor miehei*, and *Mucor javanicus*. Meanwhile, another group of lipase which belongs to non-specific lipase catalyses the hydrolysis/esterification of fatty acid in a random manner regardless of its position. Examples of these lipases are *Candida rugosa*, *Corynebacterium acnes*, *Staphylococcus aureus*. Additionally, lipases showing fatty acid specificity is much less common compared to other groups of lipases. *Geotrichum candidum* is the most common fatty acid specific lipase that shows preferences toward oleic acid. There are also lipases which show negative specificity. For instance, lipase from *Candida cylindracea* which discriminates against docosahexanoic acid, *G. candidum* against  $\gamma$ -linolenate in borage oil and *Mucor miehei* against polyunsaturated fatty acid GLA and DHA. Lipases of negative selectivity are often being utilized to concentrate and enrich certain polyunsaturated fatty acid (PUFA) in TAG.

Esterase can be classified into three classes mainly A-, B- and C-esterase based on its specificity towards organophosphate substrate. Esterase A (paraoxonase) is responsible for the hydrolysis of organophosphate, esterase B (carboxyl esterase, butylcholinesterase, acetylcholinesterase) is inhibited by organophosphate whereas esterase C (acetyl esterase) do not interact with organophosphate. Additionally, according to its ability to hydrolyse the artificial alpha and beta naphthyl acetate, esterase can be divided into alpha and beta esterase. Additionally, albumin protein which can hydrolyse  $\alpha$ ,  $\beta$ -naphthylacetate is also considered as a type of esterase (Bahar et al., 2012).

## Industrial Applications of Lipase and Esterase

The global industrial enzymes market value has reached USD 5 billion in year 2016 with enzyme lipases and esterases accounting for approximately 10% of the global industrial enzyme, ranking third after carbohydrases and proteases (Guerrand, 2017; Singh et al., 2016). The market is projected to expand at 6%–8% compound annual growth rate (CAGR) for the next five years, achieving around USD 7 billion in year 2020 (Guerrand, 2017). The rapid advances of the global biocatalyst market are driven by their capability to catalyse reactions with higher efficiency and stability, their chemo-, regio- and enantioselectivity as well as their eco-friendly characteristics as compared to conventional chemical process, rendering them to be attractive and versatile enzymes from the industrial point of view (Bornscheuer, 2002; Choi and Lee, 2001; de Godoy Daiha et al., 2015; Hasan et al., 2006; Singh et al., 2016). Enzyme-mediated processing technologies have therefore gained interests among academia and researchers from food and pharmaceutical industries.

Both lipases and esterases are generally considered as carboxylester hydrolases with the latter being capable of hydrolysing mostly water-soluble esters bearing short-chain acyl residues (<8 carbons). On the other hand, the former exhibits substrate specificity against water-insoluble esters, releasing long-chain fatty acid (>8 carbon atoms) as mentioned earlier (Bornscheuer, 2002; Panda and Gowrishankar, 2005; Pleiss et al., 1998; Romano et al., 2015). However, classification of both lipases and esterases remains a subject of debate to date. Several attempts have been made at differentiating lipases from esterases based on various benchmarks such as primary sequence, structural features, kinetics and use of specific inhibitors, but all these efforts ended in vain (Ali et al., 2012; Lopes et al., 2011; Romano et al., 2015). Both terms will therefore be used interchangeably in the following section. Lipases and esterases find many applications in the food, cosmetic and pharmaceutical industries not to mention detergent industry as well as oleochemical industry. The present monograph only focuses on these following sectors: emulsifier and surfactant, flavour development, functional food and ingredient and pharmaceutical drug.

## Emulsifier and Surfactant

A recent report on the global emulsifier market revealed that the market size was estimated to be USD 4.35 billion in year 2015. The market is expected to grow steadily for the next decade, owing to the increasing demand for these colloidal products in the bakery and confectionary industry (Report, 2017). Mono- (MAG), diacylglycerol (DAG) and their derivatives contribute the highest revenue share of the total industry. These products are glycerol-based fatty acid esters that help in improving the surface activity required for proper stabilization of the processed food as well as in the cosmetic industry to improve the consistency of creams and lotions. Other emulsifiers include lecithin, sorbitan esters, fatty acid lactylates and so on.

MAG are manufactured on an industrial scale through chemical glycerolysis of fats and oils which encounter several drawbacks such as low yield coupled with burnt taste and undesired colour. Enzymatic synthesis of MAG was reported to be an alternative in overcoming these limitations. Kaewthong et al. (2005) have been studying different types of lipases namely (*Pseudomonas* sp. (lipase PS), *Pseudomonas fluorescens* (lipase AK), *C. rugosa* (lipase AY), *Rhizopus delemar* (lipase D), *Mucor javanicus* (lipase M), *Rhizopus oryzae* (lipase F), *C. rugosa* (lipase OF) *Alcaligenes* sp. (lipase PL) and *Chromobacterium viscosum* (lipase LP) for the production of MAG. It is worth-mentioning that some specific MAGs (e.g. monoolein, monolaurin or monocaprin) exhibited specific activities such as anti-bacterial, anti-fungi and anti-viral activity and as antioxidant (Kyung-Hyun et al., 2010; Silalahi et al., 2014; Widiyarti et al., 2010). Chamgui et al. (2006) reported that conversion yield of 70% was achieved via direct esterification of oleic acid with glycerol using immobilized *Staphylococcus simulans* lipase. In recent years, there has been a significant shift towards enzymatic preparation of MAGs containing PUFAs through ethanolysis of different edible oils using commercial lipases as the biocatalysts (Wang et al., 2014).

Sugar fatty acid esters (SFAE) are receiving attention as non-allergic, nontoxic and biodegradable non-ionic surfactants. SFAEs possess outstanding surface-active properties such as remarkable surface tension-reducing capacity, penetrability into lipid bilayers, easiness of dispersion and remarkable emulsifying power when compared to surfactants derived from petrochemical industry



(Becerra et al., 2008; Ferrer et al., 2002; Neta et al., 2012; Sanchez et al., 2011). Zhang et al. (2014) investigated the synthesis of disaccharides of monolaurate, monodecanoate and monooctanoate using Lipozyme TLIM through transesterification. The mono-ester synthesised exhibited promising surface properties as well as antimicrobial activities against *Escherichia coli* O157:H7 (gram-negative bacteria) (Zhang et al., 2014). Besides, Neta et al. (2012) also showed the potential application of *Candida antarctica* type B (CALB) lipase immobilized on chitosan in producing fructose, sucrose and lactose esters which were capable of stabilising coconut milk emulsions. Another research study also described the synthesis of a series of oligofructose monoesters with fatty acids of different chain lengths (C8–18) to obtain superior food-grade surfactants with a range of amphiphilicity via CALB-catalysed esterification (van Kempen et al., 2013, 2014).

Lysolecithin (or lysophosphatidylcholine) is another type of attractive emulsifier by virtue of its strong hydrophilicity. It is produced from the hydrolysis of egg yolk lecithin after treatment with phospholipases A (aliphatic esterases), which is then incorporated for the formation of stable oil-in-water (O/W) type emulsions such as mayonnaise (Borrelli and Trono, 2015). The use of immobilized phospholipase A<sub>2</sub> (PLA<sub>2</sub>) in alginate-silica sol–gel matrix was found to be capable of producing egg yolk lysolecithin (Kim et al., 2001). Commercial porcine pancreas PLA<sub>2</sub> (Lecitase, Novozymes) and *A. niger* PLA<sub>2</sub> (Maxapal, DSM) have also been successfully developed for this purpose (Casado et al., 2012).

Interest in the production of structured phospholipid (PL) containing specific fatty acid residues has also increased significantly in recent years. For instance, Vikbjerg et al. (2007) reported the PLA<sub>2</sub>-catalysed synthesis of PL with caprylic acid by acyl modification of the sn-2 position in PLs. Another work conducted by Zhao et al. (2014) also reported the potential of phospholipase A<sub>1</sub> (PLA<sub>1</sub>) immobilized in Lewatit VP OC1600 in synthesising n-3 PUFA enriched-phosphatidylcholine. Besides delivering the emulsifying properties, replacement of fatty acid residues present in the native PL by fatty acids with beneficial physiological effects can lead to tailored PLs that offer intriguing marketing opportunity for manufactures of nutraceuticals.

### Flavour Development

Flavouring and aromatic compounds are generally considered as short-chain aliphatic esters which are important food additives to be added into the food, cosmetic and pharmaceutical industries. Despite the availability of these esters in natural sources (fruits), the concentration is relatively low and the composition changes upon maturation (Su et al., 2016). Enzymatic approach has therefore offered some advantages of being environmental friendly, highly selective and efficient in synthesising these aromatic esters.

Bayramoğlu et al. (2011) investigated the use of immobilized *C. rugosa* lipase in hexane to synthesise ethyl valerate (ethyl pentanoate) (fruity flavour) and high esterification yield of 67.2% was achieved as compared to previous study conducted by Karra-Chaabouni et al. (2006) with 51% of ethyl valerate being produced using *S. simulans* lipase. The isoamyl acetate (banana flavour) was also synthesised using *C. antarctica* lipase (Novozyme 435) with conversion of 80% under optimal conditions (Güvenç et al., 2002). Shieh and Chang (2001) achieved a hexyl acetate (fruity flavour) conversion of 86.6% by transesterification of hexanol with triacetin in hexane using immobilized *Mucor miehei* lipase (Lipozyme IM-77) under optimum synthesis conditions. The high conversion yield was obtained when using immobilized *Staphylococcus xylosus* lipase (80%) and its mutant (LK-SXL1) (95%) as biocatalysts to catalyse the esterification reaction between acetic acid and butanol to produce butyl acetate ester (pineapple flavour) (Ben Salah et al., 2007). Su et al. (2016) also discovered the potential application of cutinases, the smallest members of the  $\alpha/\beta$  hydrolase superfamily which displayed intermediate characteristics between lipases and esterases, in producing high yield (99.2%) of ethyl caproate (fruity flavour).

Terpene alcohol esters (such as geraniol and citronellol esters) are important components of essential oils found in several aromatic plants, giving fruity rose and lavender aroma. These esters can be produced chemically but these synthetic products formed are inferior to the natural ones (Claon and Akoh, 1994a,b). On the other hand, products of lipase-catalysed reactions have a higher quality and stability under certain conditions. Lipase-catalysed synthesis of terpene esters by direct esterification (Claon and Akoh, 1993, 1994a; Oguntimein et al., 1995; Salvi et al., 2017) and transesterification (Claon and Akoh, 1994b; Shieh et al., 1996; Yee et al., 1995) using different substrates as the starting materials in either solvent or solvent-free systems have been well described. However, some researchers claimed that lipases showed low affinity towards the synthesis of short-chain fatty acid esters of geraniol especially when acetic acid (C<sub>2</sub> carbon) was used as acyl donor during esterification (Oguntimein et al., 1995). Nevertheless, these low molecular weight geraniol esters (geraniol acetate) could be synthesised successfully with esterase derived from *Fusarium oxysporum* (Christakopoulos et al., 1998; Karra-Chaabouni et al., 1996). Macrocyclic lactones, an expensive aromatic substance of musky fragrance can also be produced using *Pseudomonas* sp. lipase (Kageyama et al., 1990) and *Starmerella bombicola* lactone acetyl esterase (Ciesielska et al., 2016) with high conversion yield.

Esterases and lipases from *Lactobacillus casei* CL96 were used significantly for hydrolysis of milk fat for the purpose of flavour enhancement in the manufacture of cheese-related products (Choi and Lee, 2001). An acylesterase from *Saccharomyces cerevisiae* was also reported to play an important part in the production of isoamyl acetate which has a major role in the determination of sake flavour during the fermentation stage (Fukuda et al., 1998).

Hydroxycinnamic acids (HCA) such as ferulic, sinapic, caffeic and *p*-coumaric acids, are widely applied in food, cosmetic and pharmaceutical industries due to their desirable flavour profiles. More interestingly, previous literature revealed that HCAs exhibit potent antioxidant properties (Graf, 1992) with powerful scavenging activities towards peroxynitrite and have good capability in oxidising low density lipoprotein (LDL) which translates to potential in treating cardiovascular diseases and neurodegenerative disorders such as Alzheimer's disease (Topakas et al., 2007). As a result, there is a growing interest in converting these ferulic acids into various valuable molecules such as catechol, vanillic acid derivatives and vanillin being the most universally used aromatic



molecules (Havkin-Frenkel and Belanger, 2009; Walton et al., 2000). Pentylferulate ester, a flavour precursor in food and cosmetic industries can be produced using feruloyl esterase (FE) from *A. niger* in water-in-oil (W/O) microemulsions (Giuliani et al., 2001) and commercial immobilized enzyme from *C. antarctica* (Chirazyme L-2) (Yoshida et al., 2006). On the other hand, feruloyl and cinnamoyl esterase, synergistically with pectinases, celluloses and xylanases can also be employed to hydrolyse esters from agro-industrial residues such as wheat bran, maize bran and fibre, rice bran, brewer's spent grain, sugar cane, bamboos, sugar beet pulp and various agro-industrial by-products, and releasing the beneficial HCAs (Asther et al., 2002; Topakas et al., 2007).

### Functional Food and Ingredient

The potential use of lipases and esterases in the production of food ingredients with functional properties is well documented. Over the past few decades, increased focus has been placed on the obesity epidemic owing to its high prevalence and considerable contribution to overweight-related disease burden. The search for cost-effective techniques for the production of functional oils such as DAG-enriched oil (Phuah et al., 2015), medium-and-long chain triacylglycerol (MLCT) oil (Akoh and Kim, 2008; Lee et al., 2012), very long-chain triacylglycerol (VLCT) oil (Speranza and Macedo, 2012), medium-chain triacylglycerol (MCT) oil (Langone and Sant'Anna, 1999; Langone and Sant'Anna, 2002) and PUFA-enriched TAG oil (Lee and Akoh, 1998) via lipase-catalysed reaction to help tackle obesity as the consequence of excessive edible oil consumption has therefore been intensified and reviewed periodically. Commercially available modified fats include 1) Caprenin (Procter & Gamble Co., United States), a low caloric fat product composed of carpylic (C8:0), caproic (C10:0) and behenic (C22:0) acid (Adamczak, 2004; Speranza and Macedo, 2012); 2) SALATrim/Benefat (Nabisco Foods Group, United States), a fat consists of a mixture of short chain fatty acids namely acetic (C2:0), butyric (C4:0) acids and long chain fatty acid like stearic acid (C18:0) with reduced calorie value (Speranza and Macedo, 2012); 3) Econa/Enova oil (Kao Corporation, Japan), a fat with two fatty acids esterified to a glycerol backbone, forming DAG with potential anti-obesity properties (Katsuragi et al., 2004); 4) Resetta™ (Nisshin Oillio Group Ltd, Japan), a fat containing both medium chain and long chain fatty acid in the same TAG molecule for overweight management (Lee et al., 2012); Neobee® (Stepan Co., United States), a fat consists predominantly of medium chain fatty acid (MCFA) with low energy value. These functional oils mentioned above can be synthesised with ease in the presence of the lipases. The current research trends on the enzymatic synthesis of these functional oils have been reviewed comprehensively by Kim and Akoh (2015).

The synthesis of human milk fat substitute (HMFS) to mimic the natural human milk fat (HMF) is of great interest when breast feeding is not possible in order to ensure a supply in infant nutrition with similar TAG composition to that of mothers milk (Soumanou et al., 2013). Ever since the commercialisation of Betapol® (Loders Croklaan, Netherlands) as the first available HMFS in the market produced using sn-1,3-specific lipase (such as Lipase RMIM), extensive researches have then been carried out in modifying milk fats with enhanced absorbability besides delivering a reduced calorie value as well as providing protection to bacterial and viral infection for new born, term and preterm infants (Zou et al., 2016). Previous literature studies indicated that the presence of long-chain saturated fatty acid at position sn-1 and sn-3 reduces the absorbability of milk fat due to the formation of insoluble calcium soaps of these fatty acids in the intestinal tract, leading to the loss of energy and calcium intake and in the worst case scenario, influence the bone mineralisation (Gerritsen et al., 2005; Li et al., 2010). Some researchers have therefore focused on the enzymatic preparation of HMFS-enriched with MCFA. For instance, Ilyasoglu et al. (2011) studied the lipase-mediated synthesis of HMFS-enriched with MCFA by Lipozyme using reaction substrates such as tripalmitin, hazelnut oil fatty acids and Neobee® fatty acids (carpylic and capric acids). In another study, Álvarez and Akoh (2015) produced capric acid enriched-HMFS in two enzymatic inter-esterification stages using Lipozyme TLIM by reacting high melting point palm stearin, high oleic oil and tricaprin. Incorporation of PUFA into milk fat at 1,3-position had also been reported to be possible through lipase-catalysed interesterification of tripalmitin and omega-3 fatty acid (Tecalão et al., 2010), interesterification between tripalmitin, hazelnut oil fatty acid and  $\gamma$ -linolenic acid (Sahin et al., 2005) and interesterification between stearidonic acid-enriched soybean oil with tripalmitin (Teichert and Akoh, 2011) in order to deliver essential fatty acids for proper brain growth and functioning in infants. The advances on lipase-catalysed processes currently conducted for the synthesis HMFS, namely types of enzyme, reaction conditions, reactor types and mode of operation, were reviewed by Soumanou et al. (2013) and the future outlook on the development of HMFS were discussed by Zou et al. (2016).

Cocoa butter is the most expensive TAG which consists of saturated fatty acid (palmitic or stearic acid) located predominantly at its 1,3-positions and unsaturated fatty acid (oleic acid) at its 2-position. The cocoa butter is widely used in the chocolate and confectionary industry owing to its unique sensory characteristic and sharp melting profile between 25–35 °C. It has therefore provided an opportunity for sn-1,3-specific lipases in the manufacturing of structured lipid (SL) with favourable physicochemical properties (such as cocoa butter substitute (CBS) and cocoa butter equivalent (CBE)). The production of CBS via transesterification or acidolysis of inexpensive oil such as palm mid fraction with tristearin or stearic acid using 1,3-specific lipases was initiated by Unilever and Fuji Oil when they filed their patent applications (Neil, 1981). The research continues in this area thereafter. Borhan et al. (2011) reported the possibility of using commercial immobilized sn-1,3-specific lipase from *R. miehei* (Lipase IM 60) in synthesising CBS through interesterification of palm mid fraction, palm stearin and MCT. The resulting interesterified fat showed comparable solid fat content to that of cocoa butter. Kim et al. (2014) produced CBE high in 1-palmitoyl-2-oleoyl-3-stearin (a major component in cocoa butter) via lipase-catalysed transesterification of high oleic sunflower oil with a mixture of ethyl palmitate and ethyl stearate.

Phenolic acids are of great commercial potential in food and pharmaceuticals as mentioned earlier. However, the partial solubility of these natural antioxidants limits their clinical effectiveness. Feruloyl esterases (FE) can act as biosynthesis tools used to produce phenolic acid sugar esters via esterification with aliphatic alcohols, resulting in the formation of more lipophilic derivatives. Lipases are unable to catalyse the esterification reaction in this scenario if aromatic cycle is parahydroxylated and lateral chain

is not saturated. Therefore, the reaction can only be carried out using FE. FE from *S. thermophile* was proven to be able to catalyse the transfer of feruloyl group to L-arabinobiose (Vafiadi et al., 2006).

Phytosterols (plant sterols and stanols), a naturally bioactive compound found in edible vegetable oil, are well known for their LDL cholesterol-reducing potential and antioxidant activity (Plat and Mensink, 2005; Zheng et al., 2012). These attractive properties of phytosterols have led to the recent commercialisation of phytosterols as Benecol® food supplement by Raisio Corporation (Finland) and the attempts of incorporating this product into various food matrixes such as margarine, mayonnaise, dairy products and others has thereafter increased significantly (Dutta, 2003). Despite its beneficial health effects, phytosterols in free form have limited interest owing to their low solubility, stability and low absorption rate (Villeneuve et al., 2005). Therefore, fatty esters of sterols are more advantageous as functional ingredients in food formulation. These phytosterol esters can be produced enzymatically using microbial lipases (Zheng et al., 2012), plant lipases (Villeneuve et al., 2005) and microbial sterol esterases (cholesterol esterases) (Vaquero et al., 2016) with high efficiency.

## Pharmaceutical Drug

Among the applications of lipases and esterases, the synthesis of enantiomerically pure active pharmaceutical ingredients (APIs) and their intermediates using these enzymes is a subject of continuing interest. It is noteworthy that most of the drugs are chiral, and only certain active stereoisomer has the desired biological activity while the other stereoisomer might pose deleterious side effects on health (Carvalho et al., 2015). One of the important properties of lipases and esterases is their ability to differentiate between enantiomers of chiral molecules, making them interesting as biocatalysts in the pharmaceutical field.

Microbial lipases are used in kinetic resolution of chiral compounds that serve as synthons in the synthesis of chiral pharmaceutical products in which hydroxyl acids and their derivatives are major target molecules because they constitute the framework of many chiral natural products and biological active agents. Lipases are used in the synthesis of chiral synthon as intermediates for the synthesis of paclitaxel (taxol) from 3-methoxytulene which exhibits a unique mode of action on microtubule proteins that are responsible for the formation of the spindle during cell division and therefore it has been used to treat various cancers especially ovarian cancer (Fukaya et al., 2016). Another study also demonstrated the potential application of lipases in production of intermediates for the synthesis of an antihypertensive drug namely Captopril which prevents the conversion of angiotension-I to angiotension-II by inhibition of an angiotension-converting enzyme. Immobilized lipases from both *Pseudomonas cepacia* and a *Pseudomonas* sp. catalysed the production of the S-isomer at greater than 32% yield and 96% optical purity (Patel et al., 1996). Lipases are also used in the production of a stereospecific isomer for the production of a  $\beta$ -blocker which contains aryloxypropanol-amine structure (such as (S)-propranolol and (S)-metoprolol) with an asymmetric carbon that can be used to treat hypertension, migraine headaches and cardiovascular related diseases (Schaechter, 2009). To enhance the bioavailability of the  $\beta$ -blocker, synthesis of metoprolol- or propranolol-saccharide conjugates by using combination of lipase and protease in two steps has proven to be a promising approach (Zheng et al., 2011). A review on lipases and its biocatalytic applications in pharmaceutical industries inter alia the synthesis of paroxetine hydrochloride, diltiazem hydrochloride, chiral  $\beta$ -azidoalcohols, vitamin D<sub>3</sub> derivatives, primary amines and amides, was provided by Angajala et al. (2016).

Esterases also play a major role in the synthesis of chiral drugs (Bornscheuer, 2002). For example, an esterase from *Trichosporon brassicae* has been used extensively for the production of optically pure (S)- and (R)-ketoprofen, which is effective in reducing inflammation and relieving pain resulting from arthritis, sunburn, menstruation, and fever (Shen et al., 2002). In another study, Kim et al. (2015) also showed that esterases derived from *Archaeoglobus fulgidus* mutants, displays high thermostability and improved enantioselectivity toward (S)-ketoprofen ethyl ester, enabling the production of (S)-ketoprofen with better pharmaceutical activity and lower side effects through hydrolysis of the ester bond of (R,S)-ketoprofen ethyl ester. In addition, research studies also disclosed the potential applications of esterases from *T. brassicae*, *Rhodococcus* sp., *Bacillus circulans* and *Pseudomonas* sp. in producing substantial amount of chiral compounds with therapeutic effect such as ibuprofen (Kim et al., 2002).

Similarly, an esterase from *Bacillus coagulans* catalyses the enantioselective hydrolysis of the racemic ester of 1,2-O-isopropylidene glycerol (IPG) (solketal ester), yielding IPG with high enantiomeric excess. IPG is an important chiral synthon in the synthesis of glyceryl phosphate, tetraoxaspirodecane and other biological active compounds such as prostaglandins, leukotrienes and  $\beta$ -blockers (Molinari et al., 1996). Carboxylesterase from *Bacillus subtilis* was also used in the resolution of (R,S)-ibuprofen methylester (Quax and Broekhuizen, 1994) and exhibited higher selectivity as compared to lipase from *C. rugosa* (Mustranta, 1992). An up-to-date application of esterases as stereoselective biocatalysts in pharmaceutical field has been reviewed thoroughly by Panda and Gowrishankar (2005) and Romano et al. (2015).

## Conclusion

The present monograph provides brief descriptions of the recent developments about the potential applications of lipases and esterases in various sectors namely pharmaceuticals, functional food or nutraceuticals, emulsifier or biosurfactants as well as flavour and fragrance. Besides, the different sources of lipases and esterases, their catalytic behaviours and selectivity have also been reviewed extensively. Nevertheless, relatively high cost of enzyme lipases and esterases coupled with low operational stability remains a major techno-economic bottleneck that limits their widespread industrial applications. Several points should therefore be taken into consideration in order to alleviate the barriers such as 1) fermentation technology, allowing for a feasible production process of

the enzyme on a large scale basis; 2) immobilisation techniques, improving the stability and activity of the enzymes besides adding to the possibility of the continuous use of the biocatalysts and 3) protein engineering, enhancing and developing the desired catalytic properties of the enzymes lipases and esterases. Furthermore, the applications of enzyme lipase and esterase particularly in the food and pharmaceutical sector can be further be explored in order to cope with the consumers demand for a safe, healthy and nutritious food.

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# Holistic Control of Fats and Oils by NMR Spectroscopy

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## Introduction

Fats and oils are important factors of our daily diet which are used for cooking, frying, and as ingredients in several manufactured foods such as margarine, chips, ice cream and cookies. Depending on their composition oils and fats show different physicochemical and nutritional properties concerning their melting point, oxidative stability and influence on consumer health. Furthermore, expensive oils show a great potential for being adulterated with the objective of increasing the profit. This is one of the many reasons why the quality control of edible oils and fats is a very important task of customer safety. Quality control of lipids has been classically undertaken by several official methods, approved by the German Society for Fat Science (Deutsche Gesellschaft für Fettwissenschaft e.V.; DGF) and the American Oil Chemist Society (AOCS), including chromatography and titration to determine the fatty acid profile, acid value, peroxide value, iodine value, secondary substances, etc. Many of these methods have been developed and standardized several decades ago undergoing only minor changes since then. Some of them are not only cumbersome and error-prone, but also time-consuming and expensive. Taking this into account, the development of a fast and non-destructive quality control is of great interest. Former researches have described the nuclear magnetic resonance (NMR) as a technique extending the analysis of edible fats and oils (Diehl and Skiera, 2013; Diehl, 1998; Skiera et al., 2012a, 2012b, 2012c; Guillén and Ruiz, 2003). NMR spectroscopy enables a rapid, simultaneous, non-invasive, and non-destructive holistic control of oils and fats requiring small amounts of sample (about 200 mg) and solvents (about 1 mL). NMR spectroscopy can be used for the quality control of different kinds of lipids from vegetable and marine source. Lipid ingredients are identified with  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy without requiring any standards. The quantification is performed by integrating the appropriate signals. These integrals are directly proportional to the molar amount of the component. Since there is no need of chemical modification such as derivatization, degradation processes of sensitive components are avoided and the sample can be recovered unmodified after the analysis.

## Fatty Acid Distribution

Fats and oils are mainly made up of 95% to 99% of triacylglycerols (TAGs). The rest is composed of oil specific secondary substances, free fatty acids (FFAs) and processing products. The concentration and relation of the different fatty acids (FAs) such as saturated and unsaturated FAs provides information about the oil type. NMR spectroscopy is a powerful tool for the determination of the FA composition in lipids.  $^1\text{H}$  NMR spectroscopy provides qualitative and quantitative information about several quality parameters such as the saturation grade, peroxides and aldehydes.  $^{13}\text{C}$  NMR spectroscopy enables a detailed look into the chemical structure of the individual FAs as well as their regioselective distribution.

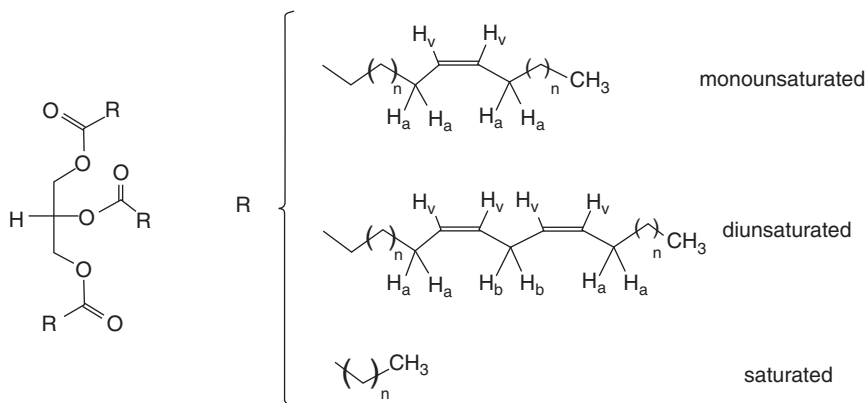
## Fatty Acid Profile by $^1\text{H}$ NMR Spectroscopy

A TAG is an ester derived from glycerol and three FAs which are categorized into saturated and unsaturated FAs. The signal assignment for  $^1\text{H}$  NMR spectra is presented in Table 1. The structure of the major TAGs is presented in Fig. 1 where from the spectroscopic

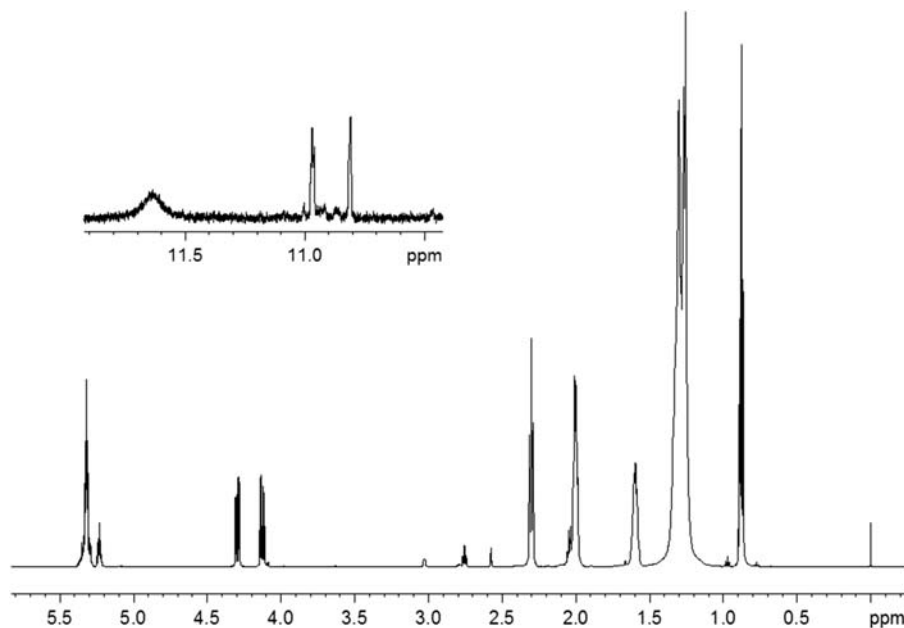
**Table 1** Signal assignment of vegetable oils in  $\text{CDCl}_3/\text{DMSO}$  (4:1);  $^1\text{H}$  NMR spectra (500 MHz)

Signal no.	Type	Functional group	Chemical shift [ppm]
1	free fatty acids (FFAs)	$-\text{COOH}$	12.0–11.1
2	hydroperoxide	$-\text{OOH}$	11.1–10.2
3	aldehyde	$-\text{CHO}$	9.8–9.0
4	Cis/trans conjugated	$-\text{CH}=\text{CH}-$	7.5–5.5
5	Allylic	$-\text{CH}=\text{CH}-$	5.5–5.3
6	Glycerin backbone	$-\text{CH}-$	5.3–5.2
7	Glycerin backbone	$-\text{CH}_2-$	4.4–4.0
8	C18:3	$-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$	2.80
9	C18:2	$-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$	2.75
10	TAG	$-\text{COO}-\text{CH}_2-$	2.4–2.2
11	TAG	$-\text{CH}=\text{CH}-\text{CH}_2-$	2.1–1.9
12	TAG	$-\text{COO}-\text{CH}_2-\text{CH}_2-$	1.7–1.5
13	TAG	$(-\text{CH}_2-)_n$	1.5–1.1
14	$\omega$ -3	$-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}_3$	0.99–0.94
15	TAG	$-\text{CH}_3$	1.0–0.7





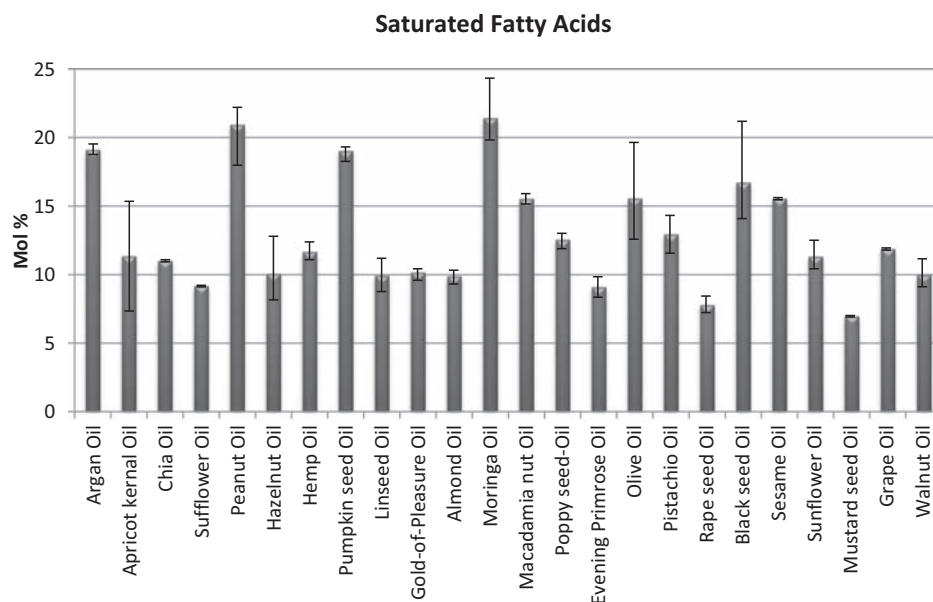
**Fig. 1** Chemical structure of TAGs in edible oils. Based on [Salinero et al. \(2012\)](#).



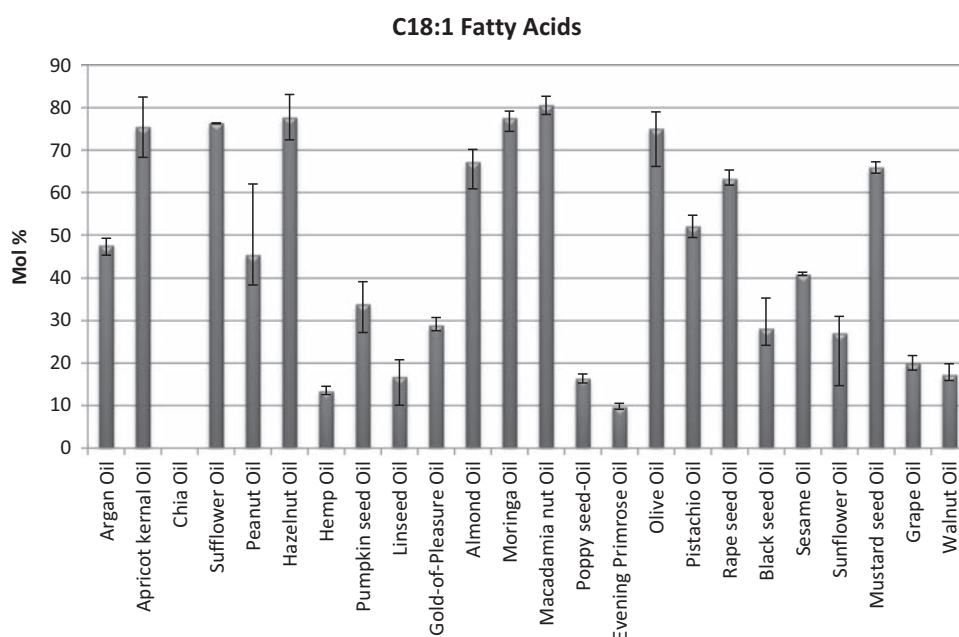
**Fig. 2** 600 MHz  $^1H$  NMR spectrum of an olive oil in  $CDCl_3/DMSO$  (4:1).

point of view, the most important hydrogens are highlighted. **Fig. 2** presents a typical  $^1H$  NMR spectrum of an edible oil. Based on the anisotropic properties, the multiplet of the vinylic hydrogens ( $H_v$ ) is shifted to  $\delta$  5.5–5.3 ppm. This signal is used for the determination of the saturation grade. With increasing distance to the  $C=C$  bond the anisotropic properties decrease continuing to separate the signals from allylic ( $H_a$ ) and bisallylic hydrogens ( $H_b$ ). By comparing  $H_a$  with  $H_b$ , mono- and polyunsaturated FAs can be differentiated. The ratio of the double bonds and the  $\alpha-CH_2$  group at  $\delta$  2.3 ppm determines the mean number of double bonds in each FA and thus the iodine value (IV). Terminal methyl groups show triplets at  $\delta$  1.0–0.7 ppm, whereby the chemical shift depends on the distance to the next double bond. Especially the methyl group of  $\omega$ -3 FAs is influenced by the anisotropy of the close double bond leading to a signal with a chemical shift of  $\delta$  0.99–0.94 ppm. To determine the molar part of  $\omega$ -3 FAs, all signals of the terminal methyl groups are normalized to 100. By splitting the integral, the value of the  $\omega$ -3 integral determines the molar ratio in %.  $\omega$ -6 and  $\omega$ -9 signals overlap and cannot be quantified separately in the  $^1H$  NMR spectrum, even by using a 600 MHz spectrometer. However, this challenge can be met by the  $^{13}C$  NMR spectroscopy. By normalizing the methyl group to 300 the relative molar ratio of several compounds such as C18:2 and C18:3 FAs can be determined. In the presented research work, FA profiles of 24 vegetable oils have been analyzed (**Figs. 3–6**). Here total saturated FAs and unsaturated FAs, especially C18:1, C18:2 and C18:3 FAs are compared in mol-%. The total saturated FAs of the analyzed vegetable oils were determined in a range of 6.98% to 21.45%, monounsaturated C18:1 FAs of <LOD to 80.66%, C18:2 FA of 0.68% to 72.22% and C18:3 FA of 0.24% to 57.50%. The characteristic FA profile is used for the determination of the lipid source.

Since some of the analyzed vegetable oils show a comparable FA profile, adulterations and contaminations cannot be detected without any statistical procedures. With the use of multivariate models like Principal Component Analysis (PCAs) the genuineness



**Fig. 3** Saturated FA of different kinds of edible oils (mean  $\pm$  deviation).

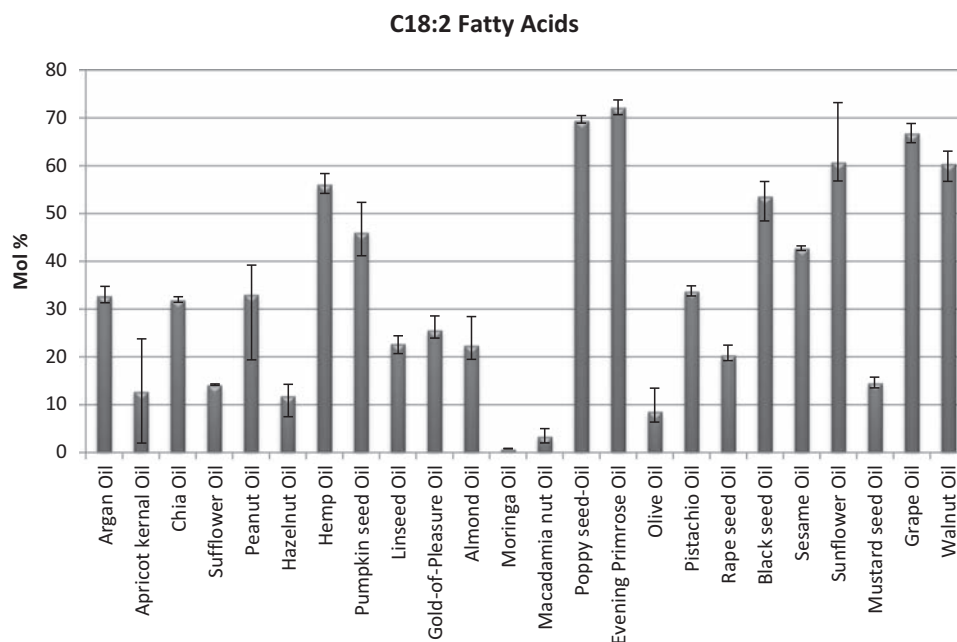


**Fig. 4** Monounsaturated FA of different kinds of edible oils (mean  $\pm$  deviation).

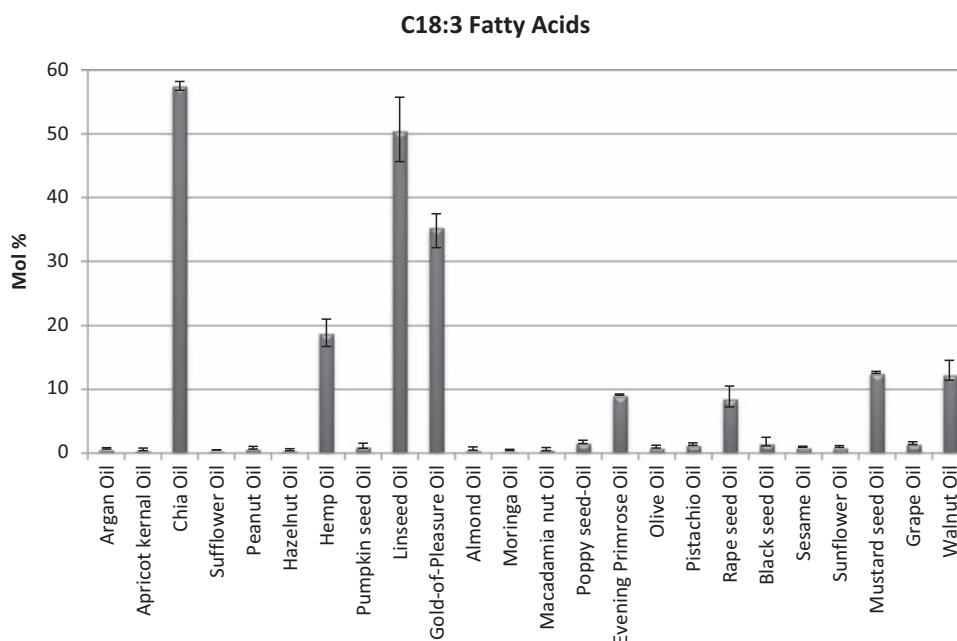
of edible oils can be distinguished by visualizing the separation of the different oil types (Fig. 7). The use of chemometric methods is presented in the section on **Adulteration Control**.

### Fatty Acid Distribution by $^{13}\text{C}$ NMR Spectroscopy

The  $^{13}\text{C}$  NMR spectroscopy provides information on the chemical structure of the TAGs and FAs showing an individual signal for each carbon. The  $^{13}\text{C}$  NMR spectra can be compared with chromatograms where each signal can be assigned to an appropriate substance. Fig. 8 illustrates a typical 600 MHz  $^{13}\text{C}$  NMR spectrum of walnut oil. In the spectrum four main regions can be identified: carbonyl region ( $\delta$  174–172 ppm), olefinic signals ( $\delta$  134–126 ppm), glycerol region ( $\delta$  74–60 ppm), and aliphatic region ( $\delta$  35–19 ppm). Since the position of the double bond within a FA causes an anisotropic shift of the neighbored carbon atoms,  $\omega$ -3 as well as  $\omega$ -6,  $\omega$ -7,  $\omega$ -9 and saturated FAs can be identified in highly resolved  $^{13}\text{C}$  NMR spectra by their separated signals. By normalizing



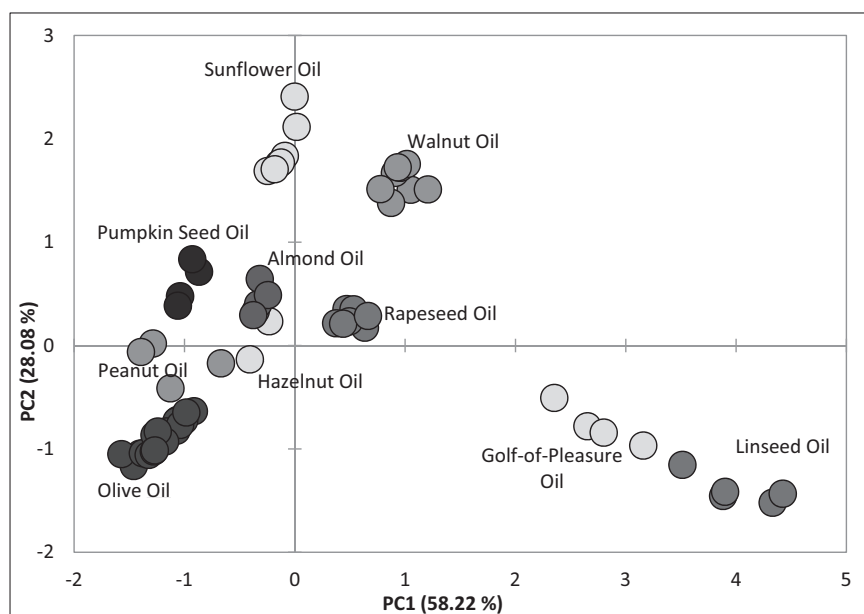
**Fig. 5** C18:2 FA of different kinds of edible oils (mean  $\pm$  deviation).



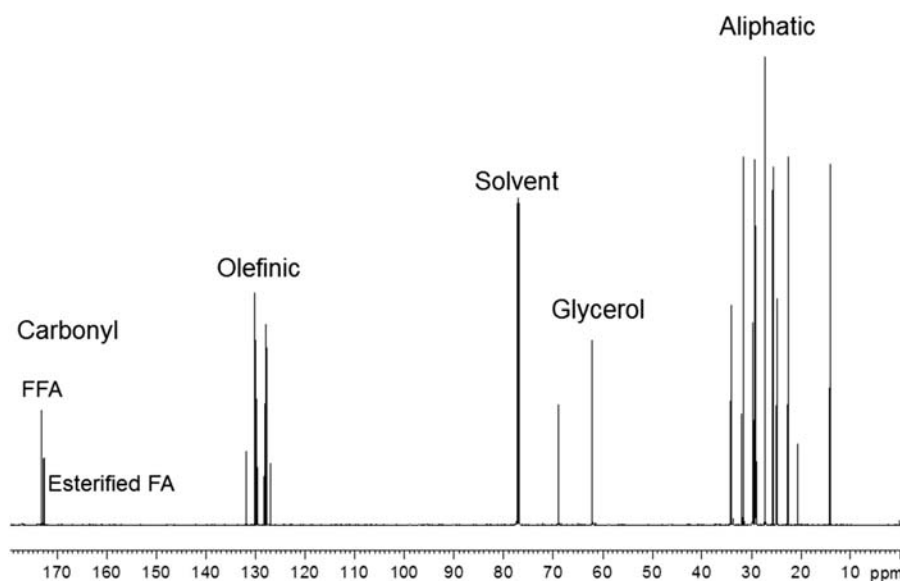
**Fig. 6** C18:3 FA of different kinds of edible oils (mean  $\pm$  deviation).

all signals of the same carbon group to 100, the molar ratio of each FA can be determined. In **Fig. 9** the FA distribution of a linseed oil is analyzed by using deconvolution for quantification. The results show that the analyzed linseed oil consists of 58.1%  $\omega$ -3, 14.9%  $\omega$ -6, 0.2%  $\omega$ -7, 17.5%  $\omega$ -9 and 9.2% saturated FA. Since the chemical structure of saturated FAs differs only in the chain length, signals of individual saturated FAs cannot be separated. Thus, saturated FAs are quantified in total.

Besides the general FA distribution, the  $^{13}\text{C}$  NMR spectroscopy provides information on the regiospecific distribution of FAs in TAGs. Since the anisotropy makes the carbonyl signals sensitive to double bonds within the FA chain, carbonyl signals of a neighboring single and double bonds show a difference of the chemical shift of approx.  $\delta$  0.5 ppm. This effect decreases according to the distance to the next double bond. A signal separation is still possible at a double bond distance up to eleven carbon atoms in the chain. Thus, the carbonyl region is very sensitive for regioselective analysis. Distinction of sn-1/3 and sn-2 are possible for TAGs, phospholipids and glycolipids (**Fig. 10**). Free and esterified FAs are also determined by evaluating the carbonyl region. Here, we



**Fig. 7** PCA scores plot on the first and the second principle components for 500 MHz  $^1\text{H}$  NMR integrals of the FA profile of ten vegetable oils.

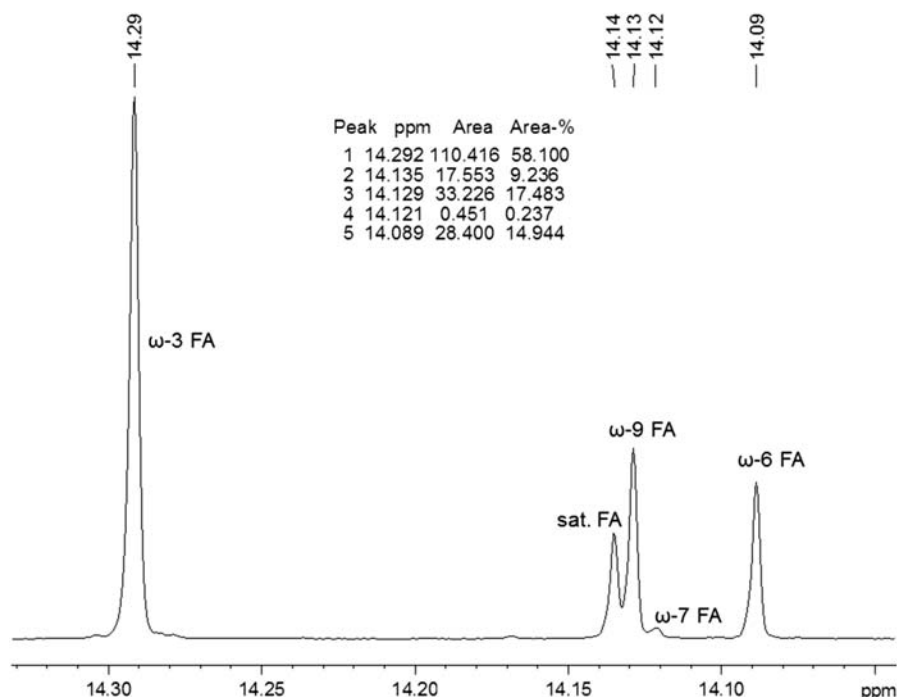


**Fig. 8** 600 MHz  $^{13}\text{C}$  NMR spectrum of walnut oil.

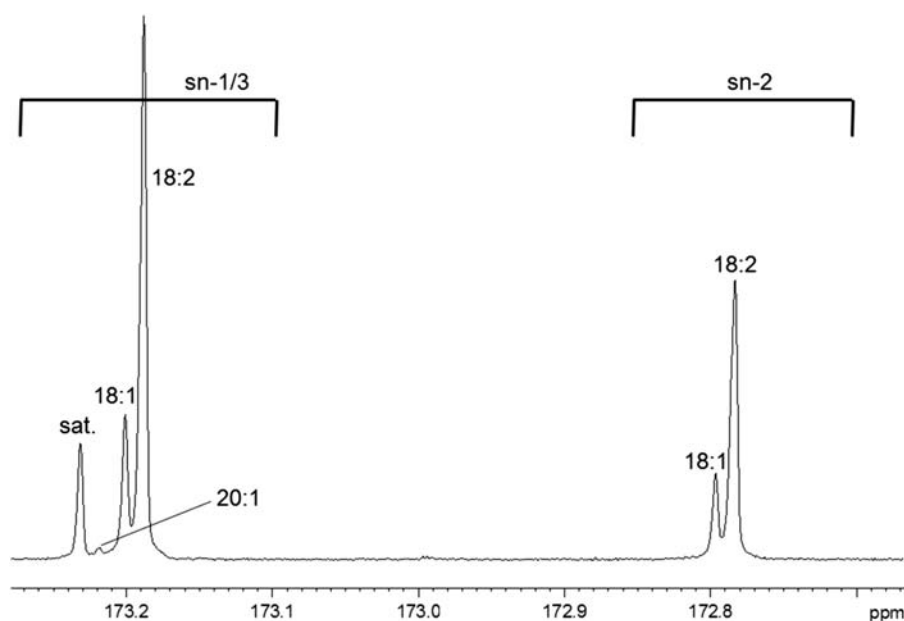
can distinguish between total free (FFA) and total esterified FAs including mono-, di- and triacylglycerides. By integrating both regions and normalizing them to 100, the molar ratio (in %) of free and esterified FAs is determinable. Furthermore, individual FFAs are assigned, as presented in **Fig. 11**. After methylation the total content of individual FAs can be examined, irrespective of whether they existed in the free or esterified form in the lipid before (**Fig. 11**).

### Trans-fatty Acids

*Trans* fatty acids have been of increasing concern to the consumers since unfavorable effects in the diet were confirmed ([Katan et al., 1995](#)). Thus, the US Food and Drug Administration (FDA) decided to put the content of *trans* FAs on the list of nutritional labeling ([Food and Drug Administration \(FDA\), 2003](#)). Food manufacturers commonly used GC to separate *cis* and *trans* FAs to examine the *trans* FA content. However, GC analysis is time-, cost- and solvent-consuming, and the results are strongly impacted by the sample pretreatment and the instrument performance. To overcome this problem, NMR spectroscopy is applied as a rapid, nondestructive method for determining the *cis* and *trans* composition of unsaturated lipids. Since resonances of neighboring hydrogens are shifted, due to the magnetic anisotropy of a double bond, to the downfield, allylic methylene groups are easily distinguishable from

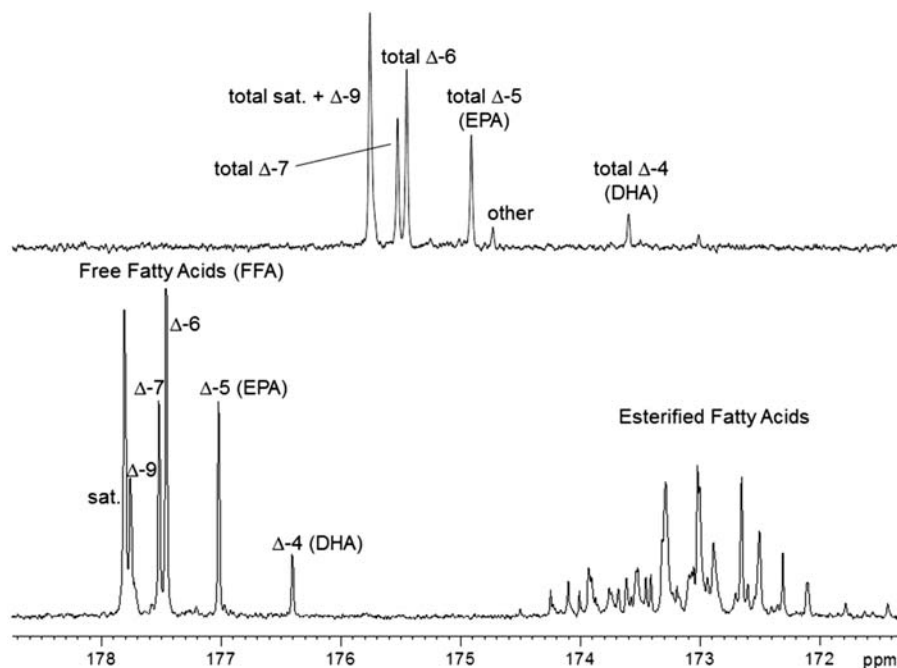


**Fig. 9** 600 MHz  $^{13}\text{C}$  NMR spectrum of linseed oil, terminal methyl group.

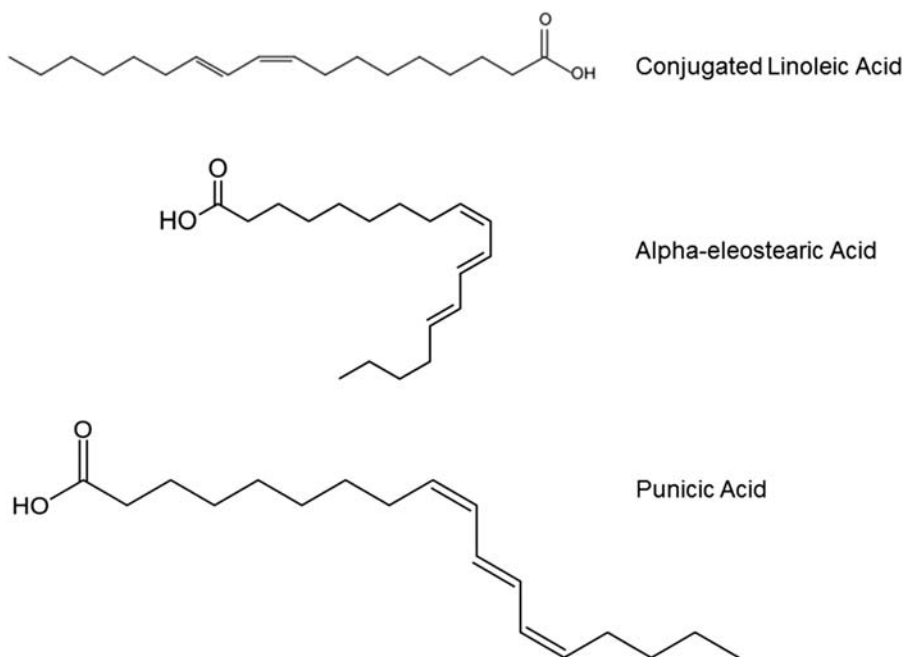


**Fig. 10** 600 MHz  $^{13}\text{C}$  NMR spectrum of a vegetable oil, carbonyl group.

saturated parts of the FA. In nature, polyunsaturated FAs show predominantly *cis* homo conjugated double bonds. However, some oils and fats show extraordinary, characteristic FAs with *trans*-double bonds. As an example, conjugated linoleic acid (CLA; **Fig. 12**) can be found in food products from grass-fed ruminants as well as in eggs after feeding chickens with CLA. In 2008, the FDA categorized CLA as generally recognized as safe ([Food and Drug Administration \(FDA\), 2003](#)). CLA contains a *cis* double bond in position C9 or C12, and a *trans* double bond in C11 or C10, respectively. *Cis* and *trans* double bonds of linoleic acid and conjugated linoleic acid can be distinguished by their  $^1\text{H}$  NMR spectra. **Fig. 13** illustrates the  $^1\text{H}$  NMR signals of the double bonds of CLA in butter and linoleic acid in hazelnut oil, respectively. Signals of *cis/trans* conjugated double bonds shift to  $\delta$  6.4 ppm – 5.5 ppm, whereby other allylic protons show a multiplet at  $\delta$  5.5 ppm – 5.3 ppm. The content of *trans* FAs can be determined if the molecular weight of the appropriate FA is known. Two other *trans* FAs which occur rarely in nature are the  $\alpha$ -eleostearic acid (C18:3 9c11t13t; **Fig. 12**) in cherry stones and bitter melon seed oil (30%–60%), and the punicic acid (C18:3 9c11t13c; **Fig. 12**) in pomegranate seeds



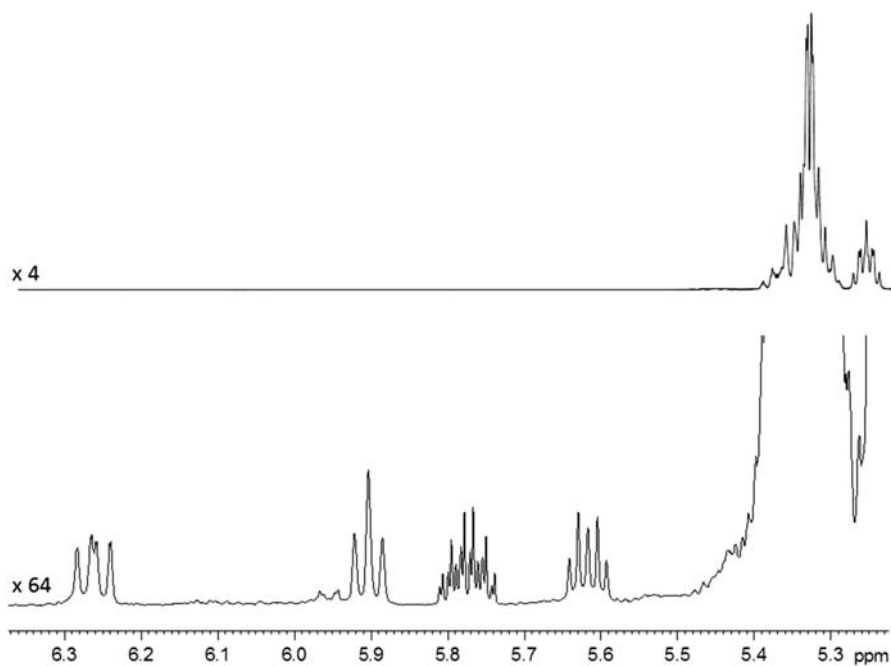
**Fig. 11** 600 MHz  $^{13}\text{C}$  NMR spectra of a fish oil, before (bottom) and after methylation (top).



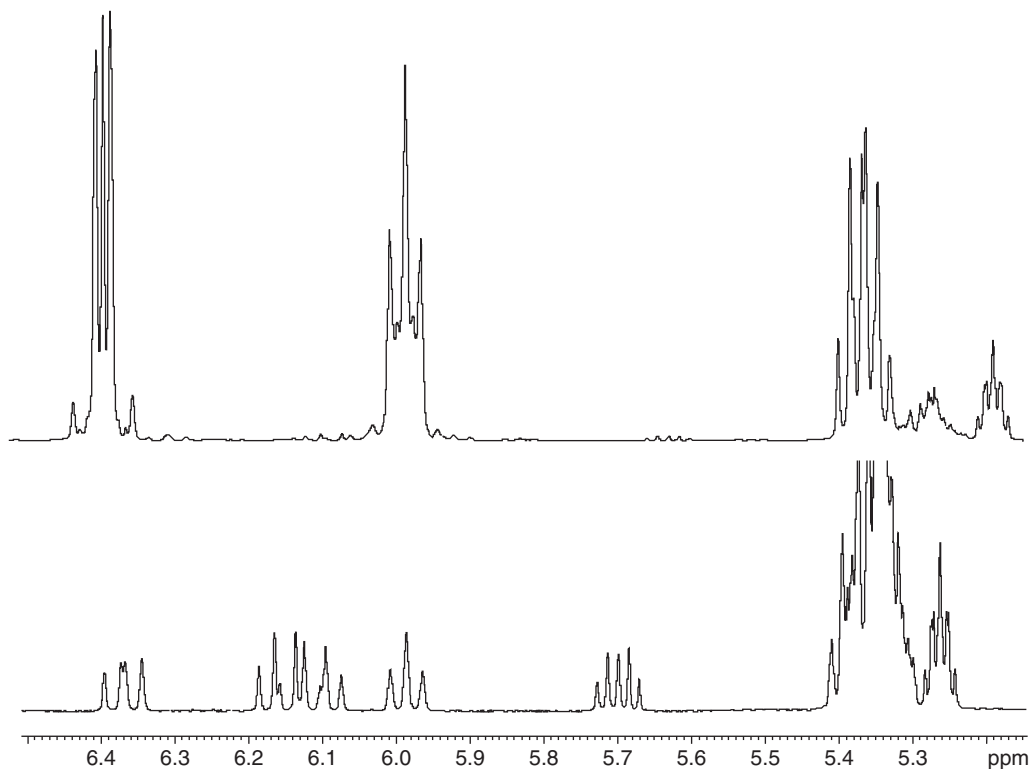
**Fig. 12** Chemical structure of *trans*-FAs.

(55%) (Saha et al., 2012; Melo, 2012). Their  $^1\text{H}$  NMR spectra are presented in Fig. 14. Each of these FAs gives a characteristic signal fingerprint. Most naturally occurring lipids exhibit only *cis* FAs which are modified by several processes like oxidation and technical processing such as microbial hydrogenation, fat hardening, deodorization and frying leading to artificial configuration known as *trans*. During these processes *cis* double bonds isomerize to the *trans* form. Thus, *trans* FAs indicate either a technical modification of the oil sample or a strong oxidation. Fig. 15 illustrates the comparison of sunflower oil before and after the refining process. Crude oils such as sunflower oil show some *trans* FAs in the region  $\delta$  6.5–5.6 ppm. These signals result from oxidation and microbiological processes showing “undefined signal mountains”. Since, in refining processes, defined *trans* FAs are formed, refined oils show defined *trans* FA signals in the appropriate  $^1\text{H}$  NMR region (Fig. 15).



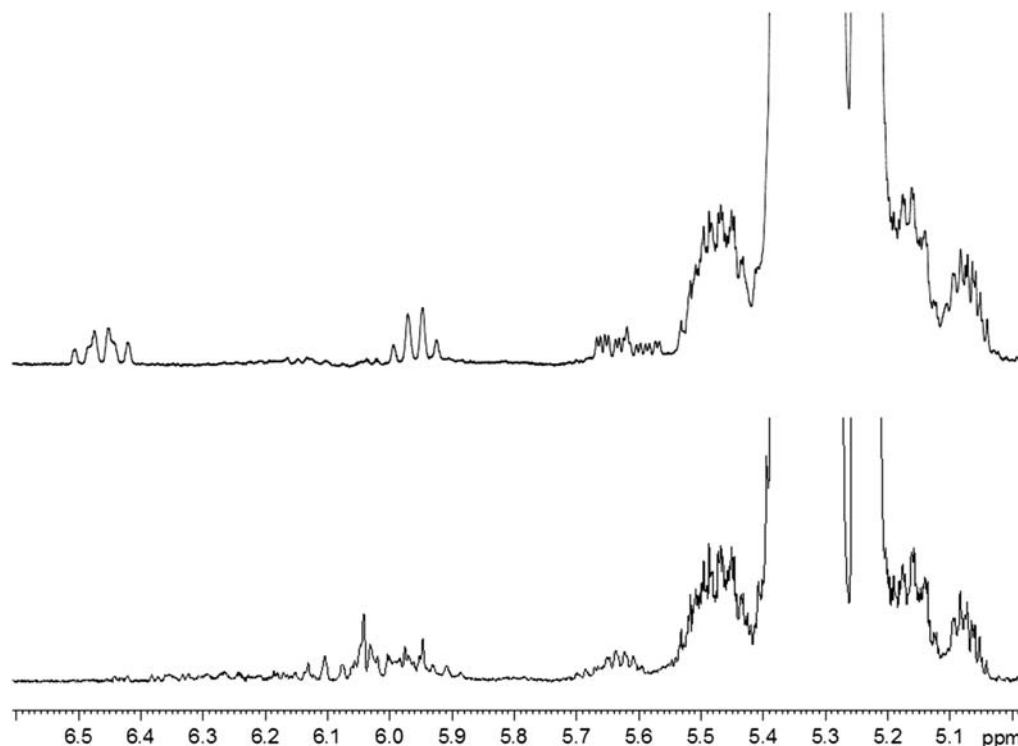


**Fig. 13**  $^1\text{H}$  NMR spectrum of hazelnut oil (top) and butter (bottom).



**Fig. 14**  $^1\text{H}$  NMR spectrum of extracted pomegranate seeds (top) and extracted cherry stones (bottom).

Since the high-resolution  $^{13}\text{C}$  NMR spectroscopy provides detailed information of the chemical structure of the FAs, it is proposed as a powerful tool for the rapid determination of *cis*- and *trans*-FAs in unsaturated lipids. Another possibility to determine the isomerization is the two-dimensional homonuclear J-resolved NMR spectroscopy (JRES) where the chemical shift is along one axis and the proton–proton coupling along the other axis. Based on the chemical shift and the coupling pattern the FA can be determined like presented in Fig. 16 where oleic acid (C18:1 9c) and elaidic acid (C18:1 9t) are compared with each other. JRES is



**Fig. 15**  $^1\text{H}$  NMR spectrum of refined (top) and crude sunflower oil (bottom).

a powerful tool for analyzing overlapped signals for which resonances and splitting patterns are unknown. By running a JRES analysis, the number of *trans* FAs can be determined.

### Quality Control

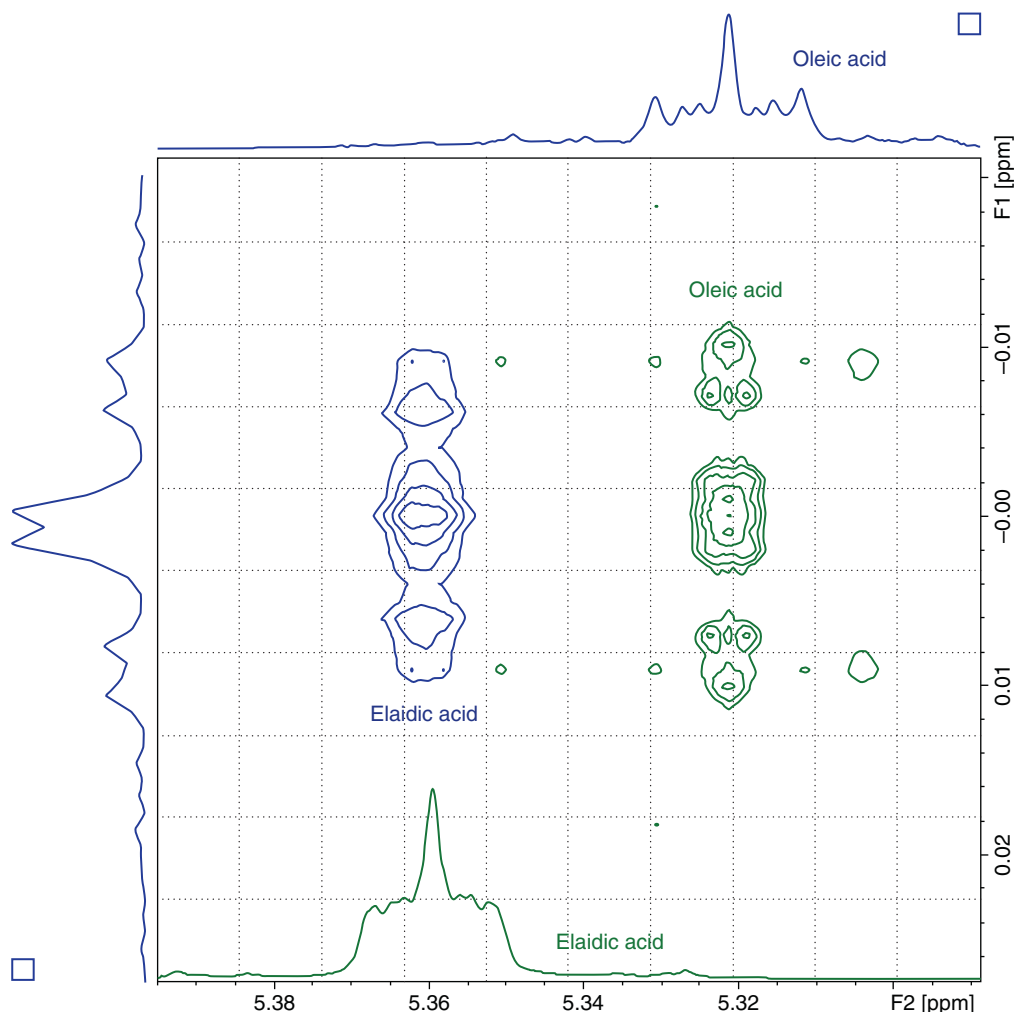
Quality control and quality assurance testing are important at every point in the food supply chain. Since quality of a product determines price, several parameters have to be assessed. Volumetric and UV/Vis spectroscopic techniques are the key elements of the analytical methods which are used for the quality control of lipids including the analysis of the peroxide value, anisidine value, acid value, saponification value and iodine value. These techniques were developed and standardized several decades ago undergoing only minor changes since then, showing several disadvantages such as poor specificity, the demand of a high amount of sample and solvents and the low robustness concerning external influences (Kamal-Eldin and Pocorny, 2005; Shahidi and Zhong, 2005). To overcome these problems, an alternative  $^1\text{H}$  NMR method was developed.

### Free Fatty Acids

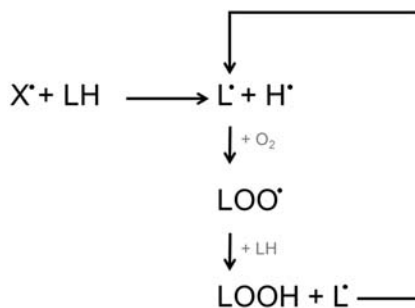
The acid value can be determined by the amount of free fatty acids in oil by integrating the carboxylic group proton ( $\text{COOH}$ ) signal which appears at  $\delta$  12–11 ppm and the methylene proton signal resonating at  $\delta$  2.4–2.2 ppm in the  $^1\text{H}$  NMR spectrum, obtained from a lipid dissolved in a solvent mixture of  $\text{CDCl}_3$  and  $\text{DMSO}-d_6$ . By analyzing a total of 305 oil and fat samples, the  $^1\text{H}$  NMR technique showed a strong correlation with the conventional titration method with the exception of hard fat which showed significant deviations (Diehl and Skiera, 2013; Skiera et al., 2012).

### Peroxides and Aldehydes

Lipid peroxidation is considered as the main process leading to oxidative damage of lipids. It is initiated by the formation and propagation of free lipid radicals, even during enzyme-catalyzed lipid oxidation. Heat energy, natural radioactivity, singlet oxygen and metals achieve the high activation energy which is required for the formation of the first free radical. By a hydrogen abstraction or addition of an oxygen-radical the chain reaction of the lipid peroxidation is initiated. Since the double bonds in polyunsaturated fatty acids (PUFAs) are adjacent to a methylene group, the methylene C–H bond becomes weaker which makes the hydrogen more susceptible to abstraction. This is the reason why PUFAs are more oxidation affected than saturated FAs. Oxygen reacts with the free carbon-centered lipid radical forming a peroxy radical which possesses enough energy to abstract a hydrogen atom converting it into a lipid hydroperoxide. The general process of lipid oxidation is presented in Fig. 17.

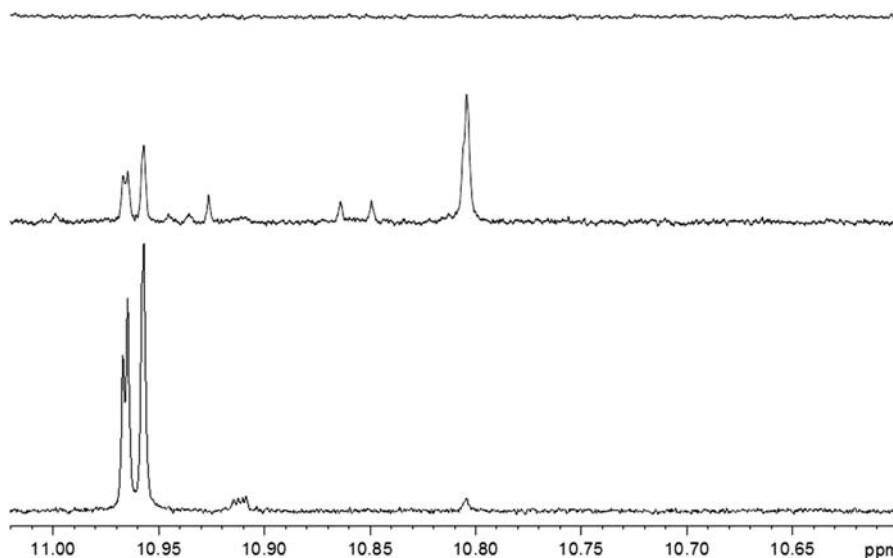


**Fig. 16** JRES NMR spectrum of oleic acid and elaidic acid.



**Fig. 17** General lipid oxidation process. Based on [Kamal-Eldin and Pocorny \(2005\)](#).

During the complex process, the formation and propagation of lipid radicals, the uptake of oxygen as well as a rearrangement of the double bonds in unsaturated lipids are involved in the formation of the first oxidation product, such as hydroperoxides, and secondary oxidation products, such as alcohols, ketones, alkanes, aldehydes and ethers. Since the chemical structure of lipids determines the structure of the hydroperoxides, several kinds of hydroperoxide isomers are formed. Based on the fatty acid profile, the number and type of hydroperoxides differ. The total amount of peroxides are examined as the peroxide value (PV). The PV is classically titrated according to Wheeler, being expressed in the units of milli equivalents of active oxygen per kilogram (meq/kg) ([Wheeler, 1932](#)). PV covers all compounds which oxidize potassium iodide under certain defined conditions ([ISO 3960, 2017](#)). This value is used as an indicator of the early oxidation stages in lipids. Over the past decades, several new methods such as colorimetric, chromatographic, and IR have been developed to determine hydroperoxides in oils and fats ([Kamal-Eldin and Pocorny,](#)



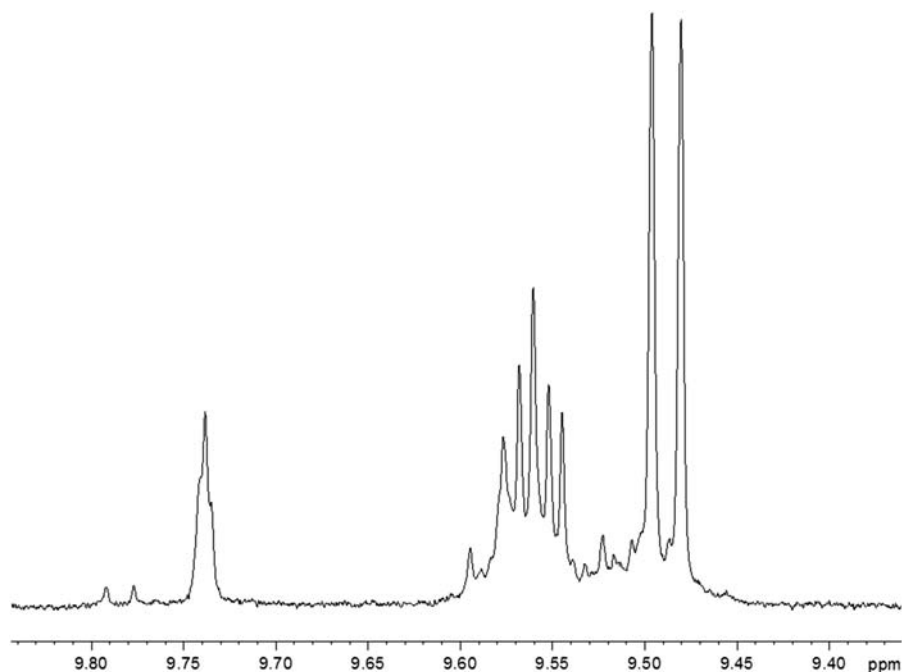
**Fig. 18**  $^1\text{H}$  NMR spectra of fresh chia oil (top), low oxidized olive oil (middle), and high oxidized almond oil (bottom); details: hydroperoxides.

2005; Yildiz et al., 2003). A very powerful tool for the analysis of the hydroperoxides is the NMR spectroscopy. Several authors found a good correlation between olefinic and aliphatic protons as well as between aliphatic and diallylmethylene protons in fatty acids and the classical PV (Saito, 1987; Shahidi and Wanasundara, 1997; Abou-Gharbia et al., 2000). Skiera et al. presented another alternative  $^1\text{H}$  NMR spectroscopic method to determine the hydroperoxide amount in edible oils by quantifying the  $-\text{OOH}$  signals (Skiera et al., 2012). By setting the  $\alpha\text{-CH}_2$ -integral value to 2000, the integral value of peroxides ( $\delta$  11.5–10.5 ppm) presents the amount of peroxides in mol-% (Fig. 18). An equation was established which related the PV to the NMR results. According to a Deming regression line the PV can be recalculated into the classical unit meg/kg. It is noticed that some oil types, especially olive oil and black seed oil seed oil, exhibit significant deviations in the PV by NMR and by titration according to Wheeler. These differences may result from cross-reactions from the titration reagents with natural oil-compounds or additives. For example, black seed oil contains natural oxidizing ingredients such as thymoquinone, a pharmacologically active terpene with significant anti-oxidative properties, which shows a strong increasing effect of the analyzed PV. Furthermore, in olive oil a high content of several phenolic compounds decreases the PV titration results. These results show that natural compounds of the oil matrix can influence the titration results significantly (Skiera et al., 2012). Moreover, a low PV does not directly indicate a low oxidized lipid. During a further oxidation step primary oxidation products form secondary oxidation products such as aldehydes which are analyzed as the *p*-Anisidine Value (AV). The knowledge of the PV and AV provides a reliable way to determine the overall oxidation stage of lipids. The AV analysis is based on the chemical reaction with *p*-anisidine forming an intensively coloured Schiff base which is nonspecifically measured by UV at  $\lambda = 350$  nm (ISO 6885, 2006). The AV depends on the aldehyde type, whereby the number of double bonds leads to different response factors for alkanals, alkenals and alkadienals of 0.5, 6, and 30, respectively. Based on this knowledge, the classical AV determination is erroneous. With NMR spectroscopy the individual aldehydes can be identified and quantified as an absolute value. By setting the  $\alpha\text{-CH}_2$ -integral value to 2000 the integral value of aldehydes presents the aldehyde amount in mol-% (Fig. 19). A further advantage of NMR spectroscopy is the simultaneous detection of primary and secondary oxidation products. Thus, the NMR spectroscopy represents a powerful tool to monitor and investigate oxidation products in edible fats and oils.

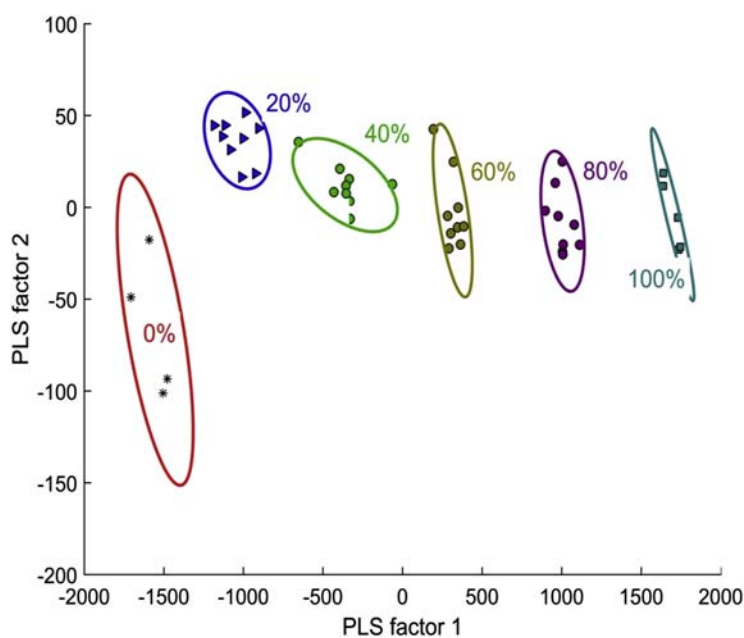
### Adulteration Control

A series of 46 blends with a total weight of 300 mg was prepared from selected olive ( $n = 5$ ) and sunflower ( $n = 5$ ) oil samples. The amount of sunflower oil in these mixtures was 0%, 20%, 40%, 60%, 80% and 100% (w/w). In each mixture the samples from both origins were selected randomly in order to assure representativeness of multivariate regression models. Recorded NMR spectra pre-processed by bucketing were correlated with “ground truth” data of the blends composition using partial least squares regression (PLS) (Fig. 19).

Scatter plot of PLS scores for the first and the second PLS dimensions clearly demonstrated the discrimination of all spiking groups (Fig. 20). Fig. 21 showed the predicted-reference plot along with 95% prediction bands for sunflower oil content in blends obtained by PLS regression. Root mean square error of regression was equal to 1.1% w/w. Limit of detection (LOD) estimated using the slope and standard deviation of response of this curve was found to be 1.1%. Quantification of olive oil adulteration is possible starting from 3.5% w/w.



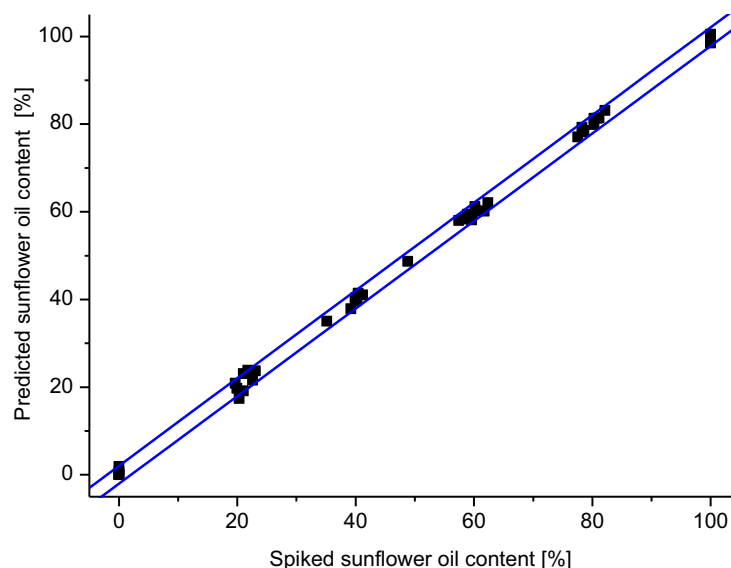
**Fig. 19**  $^1\text{H}$  NMR spectra of a high oxidized seed oil; details: aldehydes.



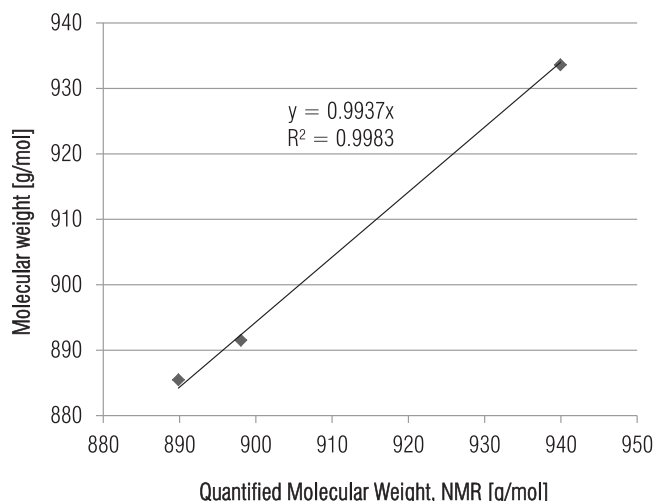
**Fig. 20** PLS scores on the first and the second PLS components for cross validation for olive-sunflower blends. Ellipsoids show 95% probability for each “spiking group” (defined as an average sunflower oil content in each group).

### Molecular Weight

Conventionally, molecular weights (MWs) of TAGs are determined with liquid or gas chromatography combined with mass spectrometry (Vorbeck et al., 1960). However, the MW can also be calculated with  $^1\text{H}$  NMR spectroscopy by taking the signals from the FAs and the glycerin backbone into account. Glycerol trioleate, Glycerol tristearate and Glycerol tridonadecanoate, whose molecular weights are known, have been analyzed with  $^1\text{H}$  NMR spectroscopy. After the quantification, the theoretical MW values have been compared with the NMR values (Fig. 22). A slope of 0.9937 and a correlation coefficient  $R^2$  of 0.9983 indicates that the NMR



**Fig. 21** Predicted-reference plot and 95% prediction bands (solid lines) obtained from spiking experiments.



**Fig. 22** Correlation of theoretical MW and with  $^1\text{H}$  NMR quantified MW.

spectroscopy is a versatile tool for the determination of molecular weights of vegetable oils. In the  $^1\text{H}$  NMR spectra the mean MW of lipids can be determined simultaneously to the analysis of several quality parameters such as free fatty acids, peroxides, aldehydes, saturation grade, fatty acid profile as well as secondary components such as phenols. All parameters are analyzed qualitatively and quantitatively in only one measuring step from one prepared sample, being ready in 3 min.

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# Lipid-Derived Flavours and Off-Flavours in Food

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## Introduction

Lipids, naturally present in food or added to food during processing, play an important role in nutrition as well as flavour and off-flavour development. Furthermore, lipid oxidation is a major cause of reducing the quality of food and its control is considered a challenge to the food industry. Generally, lipids present in food are more likely to undergo oxidative reactions resulting in the development of off-flavours, and loss of some bioactive compounds and liposoluble vitamins (Kolakowska and Bartosz, 2014; Souza and Bragagnolo, 2014; Akoh, 2017).

Generally, lipid autoxidation in food proceeds in two stages; the formation of hydroperoxides and their subsequent breakdown and production of odour-active volatile secondary lipid oxidation products (SLOP), such as alcohols, ketones, and aldehydes, among others. Polyunsaturated fatty acids (PUFA) are particularly prone to degradation due to their multiple methylene-interrupted *cis*-double bonds. Lipid oxidation is highly undesirable in food products because even low concentrations of SLOPs render distinct off-flavour to products (Jacobsen, 1999; Böttcher et al., 2015). Lipid oxidation has also generated much interest in the field of functional foods and in products that are frequently enriched with PUFA to improve their nutritional value, as in meat and meat products (Salcedo-Sandoval et al., 2013), milk and milk products (Let et al., 2003), and eggs (Petrović et al., 2012), among others. The biochemical or chemical degradation of lipids gives rise to a wide range of volatile compounds such as free fatty acids, their related esters, and lactones that are key components in the flavour of food and therefore, in the formation of aroma compounds (Wache et al., 2006). In addition, secondary products of lipid oxidation could react with peptides, proteins, and amino acids, contributing to protein oxidation that may result in loss of essential amino acids and also changes in the protein, in addition, leading to loss of functionality (Estévez, 2015; Estévez and Luna, 2016).

## Lipid Oxidation and Off-Flavour Development

The oxidation of unsaturated fatty acids is a complex phenomenon which occurs in the presence of oxygen, non-enzymatically or enzymatically. Lipid oxidation and formation of flavour-active components is catalyzed by heat, light, photosensitizers, oxygen, and transition metal ions (Mariutti and Bragagnolo, 2017). Non-enzymatic oxidation could occur via photooxidation or autoxidation. Photooxidation occurs in the presence of singlet oxygen ( $^1\text{O}_2$ ), while autoxidation occurs in the presence of triplet oxygen ( $^3\text{O}_2$ ). Enzymatic oxidation occurs by the action of lipoxygenase and the processes involved are different from non-enzymatic oxidation (Mariutti and Bragagnolo, 2017). The saturated fatty acids can enter the autoxidation process over higher temperatures during processing operations such as baking, boiling, and frying, among others. Meanwhile, only unsaturated fatty acids can be oxidised by atmospheric oxygen at ordinary temperatures (Resconi et al., 2013). The development of lipid oxidation could be followed by monitoring the primary products of fatty acid oxidation, for example, conjugated dienes and hydroperoxides, or the secondary oxidation products such as malondialdehyde (MDA), propanal, pentanal, and hexanal, among others. Usually, the thiobarbituric acid reactive substances (TBARS), reported as MDA equivalents is determined colorimetrically at 532 nm (Resconi et al., 2013).

## Autoxidation

The main mechanism of lipid oxidation in food is autoxidation which consists of the reaction of triplet oxygen with organic compounds. Triplet oxygen is a diradical species so one pair of its even number of electrons has parallel spins, and thus, it can easily react with radical species. Generally, direct reaction of oxygen with unsaturated fatty acids is not possible because their double bonds are in singlet spin state. Therefore, an initiator is necessary to remove an electron from the unsaturated lipid to generate a radical in order to facilitate the reaction between the oxygen and an unsaturated fatty acid. Lipid autoxidation is a chain reaction process induced by free radicals described by three steps of initiation, propagation, and termination. These steps have been thoroughly described by several researchers (Chaiyasit et al., 2007; Schaich et al., 2013; Shahidi and Zhong, 2010). Through the propagation stage, primary products of oxidation, namely lipid hydroperoxides, are produced. These products are unstable and break down to a number of secondary oxidation products such as ketones, aldehydes, hydrocarbons, alcohols, and volatile organic acids among others; some of which have undesirable odours with extremely low threshold values (Shahidi and Zhong, 2010). Table 1 summarizes the threshold values of relevant volatile compounds. Moreover, the products of lipid oxidation lead to loss of the nutritional value, texture, colour, and some functional properties of food (Shahidi, 2002).

**Table 1** Threshold values of compounds formed upon lipid oxidation

Compounds	Threshold value (ppm)
Hydrocarbons	90–2150
Alkenes	0.02–9
Vinyl alcohols	0.5–3
Alcohols, saturated	0.3–2.5
Alcohols, unsaturated	0.001–3
1-Alkenes	0.02–9
2-Alkenals	0.04–2.5
Furans	1–27
Alkenals	0.04–1
Aldehydes, saturated	0.014–1
Aldehydes, monounsaturated	0.04–2.5
Aldehydes, diunsaturated	0.002–0.6
trans,trans-2,4-Alkadienals	0.04–0.3
trans,cis-Alkadienals	0.002–0.006
Ketones, methyl	0.16–5.5
Ketones, vinyl	0.0002–0.007
Isolated alkadienals	0.002–0.3
Isolated cis-alkenals	0.0003–0.1

From Frankel (1985), Drumm and Spanier (1991), Shahidi (2002), and Min and Boff (2002).

### Photooxidation

The molecular oxygen in the ground state is in its triplet state ( $^3\text{O}_2$ ) which is a diradical, and could be activated by electronic excitation to a singlet state ( $^1\text{O}_2$ ). Singlet oxygen may be generated by ultraviolet light, or photosensitizers such as chlorophyll, riboflavin, and myoglobin, among others (Min and Boff, 2002). Singlet oxygen is more electrophilic than triplet oxygen, thus it is strongly reactive. For example, the reaction rate between singlet oxygen and linoleic acid is 1450 times higher than that with triplet oxygen (Min and Boff, 2002).

Singlet oxygen reacts easily with unsaturated fatty acids via cycloaddition and forms fatty acid hydroperoxides. The reaction occurs on the carbon atoms located between two double bond then cause change of position and then configuration of the double bond. Therefore, mono-, di- and triunsaturated fatty acids, respectively, oleic acid generates a mixture of two (9- and 10-hydroperoxides), linoleic acid generates a mixture of four (9-, 10-, 12- and 13-hydroperoxides), and linolenic acid generates a mixture of six hydroperoxides (9-, 10-, 12-, 13-, 15- and 16-hydroperoxides). Linolenic acid could form four conjugated and two non-conjugated hydroperoxides, while linoleic acid could form two conjugated and two non-conjugated hydroperoxides (Mariutti and Bragagnolo, 2015). The relative rates of oxidation for oleic, linoleic, and linolenic acids are shown in Table 2. The reaction rates of fatty acids upon photooxidation are faster than those during autoxidation.

### Enzymatic Oxidation

Enzymatic oxidation is based on the enzymatic activity of lipoxygenase (LOX). Lipoxygenase (LOX) is a globular protein soluble in water consisting of a single polypeptide chain with a molecular mass of 94–104 kDa in plants and 75–80 kDa in animals. It is capable of producing conjugated hydroperoxides through the oxidation of polyunsaturated fatty acids (Whitaker, 1972). And also, catalyzes the first reaction of so-called “way of lipoxygenase”. The way of lipoxygenase is of fundamental importance in the formation of all the volatile substances responsible for the aroma of vegetables and fruits that, in the case of olive oil, contributes markedly to the characterization as well as differentiation between different cultivars (Boe, 2012). A molecule of LOX contains, at the active site, one iron atom in high spin state  $\text{Fe}^{2+}$  that must be oxidized to  $\text{Fe}^{3+}$ , thus the oxidation reaction might proceed. This enzyme stereoselectively catalyzes the non-reversible oxidation of PUFA containing the group 1, 4-cis, cis-pentadiene

**Table 2** Relative rates of fatty acid oxidation

Fatty acid	Autoxidation	Photooxidation
Oleic (C18:1)	1	$30 \times 10^3$
Linoleic (C18:2)	50	$40 \times 10^3$
Linolenic (C18:3)	100	$70 \times 10^3$

Adopted from Min and Boff (2002).

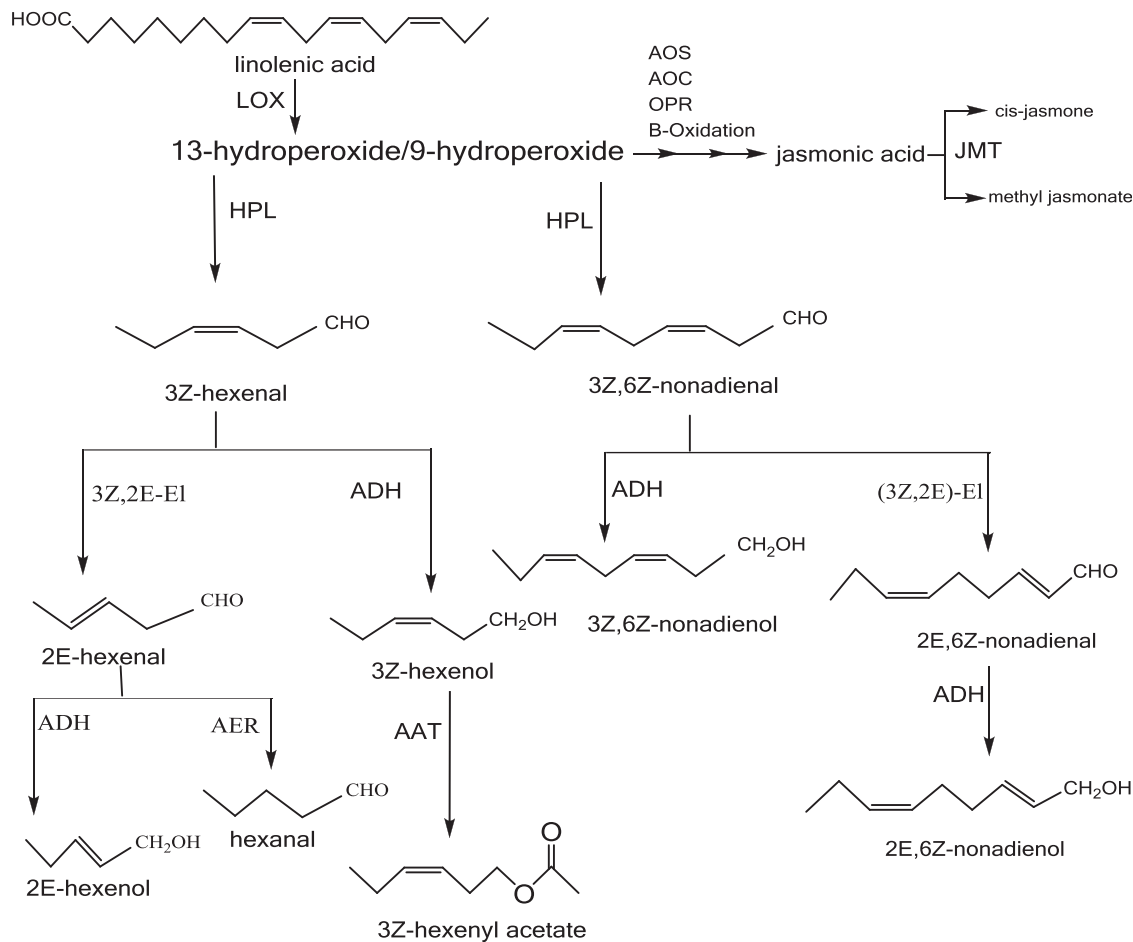
( $-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$ , commonly called malonic system), and yields a stereospecific conjugated diene hydroperoxides fatty acid (Tappel, 1963; Galliard, 1989).

## Fatty Acid-Derived and Other Lipophilic Flavour Compounds

The majority of plant volatiles are produced from unsaturated and saturated fatty acids. Fatty acid-derived straight-chain alcohols, ketones, aldehydes, acids, lactones, and esters are found in the plant kingdom in high concentrations. These are formed by three distinct processes, lipoxygenase pathway,  $\beta$ -oxidation, and  $\alpha$ -oxidation (Schwab and Schreier, 2002). Generally, in plants, fatty acids are stored as triacylglycerols and thus enzymatic oxidative degradation of lipids occurs via the action of acyl hydrolase, and then liberating free fatty acids from acylglycerols. Furthermore, identification of a number of phosphatidylglycerols, and digalactosyldiacylglycerols demonstrates the possibility of direct oxidation of the fatty acid side chain in acylglycerols (Buseman et al., 2006; Schwab et al., 2008; El Hadi et al., 2013).

## Lipoxygenase

The pathway of chain oxidation, unsaturated and saturated volatile C6 and C9 aldehydes and alcohols are very important contributors to the characteristic flavours of vegetables, fruits, and green leaves. There are four enzymes involved in the biosynthetic pathway leading to their formation; these are hydroperoxide lyase (HPL), lipoxygenase (LOX), alcohol dehydrogenase (ADH), and 3Z, 2E enal isomerase (Matsui, 2006; Stumpe and Feussner, 2006) (Fig. 1). LOX is a non-heme, iron-containing dioxygenase which catalyzes the regio- and enantio-selective dioxygenation of the unsaturated fatty acids. Numerous plant lipoxygenases (LOX) have been characterized due to the essential components of oxylipin pathway, also converting fatty acids to hydroperoxides and finally flavour compounds like 2E-hexenal, 2E, 6Z-nonadienal, and 3Z-hexenal. Volatile compounds such as methyl jasmonate,



**Figure 1** Linolenic acid-derived flavour compounds. AAT, alcohol acyl CoA transferase; ADH, alcohol dehydrogenase; AER, alkenal oxidoreductase; AOC, allene oxide cyclase; AOS, allene oxide synthase; HPL, hydroperoxide lyase; JMT, jasmonate methyltransferase; LOX, lipoxygenase; OPR, 12-oxo-phytyldienoic acid reductase; and 3Z,2E-EI, 3Z,2E-enal isomerase. Adopted from [El Hadi et al. \(2013\)](#).

*cis*-3-hexenol, and *trans*-2-hexenal are derived from C18 unsaturated fatty acids including linolenic acid or linoleic acid that undergo dioxygenation in the reaction catalyzed by LOX. These enzymes can catalyze the oxygenation of polyenoic fatty acids at C9 or C13 positions yielding two groups of compounds, the 13-hydroperoxy and the 9-hydroperoxy derivatives of polyenoic fatty acids. These derivatives could be further metabolized by several enzymes such as allene oxide synthase (AOS) and hydroperoxide lyase (HPL) which represent two lipoxygenase pathways yielding volatile compounds. The 13-hydroxyperoxy linolenic acid is converted to 12, 13-epoxyoctadecatrienoic acid (Feussner and Wasternack, 2002). Several subsequent enzymatic reactions that lead to the formation of jasmonic acid can be converted to the volatile ester, methyl jasmonate, by enzyme jasmonic acid carboxymethyl transferase (Song et al., 2005). In the hydroperoxide lyase (HPL) branch of the LOX pathway, the oxidative cleavage of hydroperoxy fatty acids through the action of HPL leads to the formation of C6 or C9 volatile aldehydes such as 3-hexenal and 3, 6-nonadienal, respectively (Akacha et al., 2005; El Hadi et al., 2013).

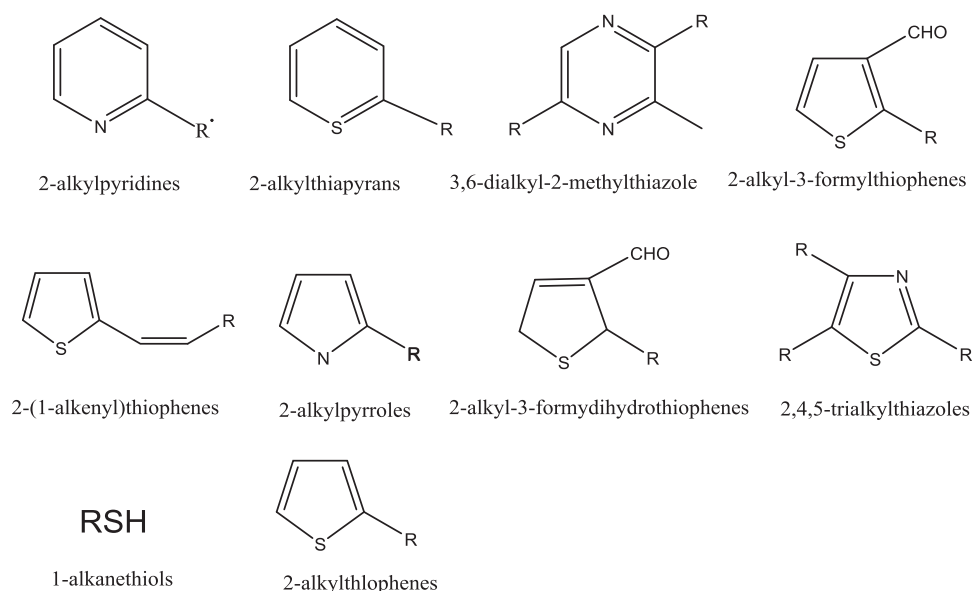
The degradation of straight chain fatty acids by both  $\alpha$ - and  $\beta$ -oxidation is considered as a major process for the formation of flavour compounds in all organisms, however, specific pathways in plants are not yet fully understood. Baker et al. (2006) described various roles for this pathway in relation to the fatty acid as well as amino acid metabolism.  $\beta$ -Oxidation results in the successive removal of C2 units (acetyl, CoA) from the parent fatty acid (Goepfert and Poirier, 2007). Sanz et al. (1997) mentioned that  $\beta$ -oxidation of fatty acids is the primary biosynthetic process providing acyl coenzyme A (CoAs) and alcohols for ester formation by losing two carbons in every round of the  $\beta$ -oxidation cycle, the fatty acid acyl-CoA derivatives are converted to shorter chain acyl CoA which require flavinadenine dinucleotide (FAD). The two classical examples are pear and apple aromas of volatile formation through  $\beta$ -oxidation pathway (El Hadi et al., 2013).

### Volatiles Compounds From Lipid-Maillard Interaction

The Maillard reaction is considered as one of the main chemical reactions which takes place during cooking of red meat and poultry. This reaction occurs between reducing sugars and amino compounds and eventually results in a large number of compounds responsible for the flavour of any species of meat (Mottram, 1994a). During the first stages of the reaction, the Amadori products are formed via glycosylamine as a result of condensation of the carbonyl group of a reducing sugar with the amino compounds. Several sugar dehydration and degradation products like furfural and furanone derivatives, dicarbonyl compounds, and hydroxyketones are formed by rearranging and dehydration of the resultant products (Mottram, 1998; Calkins and Hodgen, 2007).

The Strecker degradation of amino acids by Maillard reaction-derived dicarbonyl compounds is a vitally associated reaction (Mottram, 1998; Jayasena et al., 2013). Maillard reaction-derived carbonyl compounds react with those compounds forming intermediates which are further involved in flavour-forming reactions. At the end, a large number of important classes of flavour compounds including pyrazines, furans, pyrroles, oxazoles, thiazoles, thiophenes and other heterocyclic compounds are formed (Melton, 1999). Shahidi (1989), Mottram and Madruga (1994) reported that sulphur-compounds derived from cysteine and ribose, and carbonyl compounds are principal contributors to the flavour of meat. However, ribose is considered as the most important flavour precursor in chicken meat (Meinert et al., 2009).

Mottram (1994b) and Whitfield (1992) reported that the interaction of lipid breakdown products with Maillard reaction leads to formation of several volatiles which are identified in meat (Fig. 2). Some thiazoles with C4 to C8 n-alkyl substituents in the 2-position as well as some other alkylthiazoles with much longer 2-alkyl substituents (C13 to C15) are reported in fried chicken and



**Figure 2** Volatile compounds of lipid-Maillard interaction. Adopted from Jayasena et al. (2013) and Melton (1999).

**Table 3** Examples of volatile compounds in different types of food

Type of food	Volatile	References
Tomato juice	(E)-2-heptenal; (E)-2-hexenal; (E)-2-nonenal; (E)-2-octenal; (E,E)-2,4-decadienal; (E,Z)-2,6-nonadienal; 1-Butanol; 1-Hexanol; 2,3-Butanediol; 2-Isobutylthiazole; 6-Methyl-5-hepten-2-one; Benzaldehyde; Acetaldehyde; Dimethyl sulfide; Ethanol.	Ties and Barringer, 2012
Meat products	2-acetyl-2-thiazoline; 2-acetyl-1-pyrroline; methional; 2-furanmethanethiol; 3-methylbutanal, 2-methylbutanal; 2-methyl-3-furanthiol, bis(2-methyl-3-furyl)disulfide; (E)-2-nonenal, (E,E)-2,4-decadienal, 1-okten-3-on	Huang and Ho, 2001, Balagiannis et al., 2009
cooked poultry meat	hexanal; 2-furfurylthiol; 2(E)-nonenal; 2,4(E,E)-decadienal; 2,4(E,Z)-decadienal; 2,4(E,E)-nonadienal	Kerler and Grosch, 1997
wet-cured cooked hams	Alkenes; alkanes; aldehydes; alcohols; ketones; esters; pyrazines; chloride; terpenes; furans; amines; carboxylic acids; sulfur compounds; aromatic hydrocarbons	Ramarathnam et al., 1993, Leroy et al., 2009
dry-cured hams	2-heptanone; benzaldehyde; hexanal; limonene; hexanol; octanol; pentanol; butanol; propanone; 2-nonanone; 3-methylbutanal	Luna et al., 2006, Garcia-Gonzalez et al., 2008

heated chicken (Tang et al., 1983; Farmer and Mottram, 1994). The compound 2-octyl-4, 5-dimethylthiazole is found in chicken meat and it is also a product from interaction of lipids and Maillard reactions (Melton, 1999).

The formation of volatile compounds in different foods depend on their constituent components (Table 3). Thus flavour generation in bulk oil, oil emulsion, meat and poultry, fruits and vegetable, cereals, legumes as well as in processed foods such as coffee, roasted products by different means is dictated by a variety of factors. Some examples of different commodities and their flavour as affected by lipid constituents present are discussed in subsequent sections of this chapter.

### Flavour Compounds in Edible Oils

Unsaturated fatty acids that are oxidized by photooxidation, autoxidation or enzymatic oxidation form hydroperoxides that may decompose to low molecular weight compounds such as alcohols, ketones, hydrocarbons and aldehydes, as already mentioned (Chaiyasit et al., 2007). The decomposition of hydroperoxides (ROOH) generates volatile compounds as well as producing hydroxyl radical ( $\cdot\text{OH}$ ) and alkoxy radical ( $\text{RO}\cdot$ ), as the predominant pathway (Chaiyasit et al., 2007). Alkoxy radical ( $\text{RO}\cdot$ ) is more reactive than both peroxy radicals ( $\text{ROO}\cdot$ ) and alkyl radicals ( $\text{R}\cdot$ ) and could also react by different mechanisms: it could attack the unsaturated fatty acids or undergo  $\beta$ -cleavage. The radicals resulting from  $\beta$ -cleavage can react with several products to produce secondary products of oxidation. The main aldehydes formed by oxidation of linolenic acid are 2-butenal, propanal, 2-pentenal, 3-hexenal, 2-hexenal, 2-heptenal, 2,4-heptadienal, 2,6-nonadienal; 2,5 octadienal, and 2,4,7-decatrienal; of linoleic acid, hexanal, pentanal, heptanal, octanal, 2-octenal, 2-heptenal 3-nonenal, 2-nonenal, 2-decenal, 2,4-decadienal, and 2,4-nonadienal; and of oleic acid are octanal, heptanal, nonanal, decanal, 2- undecenal, and 2-decenal (Belitz et al., 2009).

The 4-hydroxyalkenals, one of the aldehyde classes formed during lipid oxidation, are of particular interest because these compounds are related to biological activities like chemotactic activity against neutrophils, enzyme inhibition, and inhibition of protein synthesis (Esterbauer et al., 1991). The 4-hydroxy-2-nonenal (HNE) is the main aldehyde that is formed during autoxidation of n-6 polyunsaturated fatty acids, but its mechanisms of degradation and formation is not yet completely elucidated (Esterbauer et al., 1991; Sakai et al., 2006). Mariutti and Bragagnolo (2015) have reported that the compounds of high molecular weight are responsible for changes in the viscosity and colour of the oxidized products, while compounds of low molecular weight are responsible for rancid flavour.

The oxidation of vegetable oil such as soybean oil results in the formation of hydroperoxides that degrade to form a different of volatile, short-chain secondary oxidation products (Ullrich and Grosch, 1988). Some of these volatile compounds negatively affect the quality of oil by producing undesirable off-flavours. Studies show volatile compounds in soybean oil are 2,4-hepta-dienal and 2-hexenal from linolenate hydroperoxides and hexenal, pentane 2,4-decadienal, and 2-heptenal from linoleate hydroperoxides (Selke and Frankel, 1987).

The aroma of olive oil for example is attributed to alcohols, aldehydes, ketones, hydrocarbons, esters, furans, and, maybe other unidentified volatile compounds. However, the studies reported that the major volatile compounds in most virgin olive oils in Europe are the C5 and the C6 volatile compounds. Hexanal, 1-hexanol and 3-methylbutanol, *trans*-2-hexenal are found (Aparicio et al., 1997; Kiritsakis, 1998; Angerosa, 2002). Research has shown odour thresholds and sensory descriptors of volatile compounds in olive oil (Table 4).



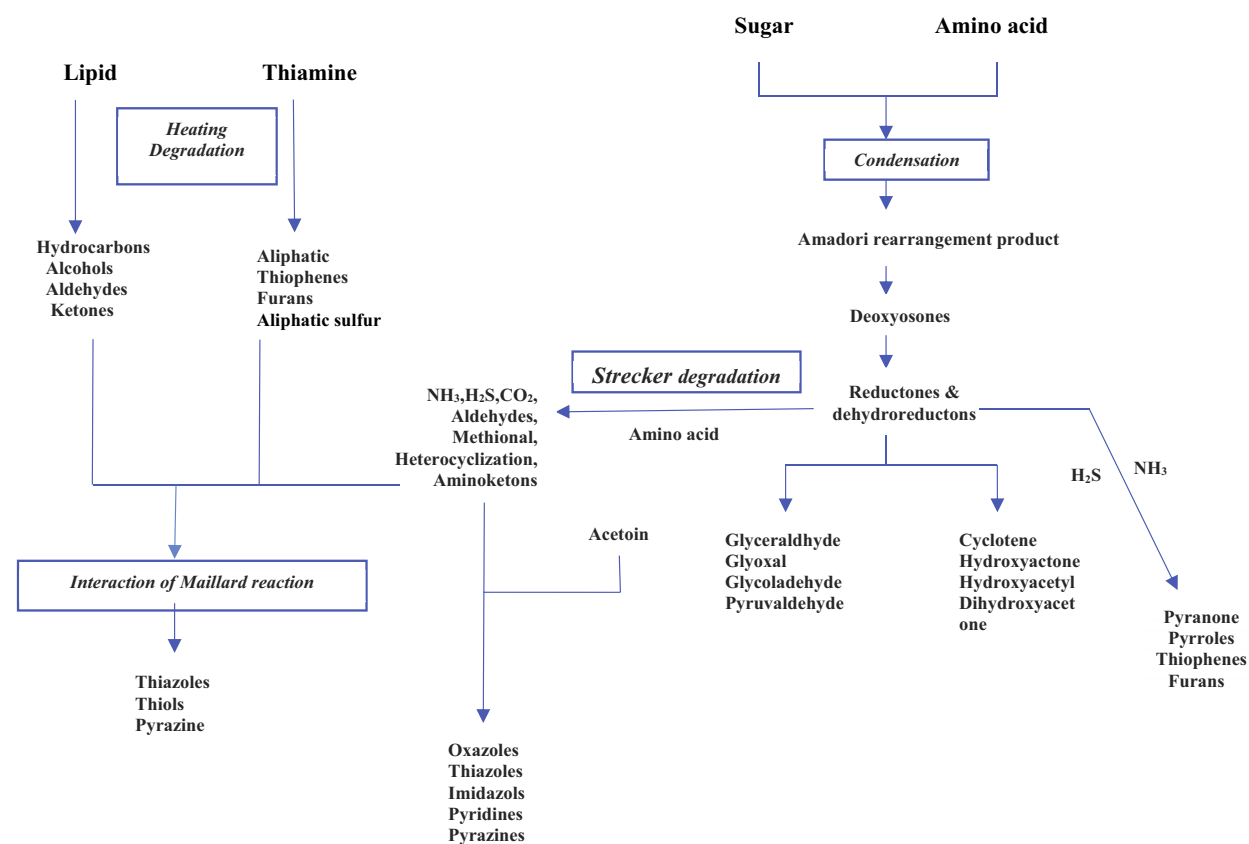
**Table 4** Odour thresholds and sensory descriptors of volatile compounds in olive oil

Volatile compounds	Odour threshold ( $\mu\text{g/kg oil}$ )	Sensory descriptor(aroma)	References
<b>Aldehydes</b>			
Acetaldehyde	0.22	Pungent, sweet	Reiners and Grosch (1998)
3-Methylbutanal	5.4	Malty	
Pentanal	240	Woody, bitter, oily	Morales et al. (2005)
Hexanal	75	Green-sweet	
<i>cis</i> -3-Hexenal	3	Green	Aparicio and Luna (2002)
Heptanal	500	Oily, fatty, woody	
Ethyl propanoate	100	Fruit, strong	
Heptan-2-one	300	Sweet, fruity	Morales et al. (2005)
Ethyl 2-methylbutyrate	0.72	Fruity	
Ethyl 3-methylbutyrate	0.62	Fruity	Reiners and Grosch (1998)
Ethyl propanoate	100	Fruit, strong	Morales et al. (2005)

### Flavour Compounds in Meat and Poultry

The development of the characteristic flavour of cooked meat is attributable to volatiles generated during heating as a result of processes such as lipid oxidation, Maillard reaction, interactions between lipid oxidation products and Maillard reaction products, and thermal degradation of thiamine, among others. **Fig. 3** Schematic illustration of volatile compounds generated by Maillard reaction between sugars and amino acids; heat degradation of lipid and thiamine (MacLeod, 1998).

Meat and meat products constitute an important source of dietary protein (Font-i-Furnols and Guerrero, 2014). The quality of these products as well as their attractiveness to consumers are, to a large extent, attributable to the palatability being a sum of gustatory and olfactory sensations. The flavour of raw meat is described as metallic, salty, and rare (bloody) with a bit of sweet aroma



**Figure 3** Schematic illustration of volatile compounds generated by Maillard reaction between sugars and amino acids; heat degradation of lipid and thiamine (Dashdorj et al., 2015; Kosowska et al., 2017).

(Soncin et al., 2007). Despite its weak flavour, meat is a rich source of compounds that serve as precursors of volatile compounds. Heat treatment initiates a series of reactions which result in the development of the characteristic flavour of meat, including lipid oxidation. Heat treatment of meat (lamb, pork, beef, and poultry) imparts non-species-specific meaty flavour, whereas warming up meat containing fat, particularly to a lesser extent triacylglycerols and phospholipids, causes development of a specific flavour of meat. Many volatile compounds are generated during thermal processing such as aldehydes, alcohols, ketones, hydrocarbons, carboxylic acids, furans, esters, lactones, pyrans, pyrazines, pyridines, phenols, pyrroles, thiophenes, thiazoles, oxazoles, thiazolines, and other sulphur or nitrogen compounds (Kosowska et al., 2017).

The species-specific flavour of meat is usually determined by mixtures of volatiles that in the case of heat-treated products includes hundreds of compounds. For instance, Mottram (1994c) had identified about 880 volatile compounds in cooked beef. The contribution of individual volatile compounds in developing the characteristic flavour, only a small part of this large number contributed to flavour development. Therefore, it is very important to distinguish aroma-active compounds from other odour inactive food constituents (Grosch, 1993). It is estimated that only 3% out of 10,000 identified volatile compounds are able of imparting odours to food products (Dunkel et al., 2014). Species specific flavour of meats are mainly influenced by the type of fats and fatty acids present in meat. Phospholipids are the major fraction of lipid that generates volatile compounds, and to a lesser extent by triacylglycerols (Meinert et al., 2007; Soncin et al., 2007). The distinct strong flavour of sheep meat (lamb or mutton) is due to the presence of volatile medium chain branched fatty acids. Brennad and Lindsay (1992) reported that 4-methyl- and 4-ethyl-octanoic acids that are present in the subcutaneous adipose tissue contribute to the species-related flavours in lamb upon hydrolysis.

The flavour of meat products is a result of several factors, the most important is the components and parameters of the production process. The main significance in finished product flavour development is ascribed to the kind of meat used as it affects the composition of volatile compounds. The major volatiles of cooked beef include nonanal, octanal (E,E)-2,4-decadienal, methional, methanethiol, 2-furfurylthiol, 2-methyl-3-furanthiol, 4-hydroxy-2,5-dimethyl-3-(2H)-furanone, and 3-mercapto-2-pentanone. These compounds also occur in chicken meat as well as cooked pork, however, their concentrations differ among species. The meaty-caramel odour noted in cooked beef is attributable to high concentration of compounds 4-hydroxy-2,5-dimethyl-3-(2H)-furanone, 2-methyl-3-furanthiol, and 2-furfurylthiol. For comparison, a lower concentration of 4-hydroxy-2,5-dimethyl-3-(2H)-furanone in pork results from significantly lower concentrations of its precursors, fructose 6-phosphate and glucose 6-phosphate. The concentration of carbonyl compounds with greasy odour notes, such as nonanal and hexanal is lower in pork than in beef (Belitz et al., 2009). The important volatile compounds of cooked poultry meat include hexanal, 2-furfurylthiol, 2(E)-nonenal, 2,4(E,E)-decadienal, 2,4(E,Z)-decadienal, and 2,4(E,E)-nonadienal (Kerler and Grosch, 1997). During oxidation stage of poultry fat,  $\gamma$ -dodecalactone and (E,E)-2,4-decadienal are formed from linoleic acid which are typical of the chicken meat flavour (Gasser and Grosch, 1991; Chen and Ho, 1998).

Ramarathnam et al. (1993) as well as Leroy et al. (2009) analyzed the wet-cured cooked ham and compared them with non-cured products. A significantly lower contents of carbonyl compounds were found in the cured products. They also found many volatile compounds originating from both the reactions in meat and from added spices or the applied smoke such as alkenes, alkanes, aldehydes, alcohols, ketones, aromatic hydrocarbons, esters, carboxylic acids, terpenes, sulphur compounds, pyrazines, chloride, furans, and amines. However, only a few of these compounds contributed to development of a typical character of a product. The main volatiles identified included terpenes (linalool, 1,8-cineole, L-carvone, menthol, cinnamaldehyde) which originated from seasonings, and also, 3-methylbutanoic acid and sulphur compounds formed upon Strecker degradation reaction (Toldra and Flores, 2007).

For the dry-cured ham, ketones alcohols, and aldehydes, had a significant effect on the characteristic flavour of the final products. The presence of 2-heptanone, benzaldehyde, hexanal, limonene, hexanol, 3-methylbutanal, octanol, pentanol, butanol, propenone, and 2-nonanone was also noted (Luna et al., 2006; Garcia-Gonzalez et al., 2008). There is an interesting group of meat products that includes raw-ripened sausages. For instance, about 51 key volatiles were identified in the Hungarian-type Salami sausage, which included amino acid degradation products (methional, phenylacetaldehyde and 3-methylbutanal), and fermentation products (acetic acid, acetaldehyde, butanoic acid, and 3-methylbutanoic acid) as well as short-chain fatty acids (Sollner and Schieberle, 2009).

The role of lipid-derived carbonyl compounds in chicken flavour has been studied by many researchers. The lean meat contains structural phospholipids and intramuscular triacylglycerols. Thus, both desirable and undesirable flavours could be generated in meat because of lipid oxidation. The reasonable thermal oxidative changes of lipids lead to generation of desirable flavour compounds as well as aromas in cooked meats (Shahidi, 2002). The flavour development of poultry meat is partly attributed to its lipids (Perez-Alvarez et al., 2010). Hundreds of volatile compounds are generated in cooked meat during lipid degradation; such compounds include aliphatic hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids, esters, some aromatic hydrocarbons, in addition to oxygenated heterocyclic compounds like alkylfurans and lactones (Mottram, 1998; Jayasena et al., 2013). About forty-one out of one hundred ninety-three total compounds reported in the flavour of chicken (roasted chicken) are lipid-derived aldehydes (Shi and Ho, 1994). According to that study, 2,4-decadienal and hexanal are relatively high of aldehydes that identified in chicken flavour and known as primary oxidation products of linoleic acid (Shi and Ho, 1994).

Many studies have confirmed that phospholipids are more important in the development of aroma volatiles during the cooking of meat than triacylglycerols (Jayasena et al., 2013). This is attributed to the presence of higher proportion of unsaturated fatty acids, such as arachidonic acid (20:4) in phospholipids (Mottram and Edwards, 1983). The levels of unsaturated fatty acids in chicken are very high compared with red meat, hence they generate more unsaturated volatile aldehydes that are vital in specific aromas of

chicken (Noleau and Toulemonde, 1987). Moreover, the aroma of fat-fried food is due to 2,4-decadienal (Mottram, 1998). Thus, aliphatic aldehydes contribute to the fatty flavours of cooked chicken meat. Shahidi (2002) reported that low amounts of  $\alpha$ -tocopherol in chicken meat is a major reason for meat flavour deterioration and formation of the undesirable “warmed over flavour (WOF)” in chicken meat products. Lipid-derived compounds in meat volatiles have greater odour threshold values (2-methyl-3-furanthiol; 2-furfurylthiol; Bis (2-methyl-3-furyl) disulphide; 2,4,5-trimethylthiazole; 2,4-decadienal; 3-mercapto-2-pentanone; 2-formyl-5-methylthiophene) as opposed to nitrogen- and sulphur-containing heterocyclic compounds that make them less aroma significant. Heterocyclic compounds possess a significant effect on aroma because of their low odour threshold values (Jayasena et al., 2013).

## Flavour Compounds in Fruits and Vegetables

The volatile lipid-derived flavour of fruits depend on the cultivar, degree of maturity, geographic region, processing methods and parameters. In general, fruit of the same cultivar grown in different geographic regions produce different volatile compounds, as does fruit from different cultivars grown under the same environmental conditions (Sacchi et al., 1998; Angerosa et al., 1999; Ridolfi et al., 2002; Benincasa et al., 2003).

For vegetables, tomatoes as an example, include over 400 flavour volatiles identified, 10 of which contribute to the aroma of ripe tomato (Petro-Turza, 1986). These volatiles include beta-ionone (Z)-3-hexenal, 1-penten-3-one, hexanal (E)-2-hexenal (Z)-3-hexenol, 2- and 3-methyl-butanol, 6-methyl-5-heptene, and 2-isobutylthiazole. A majority of these volatiles are lipid derived and mainly from the lipoxygenase (LOX) pathway. Major tomato volatiles in peel and flesh could be broken down into three categories: lipid derived, carotenoid derived, and amino acid derived (Mirondo and Barringer, 2015). Usually, tomatoes are not peeled prior to being made to juice, and also it is unknown if the presence of peel during the crushing process impacts the volatile profile, viscosity, and colour of tomato products. However, tomato peels produce a bit higher levels of lipid-derived volatiles than the fleshy part through LOX activity (Ties and Barringer, 2012).

The LOX-derived volatiles include hexanol (E)-2-hexenol, and (Z)-3-hexenol, hexanal (E)-2-hexenal, and (Z)-3-hexenal, which are associated with a fresh green flavour note in tomatoes (Ties and Barringer, 2012). In high concentrations, the fresh green flavour note changes the sensory profile and intensity of tomato flavour (Hongsoongnern and Chambers, 2008). Usually cold break tomato juice (juice processed at a temperature below 70 °C) is higher in LOX-derived volatiles because of enhanced enzymatic activity that results in the increased production of these volatiles. Therefore, cold break tomato juice made with peel is more likely to produce higher levels of the green flavour than juice made without the peel, but hot break juice exhibits decreased levels of these volatiles due to the fact that enzymes are inactivated, which does not allow production of volatiles (Buttery et al., 1987).

## Flavour Compounds in Pulses and Legumes

Off-flavours could either be developed during harvesting, processing, and storage or inherent to the pulse (Sessa and Rackis, 1977). Off-flavours inherent to the pulse can only be removed, modified, or masked, however, they cannot be prevented other than by breeding new cultivars with less off-flavour, while the developed off-flavour could be limited by tuning the processing of the seeds. Table 5 provides an example of the most important compounds associated with off-flavours in peas and soybean, the latter being the most studied type of pulse (Roland et al., 2016).

The major cause for off-flavour development during harvesting, processing and storage is the oxidation of unsaturated fatty acids such as linoleic and linolenic acids (Sessa and Rackis, 1977). This oxidation can be non-enzymatic (Pattee et al., 1983) or enzymatic (Makower and Ward, 1950; Lee and Wagenknecht, 1958). Hexanal, n-pentylfuran, 3-cis-hexenal, 2(1-pentenyl) furan, and ethyl vinyl ketone are LOX-derived contributors to green, beany, and grassy flavours of peas and soy beans (Rackis et al., 1979). A cooked bean aroma has been reported for pinto bean paste. It has low raw beany and, nutty flavour, astringency, and bitterness (Song et al., 2009). Moreover, off-flavours can be formed by the effect of heat on amino acids and sugars, like Maillard reactions, thermal degradation of phenolic acids, thermal degradation of thiamine, and via oxidative and thermal degradation of carotenoids (MacLeod et al., 1988).

## Volatile Compounds in Processed Foods

In coffee, as an example, furans have long been considered as normal components of its flavour volatiles. For instance, about 6.5% of its volatile compounds were furans (Merritt et al., 1963). In roasted coffee, the number of volatile furans identified has increased with improvements in analytical techniques to about 800 reported in 1994 (Crews and Castle, 2007).

Fatty acids such as palmitoleic acid, oleic acid, linoleic acid and  $\alpha$ -linolenic acid are precursors for five to ten carbon aroma compounds. For example, 3-hexen-1-ol (leafy), 2-hexen-1-ol, and 2-hexenal (leafy) contribute greenish as well as fresh odors in tea infusions (Takeo and Tsushida, 1980). Formation of these volatile from the oxidation of tea lipids is associated with two main pathways. The first pathway, known as lipoxygenase-mediated lipid oxidation, contributes to the flavour of tea. The second

**Table 5** Main off-flavours and associated compound families causing off-flavour in soy and peas

Off-flavours	Peas	Soy	Compound
Volatile	Green	Beany	Aldehydes
	Beany	Fatty	Ketones
	Pea	Green	Alcohols
	Earthy	Grassy	Pyrazine
	Hay-like	Rancid	Others
	Leafy	Leafy	
	Metallic	Earthy	
	Brothy	Cardboard	
	Acrid	Acrid	
	Pungent	Pungent	
Non-volatile	Fatty	Medicinal	
	Bitter	Bitter	Isoflavones
	Astringent	Astringent	Saponins
	Metallic	Metallic	Phenolic acids Peptides/amino acids

Sessa and Rackis 1977, Roland et al., 2016.

pathway is an oxidation reaction initiated by free radicals, such as autoxidation, photooxidation, and thermal oxidation. The rate of lipid oxidation increases with the degree of unsaturation of lipids. These two pathways have been thoroughly described by Ho et al. (2015). Moreover, Lipid degradation could also produce cyclic aroma compounds, for example, jasmine lactones, methyl jasmonate, and *cis*-jasmonate. These are fragrant volatiles initially identified from flowers of *Jasminum grandiflorum* as well as some green teas (Wang et al., 1996; Mosblech et al., 2009). Takeo and Tsushida (1980) reported the volatile aromas derived from lipid oxidation during the tea manufacturing process; hexanal, pentanal, jasmine, nonanal, *cis*-jasmonate, heptanal, 1-penten-3-ol (*E*)-2-hexenal (*Z*)-3-hexen-1-ol (*E,E*)-2,4-hexadienal (*E*)-2-hexen-1-ol, methyl jasmonate, and nerolidol.

## Conclusions

In order to have a food product with desirable flavour, it is important to understand how aroma compounds are derived, and the mechanisms by which flavour and off flavour compounds are generated. The formation of volatile compounds in different foods depend on their constituent components. Formation of volatile compounds is a multi-directional process as a result of some transformations linked with lipid oxidation, Maillard reaction, interactions between lipid oxidation products and Maillard reaction products, and thiamine degradation, among others.

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## Lipophilized Antioxidants

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### Glossary

**Antioxidant** A substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate.

**Bond-dissociation energy (BDE)** It is a measure of the strength of a chemical bond. The BDE for a species, AB, at room temperature is the reaction enthalpy of the bond homolysis reaction  $AB \rightarrow A + B$  and depends on the relative enthalpies of formation of reactant and product states. The BDE is the energy required to fragment the molecule AB in its ground state (i.e., the lowest energy electronic, vibrational, and rotational eigenstate) to ground state fragments A and B.

**Chain-breaking and retarder antioxidants** Chain-breaking antioxidants induce a lag phase during which the substrate is not substantially oxidized. Once the antioxidant disappears, the peroxidation rate rises sharply until it reaches the same rate as during uninhibited oxidation. Retarder antioxidants reduce the peroxidation rate without inducing a lag phase.

**Lipophilization** Grafting of a lipophilic moiety (fatty acid or fatty alcohol) on different substrates (e.g., phenolic compound, sugar, amino acid, protein), resulting in new molecules with modified hydrophilic/lipophilic balance.

**Phenolic compounds** Secondary metabolites widely found in plants, which form a part of the human diet. They include flavonoids (e.g., anthocyanins, flavonols, and flavanols) and nonflavonoids (phenolic acids such as benzoic acids and hydroxycinnamic acids, based on C1–C6 and C3–C6 skeletons, respectively; stilbenes; and derivatives).

**Deep eutectic solvent (DES)** A mix between a halide salt or another hydrogen bond acceptor and a hydrogen bond donor (HBD), resulting in a solvent with a room temperature melting point. The most common DESs are formed by choline chloride with cheap and safe HBD, the most popular ones being urea, ethylene glycol, and glycerol, but other alcohols, amino acids, carboxylic acids, and sugars have also been commonly used.

**Ionic liquids** Ion pairs that are liquid at ambient temperature. They consist in an association between an organic cation (such as an imidazolium or pyridinium) and a coordinating anion. Generally, cations are dissymmetric and large quaternary ammoniums or phosphoniums with various substitutions, whereas anions can be inorganic or organic.

### Nomenclature

BDE Bond-dissociation energy

CCL Critical chain length

DES Deep eutectic solvent

HLB Hydrophilic/lipophilic balance

ILs Ionic liquids

ROS Reactive oxygen species

## Introduction

Lipophilized antioxidants are amphiphilic molecules constituted of a lipid moiety covalently linked to a polar group possessing antioxidant properties, which when present at low concentrations compared with those of an oxidizable substrate, are able to delay or prevent oxidation of that substrate (Halliwell, 1990; Figueroa-Espinoza and Villeneuve, 2005). Antioxidants are employed to limit lipid oxidation of unsaturated fats and oils, the concomitant formation of toxic molecules, and degradation of organoleptic properties, to preserve the quality in food, cosmetics, and pharmaceutical products, which most of them exist as complex heterogeneous lipid dispersions. Meanwhile, because of some antioxidants' relatively low solubility in aprotic media, their application in oil-based products is limited. Hence, the importance of their functionalization by lipophilization can be performed chemically, enzymatically, or chemoenzymatically, through the covalent grafting of a lipid moiety onto the polar antioxidant. Depending on the structure of the latter, a multitude of lipophilized antioxidants can be synthesized; e.g., phenolic acids, flavonoids, tocopherols, and ascorbic acid. Lipophilized phenolic compounds, also called phenolipids, are antioxidants, which become surface-active molecules while preserving or even improving their physicochemical and biological properties over their parent phenolic compounds, which are known to possess antioxidant, antimicrobial, antiviral, antiinflammatory, and anticarcinogenic properties (Fung et al., 1985; Rice-Evans et al., 1997; Tanaka, 1997; Sergent et al., 2010; Yi-Hang et al., 2017).

The synthesis of lipophilized antioxidants is a strategy that allows to design powerful and custom-made surface-active antioxidants to protect heterogeneous systems (lipid dispersions such as emulsions, micelles or liposomes, or cultured cells) against oxidation and food and nonfood products against microorganism spoilage (Figuerola-Espinoza and Villeneuve, 2005; Laguerre et al., 2011; Suárez-Quiroz et al., 2013a,b; Suriyarak et al., 2013; Suriyarak et al., 2014; Durand et al., 2015; Decker et al., 2017; Durand et al., 2017b). This article focuses on lipophilized antioxidants derived from phenolic compounds, their physicochemical and biological activities, and their applications.

## Lipophilized Antioxidants in Nature

Alkyl esters of phenolic compounds are naturally present in plants, although long-chain esters are less common. For example, hexacosanyl and triacontanyl caffeates were isolated from the stem bark of *Pongamia glabra* Vent. (Leguminosae) (Saha et al., 1991), two esters of docosanol (n-docosanol-E-*p*-coumarate and caffeate) were isolated from *Myoschilos oblongum* (Santalaceae) roots (Piovano et al., 2003), tetradecyl ferulate was isolated from *Ocimum sanctum* L. (Lamiaceae) (Sonar et al., 2017), and methyl caffeate and a mixture of n-alkyl *p*-coumarates (C<sub>20</sub>, C<sub>22</sub>, C<sub>24</sub>) were isolated from the aerial parts and the roots, respectively, of *Artemisia assoana* Willk (Asteraceae; arid mountain regions of south Europe) (Martínez et al., 1987).

According to Snook et al. (1994), hexadecyl, octadecyl, and eicosyl *p*-coumarates could play a repellent role in the herbivore insect resistance of the sweet potato latex (*Ipomoea batatas* (L.) Lam.; Convolvulaceae) and contribute to the higher viscosity of the root latex compared with that of the vine latex. Bernards and Lewis (1992) identified nine alkyl ferulates in suberizing potato tuber tissue, after extraction from wounded tubers. C<sub>16</sub>–C<sub>28</sub> ferulates were identified, two being odd chain ferulates with C<sub>19</sub> and C<sub>21</sub> alkyl chains. Ferulate esters were at or near nondetectable levels in unwounded tubers, and their levels increased after 3–5 days following wounding, reaching a maximum after 11–15 days. Both C<sub>16</sub> and C<sub>18</sub> ferulates were more abundant than their longer-chain analogs, especially at 11 days after wounding. Ferulate accumulation was restricted to the wound periderm, suggesting that its formation is thus temporally and spatially correlated with suberin formation in the wound healing potato tubers playing a protecting role.

Gibbons et al. (1999) isolated a long-chain ester of caffeic acid (docosyl caffeate) from the halophytic plant *Halocnemum strobilaceum* (Pall.) M. Bieb. (Chenopodiaceae). Interestingly, these authors proposed that this kind of compounds may play a role in the stress management of halophytic plants by guaranteeing water retention within the plant cells: the alkyl chain would be anchored within the cell membrane and the hydrophilic portion would remain within the cell, thus retaining a hydration shell. Thus, phenolic lipophilized antioxidants in plants would play a protecting role against insects, microbial attack, or environmental stress.

## Lipophilization of Phenolic Compounds

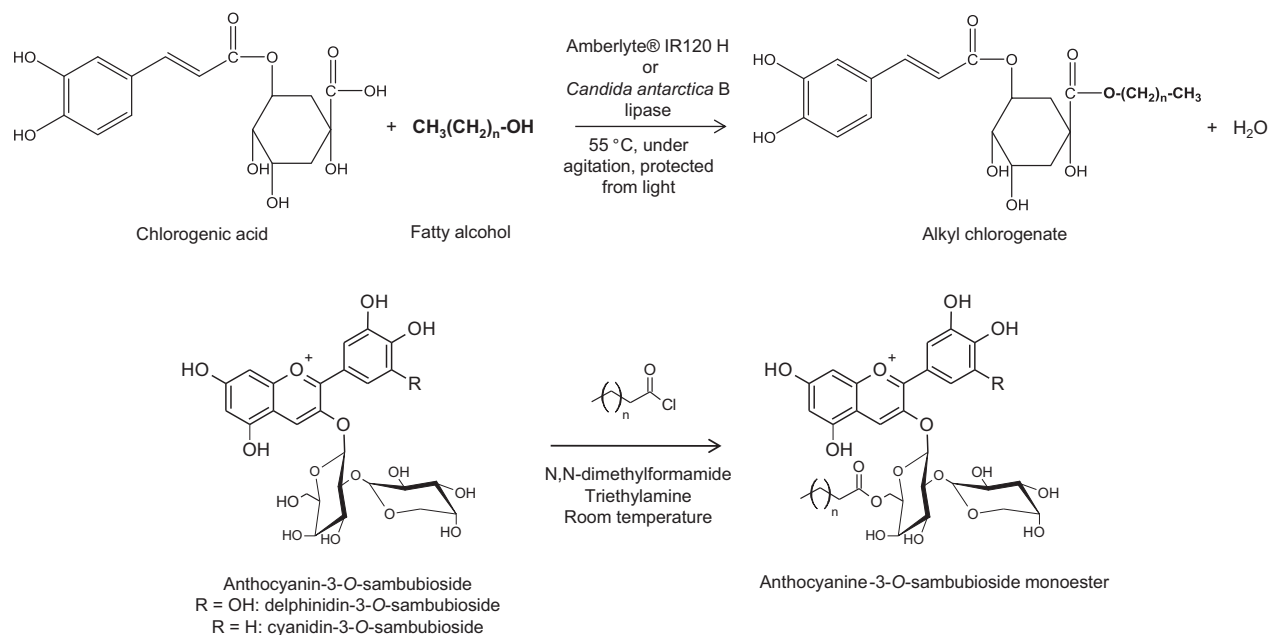
Lipophilization of phenolic compounds to obtain phenolipids can be achieved chemically, enzymatically, or chemoenzymatically, by esterification, amidation, etherification, or transesterification. For example, phenolipids can be obtained by esterifying the carboxylic or a hydroxylic group of a phenolic compound with a fatty alcohol or a fatty acid, respectively (Figs. 1 and 2), or with a triacylglycerol or a phospholipid (López Giraldo et al., 2007). These reactions can be achieved in the presence of an organic solvent, in an excess of alcohol (playing both the role of solvent and reactant), or in microemulsions. Enzymatic lipophilization has been achieved by using enzymes of the carboxylic ester hydrolase family (lipases, tannin acyl hydrolases, feruloyl esterases, cutinases) (Figuerola-Espinoza and Villeneuve, 2005). More recently, to enzymatically lipophilize antioxidants, ionic liquids (ILs) and deep eutectic solvents (DESs) have been used as a green alternative medium to conventional organic solvents, while creating an efficient and low moisture reaction system where both phenolic compounds remain soluble and the enzyme remains active (Fig. 3) (Katsoura et al., 2009; Yang et al., 2012; Durand et al., 2013). The interest in using ILs or DESs lies in their particular physicochemical properties (e.g., low vapor pressure, high thermal stability, reduced toxicity, biodegradability, etc.).

Table 1 presents a nonexhaustive list of lipophilized antioxidants reported in literature. Examples of lipophilized flavonoids have been described and reviewed by Chebil et al. (2006). This article will focus more on lipophilized phenolic acids.

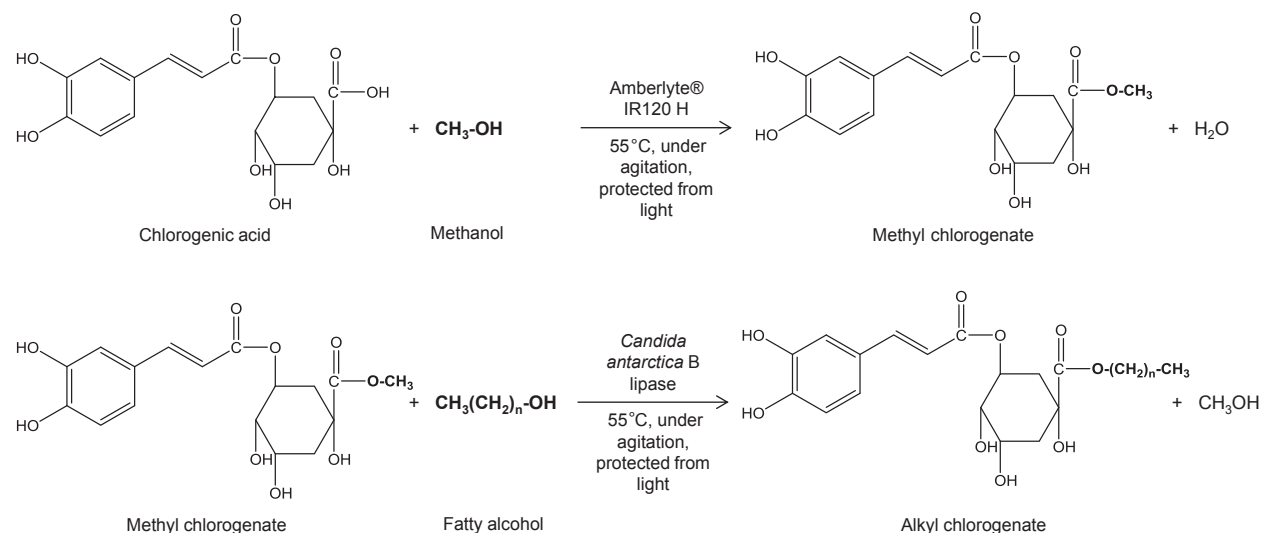
## Physicochemical and Biological Properties of Lipophilized Antioxidants

Lipophilized antioxidants exert antioxidant activity against lipid oxidation in heterogeneous systems and possess biological activities (antimicrobial, antiviral, antiinflammatory, anticarcinogenic, etc.), making them interesting multifunctional molecules for food and nonfood applications, as they are also surface actives and depending on the parent phenolic molecules, they can possess color properties.

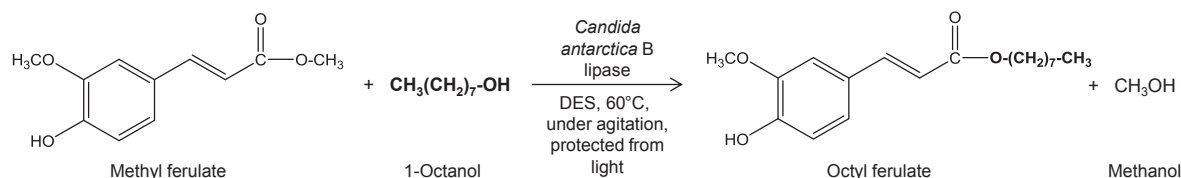
The study of physicochemical and biological activities of a broad range of homologous series of lipophilized antioxidants in heterogeneous systems (e.g., dispersed lipids, microorganisms, cultured cells) showed that the relationship between the activity and hydrophobicity follows a parabolic-like trend or a nonlinear behavior named “cut-off effect,” the term that was first adopted



**Figure 1** Direct lipophilization reaction of chlorogenic acid (5-*O*-caffeoyl quinic acid) and of anthocyanin-3-*O*-sambubioside. Adapted from López Giraldo L.J., Laguerre, M., Lecomte, J., et al., 2007. Lipase-catalyzed synthesis of chlorogenate fatty esters in solvent-free medium. *Enzyme Microb. Technol.* 41, 721–726 and Grajeda-Iglesias, C., Salas, E., Barouh, N., Baréa, B., Figueroa-Espinoza, M.C., 2017. Lipophilization and MS characterization of the main anthocyanins purified from hibiscus flowers. *Food Chem.* 230, 189–194.



**Figure 2** Chemoenzymatic lipophilization of chlorogenic acid (5-*O*-caffeoyl quinic acid). Adapted from López Giraldo L.J., Laguerre, M., Lecomte, J., et al., 2007. Lipase-catalyzed synthesis of chlorogenate fatty esters in solvent-free medium. *Enzyme Microb. Technol.* 41, 721–726.

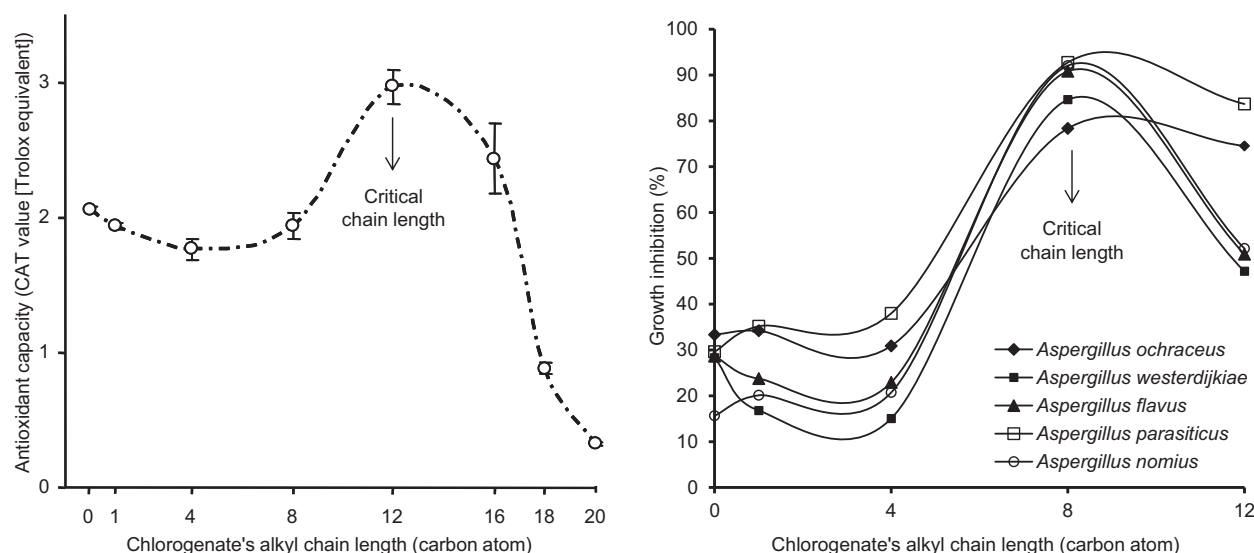


**Figure 3** Enzymatic alcoholysis of methyl ferulate with 1-octanol in deep eutectic solvent (DES) constituted of choline chloride, urea or glycerol and water. Adapted from Durand, E., Lecomte, J., Barea, B., et al., 2013. Evaluation of deep eutectic solvent-water binary mixtures for lipase-catalyzed lipophilization of phenolic acids. *Green Chem.* 15, 2275–2282.

**Table 1** Some examples of reported lipophilized antioxidants.

Lipophilization			Solvent			Phenolic acid														Flavonoids					Reference				
Chemical	Enzymatic	Chemo-enzymatic	Without solvent	Organic solvent	Deep eutectic solvent	Ionic liquid	Caffeic	Cinnamic	Coumaric	Chlorogenic	Dihydrocaffeic	3,4-dimethoxycinnamic	Ferulic	Gallic	Gentisic	Hydroxybenzoic	p-Hydroxycinnamic	Protocatechuic	Rosmarinic	Sinapic	Syringic	Vanillic	Cyanidin-3-O- sambubioside	Delphinidin-3-O-sambubioside	Malvidin-3-O-glucoside	Naringin	Procyanidin B4	Rutin	
X				X									X																Bernards and Lewis (1992)
	X		X				X	X			X	X	X																Guyot <i>et al.</i> (1997)
	X			X										X	X	X					X	X							Buisman <i>et al.</i> (1998)
	X		X	X			X	X	X				X			X													Stamatis <i>et al.</i> (1999)
	X			X									X																Compton <i>et al.</i> (2000)
	X		X	X						X				X	X	X					X	X							Guyot <i>et al.</i> (2000)
X				X			X						X	X			X			X									Chalas <i>et al.</i> (2001)
	X												X																Giuliani <i>et al.</i> (2001)
	X		X					X	X				X																Stamatis <i>et al.</i> (2001)
	X			X				X	X				X																Topakas <i>et al.</i> (2003)
	X					X	X	X	X				X							X									Katsoura <i>et al.</i> (2009)
X																			X										Lecomte <i>et al.</i> (2010)
X			X															X											Reis <i>et al.</i> (2010)
X				X														X											Grajeda-Iglesias <i>et al.</i> (2016)
	X					X							X																Chen <i>et al.</i> (2011)
						X					X																		Yang <i>et al.</i> (2012)
	X		X	X																						X		X	Kontogianni <i>et al.</i> (2003)
X				X																					X		X		Cruz <i>et al.</i> (2015)
	X																								X				Cruz <i>et al.</i> (2016)
X				X																			X	X					Grajeda-Iglesias <i>et al.</i> (2017)

by Ferguson (1939). Balgavý and Devínský (1996) reviewed several hypotheses of the cut-off effect in biological activities of long-chain amphiphilic compounds and observed that the cut-off effect is a general phenomenon observed in various biological and toxic activities for almost all tested amphiphile homologous series. As a rule, the same substances display the cut-off effect in different biological activities. This cut-off effect for antioxidant activity also occurs in heterogeneous systems as lipid dispersions, cellular models, and microorganisms (Laguerre *et al.*, 2009). That means that the physicochemical or biological activities rise progressively with increasing alkyl chain length until a threshold or critical point, beyond which the activity collapses. Furthermore, for the same homologous series of phenolipids, the critical chain length may vary with the studied system, and the optimal chain length of the alkyl group varies with the nature of the parent antioxidant. An example of this is shown in Fig. 4: lipophilization



**Figure 4** Antioxidant capacity (in an emulsified system) and antifungal effect (at 1.5 mg/mL) against different *aspergilli* of 5-*O*-caffeoyl quinic acid and its esters, in function of their alkyl chain length. Adapted from Laguerre, M., Giraldo, L.J.L., Lecomte, J., et al., 2009. Chain length affects antioxidant properties of chlorogenate esters in emulsion: the cutoff theory behind the polar paradox. *J. Agric. Food Chem.* 57, 11335–11342; Suárez-Quiroz, M.M.L., Alonso Campos, A., Valerio Alfaro, G., et al., 2013a. Anti-*Aspergillus* activity of green coffee 5-*O*-caffeoyl quinic acid and its alkyl esters. *Microb. Pathog.* 61–62, 51–56.

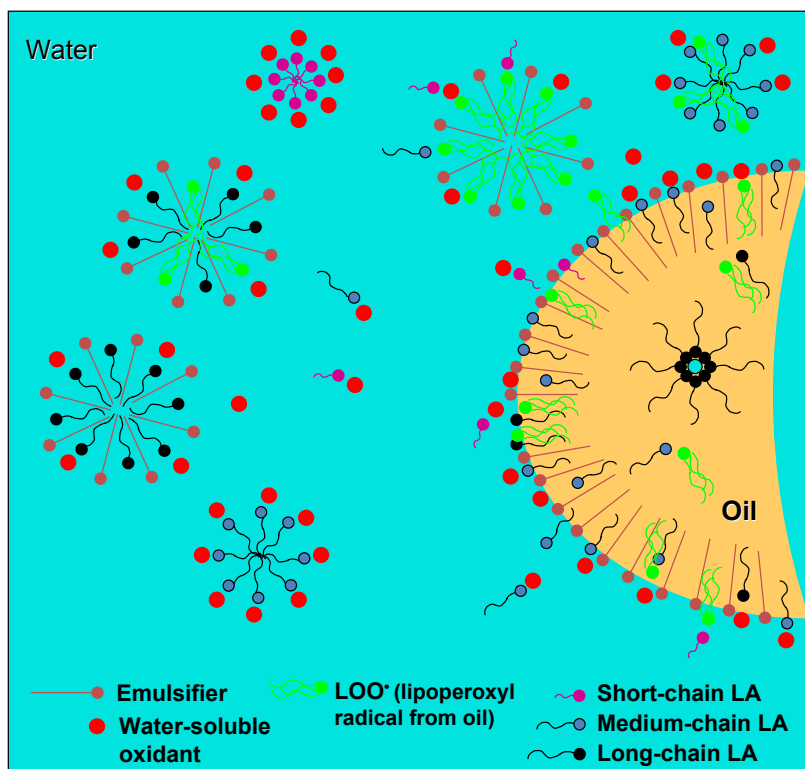
improved the antioxidant (in O/W emulsion) and antifungal properties of 5-*O*-caffeoyl quinic acid (chlorogenic acid), which increased with the ester alkyl chain length, exhibiting a cut-off effect at 12 and 8 carbons, respectively (Laguerre et al., 2009; Suárez-Quiroz et al., 2013a). In the same O/W emulsion, rosmarinates exhibited a cut-off effect centered on an eight-carbon chain length (Laguerre et al., 2010). The partitioning behavior and location (aqueous, oil, and/or interfacial phase) of lipophilized antioxidants in emulsions govern their antioxidant efficiency.

The partition behavior of chlorogenic acid and its alkyl esters between the continuous phase and the discontinuous phase in the O/W emulsion showed that the concentration of chlorogenates into the aqueous phase decreases as the alkyl chain length increases, until a threshold was reached for the dodecyl chain. Short-chain chlorogenates would be in suspension and the long-chain ones may form micelles on the aqueous phase (Laguerre et al., 2009). C<sub>12</sub> chlorogenate's suitable surface activity allows it to be more fusogenic and to have a good affinity with the lipid–water interface close to the site of lipid oxidation.

More interestingly, this “cut-off” phenomenon was also observed in more complex systems such as living cells (human dermal fibroblasts) with a threshold for the C<sub>12</sub> chlorogenate that lowered the oxidative stress in cultured fibroblasts by decreasing the reactive oxygen species (ROS) located in the cytoplasm (Laguerre et al., 2011). These results suggest that the suitable esterified alkyl chain length confers to the parent molecule the highest affinity for a interfacial membrane (emulsion) or a biological membrane (microorganisms or fibroblasts). Similar behavior was observed when testing alkyl rosmarinates that showed a critical chain length at C<sub>8</sub> for their antioxidant activity in emulsified system (Laguerre et al., 2010), at C<sub>4</sub> for their antioxidant capacity in chitosan-coated liposomes (Panya et al., 2010), and at C<sub>10</sub> for their antioxidant activity in ROS-overexpressing fibroblasts, as these esters could cross cell membranes to enter into the fibroblasts to target mitochondria (a major site of ROS production) and act as ROS scavenger within the cell. C<sub>16</sub> and C<sub>18</sub> rosmarinates formed extracellular aggregates, whereas rosmarinic acid and C<sub>4</sub> rosmarinate distributed within the cytosol. These results showed that the in cellulo antioxidant activity of lipophilized antioxidants depends on their ability to cross the cell membrane and on their subcellular location. The antioxidant activity on ROS-overexpressing fibroblasts of a series of 1,2-diacylglycerol rosmarinates with different alkyl chain lengths from 5 to 18 carbons confirmed again the occurrence of a cut-off effect at C<sub>10</sub> (Durand et al., 2015).

The antioxidant and biological properties in heterogeneous system of lipophilized antioxidants are thus dictated by their reducing activity, the parent molecule toxicity, their hydrophobicity, and the position of the lipophilic group in the parent molecule. The hydrophilic/lipophilic balance of lipophilized antioxidants influences their partitioning in every phase constituting the dispersed system and their capacity to self-aggregate, e.g., to form micelles, alone and/or with other molecules present, e.g., in the continuous, discontinuous, or interface in an emulsion, or in membranes of microorganisms or cells.

The key to synthesize the optimal lipophilized antioxidant in a rational manner, to maximize its physicochemical and biological properties, would be to graft the proper lipophilic group (corresponding to critical chain length [CCL]) while maintaining intact the reactive moiety. This CCL would allow the lipophilized antioxidant to efficiently move toward the oxidation sites (e.g., the interface in an emulsion or a membrane for liposomes and cells), avoiding, if the grafted alkyl chain is too long, its self-aggregation in the continuous phase (e.g., forming micelles) or its internalization in the discontinuous one (e.g., into the lipid core of the oil droplet in emulsions and micelles). The length of the alkyl chain could also influence the mode of action of the lipophilized antioxidant: chain breaker or retarder (Grajeda-Iglesias et al., 2016). It was observed that C<sub>1</sub> to C<sub>8</sub> protocatechuates would have a chain-breaking



**Figure 5** Proposed scheme of the lipophilized antioxidants (LA) distribution in an oil-in-water emulsion. Adapted from Grajeda-Iglesias, C., Salas, E., Barouh, N., et al., 2016. Antioxidant activity of protocatechuates evaluated by DPPH, ORAC, and CAT methods. *Food Chem.* 194, 749–757.

behavior and  $C_{10}$  to  $C_{16}$  protocatechuates a retarder one in an oil-in-water microemulsion antioxidant test (Laguerre et al., 2008). An illustration of how lipophilized antioxidants could distribute in an oil-in-water emulsion is presented in Fig. 5. This system is not static as an interparticle transfer exists for both oxidants and lipophilized antioxidants in oil-in-water emulsions. The molecules can get transferred by three mechanisms: diffusion, collision–exchange–separation, and micelle-assisted transfer (Laguerre et al., 2017). According to the alkyl chain length, lipophilized antioxidants can distribute between the water phase, the interface (as they are surface actives (Yuji et al., 2007)), and the oil drop, as individual molecules or as aggregates, depending on their solubility. They can form micelles or comicelles with other molecules present in the corresponding phase. They can also move from one phase to another, depending on their own mobility and solubility, either alone (short and medium alkyl chain) or transported by micelles (formed by amphiphilic molecules present in the system: added emulsifier and/or medium and long alkyl chain lipophilized antioxidants). The existence of micelles formed by an excess of emulsifier and/or long alkyl chain lipophilized antioxidants in the continuous phase of an oil-in-water emulsion is possible, and the transfer of these molecules from the oil droplet to the continuous phase by an excess of emulsifier has been proposed (Panya et al., 2012). We could also imagine that lipoperoxyl radicals, being slightly more polar than triglycerides, could approach the interface and then migrate into the aqueous phase transported by the surfactant micelles, formed by the excess of emulsifier and/or lipophilized antioxidants, and thus be stabilized forming “mixed” micelles or comicelles. In addition, it is also possible that lipophilized antioxidants in surfactant micelles could inactivate aqueous phase free radicals that originate from hydrogen peroxide or surfactant peroxides (Kubo et al., 2002c).

In conclusion, lipophilization would be a double-edge sword; if the grafted aliphatic chain is too short or too long, the resulting antioxidant activity will not be optimal (Laguerre et al., 2013). The factors to take into account to predict antioxidant capacity in homogeneous and/or heterogeneous systems of lipophilized antioxidants are as follows:

- To maintain the reactive moiety of parent antioxidants by grafting the nonreactive lipophilic moiety in the proper position.
- The presence of an *o*-dihydroxy group (catechol) in the structure of the parent molecule and/or the occurrence in the parent molecule of any structure that extends the conjugation of phenolic hydroxyls, i.e., enlarging the unpaired electron delocalization area and lowering the bond-dissociation energy, making the parent antioxidant more reactive.
- The intrinsic chemical reactivity of lipophilized antioxidant toward free radicals.
- The lipophilized antioxidant surface activity (hence hydrophobicity).
- The lipophilized antioxidant mobility to easily diffuse to the site of action and to properly position in heterogeneous systems; e.g., compartmentalized systems such as emulsions, membranes, or living cells.



## Biological Properties of Lipophilized Antioxidants

Some examples of biological properties of lipophilized antioxidants are shown in Table 2.

Phenolic compounds' antimicrobial and antiviral activities can be improved by lipophilization (Kubo et al., 2004; Suárez-Quiroz et al., 2013a,b; Suriyarak et al., 2013; Durand et al., 2017b). Grafting a lipophilic moiety to a phenolic compound allows the bioactive hydrophilic molecule to penetrate the lipid barrier that it encounters in various systems (microbial, living cells, etc.).

As for antioxidant activities, biological activities of long-chain surface-active substances also show a nonlinear dependence on the chain length or cut-off effect. For example, the antimicrobial cut-off effect of gallates has been largely studied (Kubo, 1999; Fujita and Kubo, 2002; Kubo et al., 2002a,b,c). The antibacterial activity of alkyl gallates against a Gram-positive bacterium (*Bacillus subtilis*) was observed to be a parabolic function of their lipophilicity and presented a cut-off effect with an alkyl chain of 9 and 10 carbons (Kubo et al., 2004). In Gram-positive bacteria, various enzymes from the electron transport chain (ETC) are embedded in the cytoplasmic membrane, which possesses an inner and outer hydrophilic surface and a hydrophobic interior. Alkyl gallates would penetrate the membrane lipid bilayer portion, thus inhibiting the ETC by interfering with the redox reactions. In most of Gram-negative bacteria, gallates would not reach the ETC in the cytoplasmic membrane because they would be incorporated into the Gram-negative outer membrane (lipopolysaccharide layer) (Kubo et al., 2002a). This is in accordance with the results of Suárez-Quiroz et al. (2013b) who observed that dodecyl chlorogenates were active against some Gram-positive bacteria (*Bacillus cereus*, *Clostridium sporogenes*, and *Listeria innocua*) but inactive against three Gram-negative ones (*Escherichia coli*, *Pseudomonas fluorescens*, and *Salmonella enterica*). Long-chain alcohols possess a more important antimicrobial activity on Gram-positive bacteria, yeasts, and molds, than against Gram-negative bacteria (Kubo et al., 1995). The presence on Gram-negative bacteria of an outer lipopolysaccharide layer could entrap hydrophobic antibacterial agents, delaying their adverse effect on the electron transport chain.

In contrast, the antifungal activity of alkyl gallates (C<sub>3</sub>–C<sub>13</sub>) and protocatechuates (C<sub>3</sub>–C<sub>14</sub>) would be due to their ability to act as nonionic surfactants disrupting the membrane in yeasts, such as *Saccharomyces cerevisiae* (Kubo et al., 2002c; Nihei et al., 2004). They observed a cut-off effect with the nonyl esters. The pyrogallol moiety of alkyl gallates would adsorb onto the surface of *S. cerevisiae* by forming an intermolecular hydrogen bond with the hydrophilic portion of the membrane surface, and the alkyl chain would create a disorder in the fluid bilayer of the membrane by disrupting specific target proteins such as cell-surface receptors or signal transduction proteins.

The antiviral activity versus AIDS' virus HIV-1 of ferulic acid and its esters was studied by Sonar et al. (2017). These authors observed that the esterification of ferulic acid with a long alkyl chain (C<sub>10</sub> to C<sub>18</sub> and C<sub>18:1</sub>) is essential for the inhibition of RNase H function, as neither ferulic acid nor methyl ferulate were efficient. C<sub>14</sub> and C<sub>18:1</sub> ferulates were the most actives. Furthermore, molecular modeling studies together with Yonetani-Theorell analysis demonstrated that N-oleylcaffeamide is able to bind to both the allosteric sites: one close to the nonnucleoside reverse transcriptase inhibitor (NNRTI)-binding pocket and the other close to ribonuclease H (RNase H) catalytic site, thus inhibiting viral replication.

Lipophilized antioxidants could also be used as antiinflammatory drugs. For this, it is important to determine their toxicity profile and mechanism of action. For example, Pereira et al. (2017) evaluated the cytotoxicity of C<sub>8</sub> to C<sub>16</sub> protocatechuates against cancer and noncancer cell lines and on the macrophages cell line RAW 264.7, which are among the most widely used in vitro models for the study of antiinflammatory drugs. It was found that these lipophilized antioxidants are selectively toxic to macrophages. The most toxic protocatechuates against cancer cells and macrophage cell line were the C<sub>10</sub>–C<sub>14</sub> and the C<sub>8</sub>–C<sub>14</sub>, respectively. These last provoked the nuclear morphological changes on macrophages (shrunk nuclei, and chromatin condensation and fragmentation), loss of mitochondrial membrane potential, and increase in intracellular ROS. This toxicity would be a consequence of their higher partition coefficient and thus their higher accumulation in cells. C<sub>8</sub> protocatechuate was the most toxic compound, toxicity started to decrease with C<sub>10</sub>, and mild toxicity was found up to C<sub>14</sub>. Furthermore, C<sub>10</sub> and C<sub>12</sub> esters were the only ones that, at the higher

**Table 2** Some examples of reported biological properties of lipophilized antioxidants

Compound	Biological properties	References
Ferulates (C <sub>16</sub> to C <sub>28</sub> )	Protection role as constituents of suberin in injured plant tissues	Bernards and Lewis (1992)
<i>p</i> -Coumarates (C <sub>16</sub> , C <sub>18</sub> , C <sub>20</sub> )	Antiherbivore role for latex (plant insect resistance)	Snook et al. (1994)
Protocatechuates (C <sub>8</sub> , C <sub>9</sub> )	Antifungal	Kubo (1999)
Caffeates and gallates (C <sub>3</sub> , C <sub>8</sub> )	Anticancer properties	Fiuza et al. (2004)
Gallates	Antibacterial, antifungal	Fujita and Kubo (2002); Kubo et al. (2002c); Kubo et al. (2004)
Chlorogenates (C <sub>1</sub> , C <sub>4</sub> , C <sub>8</sub> , C <sub>12</sub> )	Antibacterial, antifungal, and antimycotoxigenic activity	Suárez-Quiroz et al. (2013a,b)
Rosmarinates (C <sub>8</sub> , C <sub>10</sub> , C <sub>12</sub> )	Antibacterial activity	Suriyarak et al. (2013)
Ferulates (C <sub>10</sub> , C <sub>12</sub> , C <sub>14</sub> , C <sub>16</sub> , C <sub>18</sub> , C <sub>18:1</sub> )	Inhibitors of AIDS' virus HIV-1 reverse transcriptase	Sonar et al. (2017)
Procatechuates (C <sub>1</sub> –C <sub>16</sub> )	Antiinflammatory activity	Pereira et al. (2017)
<i>p</i> -Coumarates	Anticancer properties	Menezes et al. (2017)

tested concentrations, provoked loss of membrane integrity. These results are in line with those from Durand et al. (2017a) who investigated, via small angle X-ray scattering approaches in model membrane liposomes, the interaction and location within the lipid bilayer of a homologous series of rosmarinates; C<sub>8</sub> rosmarinate shows the highest affinity for the membrane, whereas C<sub>10</sub> rosmarinate constitutes a critical chain length from which the penetration depth decreased. These interesting results show that lipophilized antioxidants can be used as antiinflammatory drugs.

## Conclusion

Lipophilized antioxidants are amphiphilic molecules possessing a myriad of interesting physicochemical and biological properties, including antioxidant (in food and nonfood products and in cells), emulsifying, antimicrobial, antiviral, anticarcinogenic, and antiinflammatory properties. The ability of these bioactive molecules to cross or to interfere with lipid interfaces or membranes depends at once on the structure of their polar head and of their lipophilic tail. Finding the optimal lipophilic moiety chain length to lipophilized antioxidants is crucial to maximize their physicochemical properties and overall biological effects, and it has to consider both the parent molecule structure and the nature of the heterogeneous system where it will be applied, may this last be a cell or organism or a food or nonfood product.

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## Meat Color: Factors Affecting Color Stability

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### Glossary

**Aging** A process of holding carcasses or a part of it at refrigeration temperatures for improving sensorial characteristics, particularly tenderness.

**Antioxidant** A synthetic or natural molecule that inhibits the oxidation of other molecules.

**Electrical stimulation** A practice whereby an electric current is transmitted through the carcass immediately after slaughter to avoid cold shortening.

**Haem or heme** A cofactor consisting of an  $\text{Fe}^{2+}$  ion contained in the center of a porphyrin.

**Myoglobin** An oxygen-binding protein found in muscle tissue of vertebrates, which serves as a local oxygen reservoir.

**Oxidation** This is the loss of electrons or hydrogen or gain of oxygen and an increase in oxidation state by an atom, molecule, or ion.

**Reduction** This is the gain of electrons or hydrogen or loss of oxygen and a decrease in oxidation state by an atom, molecule, or ion.

**Rigor** Stiffening of postmortem muscles due to irreversible binding of myosin to actin after exhaustion of ATP reserves.

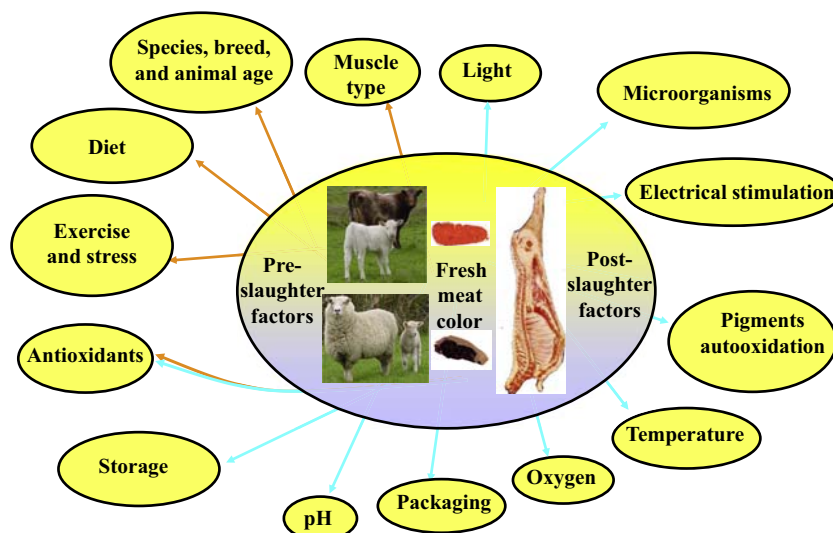
**Water-holding capacity** An ability of postmortem muscle to retain its water when some external force or pressure is applied to it.

### Introduction

The color of meat at any time after the animal has been slaughtered is a combination of the composition of the muscle of origin, the process where that muscle turns into meat and the conditions under which that meat is stored. Meat color is largely a result of the amount and state of the oxygen-binding protein, myoglobin (Mb), and the inevitable oxidation of the iron within it over time. As color is an important quality characteristic and a key determinant of shelf life, there have been a number of technologies developed to slow the oxidation process.

### Myoglobin in Muscle

Variation in meat color is dependent on many animal factors (species, breed, sex, age, diet, and activity) and the muscle type (Fig. 1). These affect both the amount of Mb (the native pigment, also known as deoxymyoglobin) and whether it is oxymyoglobin (OxyMb), deoxymyoglobin, or metmyoglobin (MetMb). The increase in MetMb over time is the primary cause of deterioration in muscle color.



**Figure 1** Factors affecting the color of fresh meat.

## Autoxidation of Meat Pigments

The general understanding of MetMb accumulation is that the net amount of MetMb formed is the result of its formation by the autoxidation of OxyMb and Mb and its reduction by MetMb reducing activity (Bekhit and Faustman, 2005; Bekhit et al., 2013). Mb autoxidation has a half-life of hours under in vivo pH, ionic strength, and temperature conditions (Livingston et al., 1986). The binding of O<sub>2</sub> triggers a globin conformational adjustment so that the orientation of the proximal imidazole, which is in contact with heme, changes to enhance the  $\pi$  orbital overlap with the iron (Giddings, 1977). At a physiological pH (7.2), the Hb association/dissociation rate is too low to produce any detectable autoxidation, but at normal pH (5.8) of meat the autoxidation rate is significant (Adams, 1976). Gotoh and Shikama (1974) estimated that the autoxidation half-life of bovine OxyMb at pH 5 was 2.8 h at 25°C and 5 days at 0°C. OxyMb has a high resonant structure that makes it more stable to oxidation than deoxymyoglobin (Giddings, 1974).

Renner et al. (1992) reported that the autoxidation of purified OxyMb separated from LD and PM muscles over a wide pH range (5–9), temperature (20–50°C), and ionic strengths (0–500 mM) was dependent on the postmortem time and muscle type. The autoxidation rates for the muscles were not different when the OxyMb was extracted at 2 h postmortem but at 192 h postmortem the autoxidation of the PM OxyMb was higher than that from the LD muscle.

Heme pigments may be more active as catalysts when iron is in the ferric state (Greene and Price, 1975). Inherent differences in MetMb accumulation (rates of autoxidation) have been claimed to be the primary factor for the differences in color stability between different muscles (O’Keeffe and Hood, 1982). On the other hand, Ledward et al. (1977) and Ledward (1985) reported that the rate of autoxidation between a range of muscles was similar and that the MetMb reducing system was the most important factor regulating the color. Increased oxidation of Mb postmortem in the absence of antioxidant and low reducing activity is the main cause of discoloration (Bekhit et al., 2007, 2013).

## Muscle Type

The biochemical and physiological characteristics, as well as the visual appearance of muscle, reflect the properties of the fiber types within a muscle (Karlsson et al., 1999). On the basis of histochemical staining or molecular techniques, Harper (1999) divided muscle fibers into three broad categories: glycolytic (fast-twitch B), slow oxidative (slow-twitch), and intermediate (oxidative–glycolytic, fast-twitch A). Muscle fiber types contain different concentrations of Mb, oxidative and glycolytic enzymes, and different contractile proteins (Klont et al., 1998). The slow oxidative (slow-twitch) fibers, which are typically red, have high concentrations of mitochondria, Mb, and blood vessels, but the fiber diameter is small. The fast oxidative–glycolytic (fast-twitch A) muscles are pink, having intermediate concentrations of mitochondria, Mb, and blood vessel supply. The fast glycolytic (fast-twitch B) fibers are white, having low concentrations of mitochondria, Mb, and fewer blood vessels. Different muscle fibers have different amounts of MetMb reducing activities (Echevarne et al., 1990; Reddy and Carpenter, 1991). The proportions of fiber types within a muscle vary with the action of the muscle. Animal muscles used for locomotion appear darker in color because they usually contain higher concentrations of Mb than support muscles, e.g., 12 mg/g wet tissue in extensor carpi radialis compared to 6 mg/g wet tissue in LD (Cross et al., 1986). Muscles containing high Mb concentrations appear darker than those with less Mb. Differences in OCR and Mb autoxidation rate with muscle types can explain variation in color stability between different muscles (Renner and Labas, 1987). Renner (1984) reported that the catabolism of NAD differs according to the muscle type. Madhavi and Carpenter (1993) found that LD was more color stable than PM. They also reported that the LD muscle had a higher MetMb reducing activity, high NAD content, and low OCR compared to the PM muscle.

## Muscle to Meat

Meat color is also affected by the rate of decline in temperature and pH postmortem and the ultimate pH of the meat. During this postmortem period, the balance between prooxidative and antioxidative factors favors oxidation (Morrissey et al., 1998; Bekhit et al., 2007).

## The Rate of pH Fall

The conversion of muscle to meat involves anaerobic glycolysis to lactic acid to maintain ATP levels (O’Halloran et al., 1997). The rate of glycolysis and the resulting pH decline is affected by animal species, preslaughter stress (Warriss, 2000), and postmortem temperature (Tornberg et al., 2000). Abnormal rates of pH fall may reduce the quality of the meat produced and can play a role in determining the color stability of meat by modifying the perceived color independently of MetMb formation (Ledward, 1985). A rapid pH fall, while the carcass temperature is high, can result in a low ultimate pH (>5.5) and a lighter meat appearance due to decreased water-holding capacity and increased unbound water. This is seen in pale soft exudate (PSE) pork. A slow rate of pH fall, while the carcass temperature is high, results in a higher ultimate pH (>5.8) and darker meat color in beef and lamb and dark firm dry (DFD) pork.



The unusual pH and water-holding capacity of PSE and DFD meat produce abnormal colors in fresh meat (Faustman and Cassens, 1990a; Ledward, 1992). They also display different color stabilities during retail display of pork with PSE, the least stable, and DFD, the most stable. The poor stability of PSE meat is probably due to increased Mb autoxidation and the decreased MetMb reduction with the lower pH (Owen and Lawrie, 1975).

The rate of change of temperature and pH of the muscle during postmortem glycolysis can also influence the color of fresh meat independent of MetMb formation (Ledward, 1985). The oxygen consumption rate (OCR) in rapidly chilled meat is likely to be higher than in slowly chilled meat and may be responsible for the formation of the higher contents of MetMb during storage (Atkinson and Follett, 1973). Higher OCR is found in high pH meat and contributes to the dark color associated with such meat (Ledward, 1985).

## Electrical Stimulation

Electrical stimulation is used in the meat industry to avoid the incidence of cold shortening that causes meat toughness. The temperature–pH profiles are very important factors to consider when electrical stimulation (ES) is applied to carcasses to improve the tenderness of meat. For example, during ES, the pH of meat falls approximately 0.35 units per min (Lawrie, 1998), which represents a 100- to 150-fold increase over the normal rate. The importance of ES on meat color originates from its effect on the pH–temperature relationship and, depending on the ES intensity, it can also affect the muscle tissue structure.

Electrical stimulation per se has generally been associated with improvement of the fresh meat appearance by imparting a lighter bright red color on the meat surface (Savell et al., 1978; Tang and Henrickson, 1980; Eikelenboom et al., 1985; Smith, 1985; Unruh et al., 1986). Moore and Young (1991) found that the color stability of ES chilled lamb during display was better than that of non-ES lamb. However, the advantage disappeared after 2 weeks aging in a vacuum pack at  $-1.5^{\circ}\text{C}$ . In contrast, Powell et al. (1996) reported that consumers did not perceive differences in the rate of discoloration between ES and non-ES SM steaks. However, significant consumer preferences were found for ES compared to non-ES meat that were associated with pH differences (5.08–6.0) and between meat aged for 5 days and 33 days.

Mareko (2000) studied the effect of mild ES (75 V, 15 Hz, 20 s) versus intense ES (75 V, 15 Hz, 40 s) on the quality of LD muscles of beef carcasses. Mild ES meat exhibited significantly higher  $a^*$  (redness, CIE- $L^*a^*b^*$  system) than intense ES. Both  $a^*$  and  $b^*$  values increased after 7 days of vacuum packing at  $2^{\circ}\text{C}$  with significant higher values in mild ES compared to intense ES. A lighter color of meat was produced from low voltage ES compared to non-ES carcasses (Hildrum et al., 2000).

Unwanted and negative effects of ES on color have also been reported (Petch and Gilbert, 1997). Ledward et al. (1986) reported a reduction in the color stability of deep muscles such as SM, while surface muscle such as LD was unaffected. The greater color stability of LD was related to its exterior position on the carcass, which allowed it to cool rapidly. Studies by Ledward (1985) and Ledward et al. (1986) pointed out that the negative effect of ES on the color stability of beef muscle was more pronounced in meat, which has been aged compared to fresh meat. Others did not report any effect of ES on the color of beef SM muscle (Sammel et al., 2002). While Renere and Bonhomme (1991) found a positive effect for ES and hot boning on lamb LD muscle, Jeremiah et al. (1997) found no effect of ES and hot boning on the color or color stability of lamb muscles (LD and SM).

## Rigor Temperature

Color and color stability has been reported to be affected by the temperature at which the muscle enter rigor (Ledward, 1985). This is relevant to meat processing, particularly those that use ES and hot boning. Although those muscles, which entered rigor at higher temperatures as a result of ES, were less stable, there is evidence of significant improvements in color parameters (chroma and  $L^*$ ) if rigor occurred at the extreme temperatures of  $30\text{--}35^{\circ}\text{C}$  has been reported (Ledward, 1985; Farouk and Swan, 1998; Young et al., 1999; Geesink et al., 2000). However, the effect was lost with vacuum packaging and aging (Farouk and Swan, 1998; Geesink et al., 2000).

## Ultimate pH

The ultimate pH ( $\text{pH}_u$ ) is an important general indicator of meat quality and a specific indicator of meat color. A significant correlation between beef color and  $\text{pH}_u$  has been reported (Purchas et al., 1999). The  $\text{pH}_u$  was found to correlate with  $a^*$  and  $b^*$  components of color but not with  $L^*$  in beef (Warner, 1989).  $\text{pH}_u$  for normal meat ranges from 5.4 to 5.8 (Faustman and Cassens, 1990a,b). At lower and higher pH values, abnormal meat conditions and color can be found. It is generally regarded that meat with a low pH discolors more rapidly than meat with a high  $\text{pH}_u$  (Ledward, 1985; Zhu and Brewer, 1998). For example, it has been reported that beef with  $\text{pH}_u$  of 5.6 was less color stable than beef with a  $\text{pH}_u$  higher than 5.80 (Ledward et al., 1986).



Renner (1999) proposed that the poor stability at low pH was caused by an increased rate of Mb autooxidation, while the enzymatic reduction was severely reduced. In addition to the acceleration of the Mb autooxidation rate at a low pH (Gotoh and Shikama, 1974), the nonenzymatic MetMb reduction was decreased especially as the pH reached less than pH 6.0 (Zhu and Brewer, 2003).

Possible mechanisms for a low pH accelerating the oxidation of Mb or OxyMb to MetMb and causing meat discoloration are as follows:

- Denaturation of the globin protein moiety that protects the heme groups. This causes the disassociation of O<sub>2</sub> from the heme, as well as the oxidation of the iron molecule (Cross et al., 1986).
- The low stability of OxyMb at low pH. For example, the half-life of beef OxyMb at 0°C was 71 times higher at pH 9.0 than OxyMb at pH 5.0. At 25°C, the half-life of OxyMb was 60 times higher at pH 9.0 than at pH 5.0 (Gotoh and Shikama, 1974).
- Acceleration of the protonation of bound O<sub>2</sub> and the formation of reactive oxygen species (ROS) that are potent prooxidants (Livingston and Brown, 1981).
- Iron-catalyzed oxidation that has been reported to be pH-sensitive and the most active under acidic conditions (Lee and Hendricks, 1997).

High pH<sub>u</sub> increases the water-holding capacity, and OCR (Ledward, 1985) causing dark color due to swelling of the fibers results in closer spacing of the fibrils leading to less light scattering and, as a consequence, the meat is more translucent. Under such conditions, the light penetrates deeper into the meat and hence, the Mb absorbs the light stronger, causing the darker color of the meat. The increased water-holding capacity and a more closed myofibril structure resist deep O<sub>2</sub> diffusion that, in addition to the high OCR, makes deoxymyoglobin the dominating pigment. Hence, this meat has a dark purple color (Warner, 1989).

The rate of OxyMb formation at pH 8 can be three to four times that at pH 5–7 (Schwimmer, 1981). Lowering the pH from 7.4 to 5.7 during the muscle to meat conversion process tends to denature some of the active reducing enzymes and cause a decrease in OCR (Schwimmer, 1981). In support of this view, Asghar et al. (1990) found that the presence of mitochondria and microsomes in sarcoplasmic extracts increased OxyMb and decreased MetMb at high pH, but at a low pH their presence had little effect.

## Storage Conditions

After the muscle has turned into meat, the carcass is processed into individual cuts of meat and often stored under chilled conditions. During this when meat is first exposed to air the color changes from the purple of deoxymyoglobin to the bright cherry-red color of oxymyoglobin, a process known as blooming. After this time the color deteriorates mainly as a result of oxidative processes.

## Oxygen

The brightness and saturation of a developed red color depends on the depth of the zone in which O<sub>2</sub> can penetrate into the tissue and oxygenate the Mb. Oxygen penetration increases linearly with time after meat is cut and is influenced by animal species and muscle type (MacDougall and Taylor, 1975). The depth of the O<sub>2</sub> penetration into the meat tissue is determined by (1) the rate of O<sub>2</sub> diffusion into the tissue, (2) OCR of the tissue, and (3) the pressure of the O<sub>2</sub> at the surface (O’Keeffe and Hood, 1982). At low O<sub>2</sub> levels, off-colors develop in meat. This effect can be observed in a freshly cut cross-section of an aged beef steak. The pigment at the surface, where O<sub>2</sub> is readily available, is OxyMb (bright red) resulting from the mass action effect of O<sub>2</sub>, which has diffused into this region. As the amount of available O<sub>2</sub> diminishes, available Mb is converted into MetMb. This is manifested as thin brown layer a few millimeter below the original surface. As the O<sub>2</sub> is exhausted in this region, no O<sub>2</sub> is available to migrate to the next region, center of the slice, so that this region (the center) imparts the purplish color of Mb. The formation of MetMb is optimal when the O<sub>2</sub> partial pressure is 6 mm Hg (at 0°C) or 7.5 mm Hg (at 7°C) (Ledward, 1970).

The OCR decreases with time postmortem due to the depletion of substrates and coenzymes and degradation of enzymes involved in mitochondrial respiration (DeVore and Solberg, 1974). The OCR of LD muscle was decreased to 30% of its initial value after 2 days of postmortem and to 15% after 10 days of postmortem (O’Keeffe and Hood, 1982). This can lead to higher O<sub>2</sub> penetration (Feldhusen et al., 1995) and faster blooming on exposure to air. Thus, many researchers believe that OCR is the main factor controlling the color stability (Echevarne et al., 1990; Lanari and Cassens, 1991). Inhibiting mitochondrial respiration by the addition of rotenone or lowering the pH of prerigor beef muscle homogenates produced a bright red color (Cornforth and Egbert, 1985), which supports the hypothesis. Dark color associated with prerigor meat is due to the active mitochondria that consume O<sub>2</sub> and maintain Mb in their deoxygenated form (purple color).

## Storage Atmosphere

Modifying the atmosphere surrounding meat can considerably influence the storage life (meat color) of fresh meat. Modified atmosphere packaging (MAP) in combination with optimum temperature control and processing hygiene is the most effective means of extending the storage life of fresh chilled meat and maintaining the product quality and safety (Jeyamkondan et al., 2000; Mancini et al., 2009). MAP commonly utilizes different amounts of CO<sub>2</sub>, O<sub>2</sub>, and N<sub>2</sub>.

Vacuum packaging can be regarded as a special case of MAP, since creating anoxic conditions modifies the package atmosphere. It is well known that the color of fresh meat deteriorates with storage time and that vacuum packaging improves the color of raw meat on exposure to air. However, any improvements in the color are short lived and the rate of change in color of meat previously vacuum packed is higher than nonvacuum-packed meat.

When O<sub>2</sub> is included in the MAP gas mixture the Mb is maintained in the oxygenated form. It has been reported that packages containing high concentrations of O<sub>2</sub> (80% O<sub>2</sub> and 20% CO<sub>2</sub>) can improve the storage life of beef by 2 weeks while maintaining acceptable red color at 1.5°C (Gill and Jones, 1994).

The use of CO<sub>2</sub> has the advantage that its inhibitory effect increases with decreasing meat temperature (Jay, 1992). It has been shown that a combination of high CO<sub>2</sub> MAP (100%) and low temperature (−1.5°C) can improve the shelf life of meat products (Gill and Jones, 1992). However, at that high CO<sub>2</sub> percent the color is purple and the consumers may not accept it unless they have been educated. CO<sub>2</sub> is bacteriostatic, but the exact mechanism of microbial inhibition is not known (Jeyamkondan et al., 2000). Nitrogen is usually used as an inert filling gas.

## Temperature

The influence of temperature on meat color deterioration is well known. An increase from 0 to 5°C doubles the rate of discoloration (Taylor, 1981), and the degree of discoloration after 96 h storage at 10°C caused two to five times the discoloration of storage at 0°C depending on the muscle (Hood, 1975). The Q<sub>10</sub> (the temperature coefficient) for OxyMb oxidation is 1.25–2.5 times that of chemical or enzymatic reactions (Faustman and Cassens, 1990a). High temperatures favor greater scavenging of O<sub>2</sub> by residual respiratory enzymes, which leads to low O<sub>2</sub> tension and facilitates Mb autooxidation. Lipid oxidation also increases with elevated temperatures, which contributes to the nonenzymatic Mb oxidation (Renner, 1999).

The impact of storage temperature is dependent on the packaging and muscle type. For example, when beef meat is stored in air, the increased rate of discoloration by a high storage temperature was proportionately the same for all muscles (PM, GM, and SM) or higher for muscles (SM) with more color stability (Ledward, 1985; O’Keeffe and Hood, 1980-1981a,b). When the meat was stored in an O<sub>2</sub> free atmosphere, the increase in storage temperature resulted in a higher discoloration rate in a muscle (PM) with intrinsically less color stability (O’Keeffe and Hood, 1980-1981a,b).

Increased temperature accelerates Mb and OxyMb oxidations resulting in increased MetMb formation in meat through the following mechanisms:

- Increased oxidative processes by increasing the rate of prooxidant reactions and accelerated lipid oxidation (Faustman and Cassens, 1990a).
- Decreased oxygen solubility that favors the dissociation of O<sub>2</sub> from OxyMb. The less stable deoxymyoglobin has a greater tendency toward oxidation (Giddings, 1977; O’Keeffe and Hood, 1982).
- Increased OCR (Urbain and Wilson, 1958; cited from O’Keeffe and Hood, 1982) that results in a corresponding decrease in the depth of the O<sub>2</sub> penetration.
- Increased microbial growth.

## Lipid—Heme Reciprocal Autoxidation

The relationship between lipid oxidation and muscle pigments has been substantiated by many studies (Faustman et al., 1989, 1992). Heme pigments in meat tissue catalyze the oxidation of muscle tissue lipids and result in a rancid odor and flavor. Free radicals produced in oxidizing lipids can oxidize and decompose the heme pigments (Bekhit et al., 2013). These two reactions appear to be interdependent in that inhibition of either reaction will result in inhibition of the other (Greene and Price, 1975). A time lapse has been observed between initiation of pigment oxidation and initiation of lipid oxidation, which suggests that conversion of Mb to MetMb is necessary to accelerate lipid oxidation catalysis (Koizumi et al., 1973).

The development of a MetMb layer under the surface of meat depends on the partial O<sub>2</sub> pressure and therefore one may expect that a high O<sub>2</sub> pressure should stabilize the color since MetMb formation requires a low O<sub>2</sub> pressure. According to Wang (1962), the rate of OxyMb oxidation is slower than the corresponding process for free heme by a factor of about 10<sup>8</sup>. Hence, maintaining the Mb in the oxygenated form reduces the autoxidation rates dramatically. However, high O<sub>2</sub> pressure increases the rate of lipid oxidation and may counteract the beneficial effects on color gained by that treatment.

The ferric forms of Hb and Mb were reported to be very active catalysts of lipid oxidation (Kaschnitz and Hatefi, 1975). Likewise, the activity of OxyMb as a catalyst was reported to be similar to that of MetMb, especially at lower concentration ratios of heme pigments to unsaturated lipid (Koizumi and Nonaka, 1975). Greene and Price (1975) concluded that heme pigments may be more active catalysts of lipid oxidation when ferric and that nonheme iron may be more active catalyst in the ferrous state. Ferrous nonheme acts as a prooxidant in cooked meat, whereas MetMb exhibits little or no prooxidant activity (Love and Pearson, 1975). Thus, iron (free and protein bound), heme, and nonheme (oxidized or reduced) have the ability to oxidize unsaturated fatty acids in meat (Gandemer, 1998).

## Effect of Antioxidants

Oxidative processes contribute to the deterioration in the color of displayed fresh retail meat (Decker et al., 1995). Synthetic antioxidants, such as butylhydroxytoluene (E 321) and butylhydroxyanisole (E 320), are currently permitted as food additives. These antioxidants, however, have limited applications because of their low water solubility and low penetration of intact muscles (Lee et al., 1998). Their safety is also in question (Kansci et al., 1997).

Vitamin C is regarded as a safe antioxidant and has been under investigation in many meat systems. However, the ability of vitamin C to maintain the color or improve the color stability of raw meat depends on the method of incorporation into meat. Dipping or spraying meat with 2.5% or 5% ascorbate solution inhibited the formation of MetMb but impaired the blooming (Hood, 1975; Harbers et al., 1981). Preslaughter injection of ascorbate improved the color stability and the shelf life of PM, GM, and SM but not LD (Hood, 1975), whereas jugular infusion of preslaughter extended the shelf life of PM, GM, and LD (Schaefer et al., 1995).

Vitamin E is able to delay the oxidative process and to improve fresh meat color. Dietary supplementation with vitamin E improved beef color (Lanari et al., 1993; O'Grady et al., 2001a; Mitsumoto et al., 1995). Steaks from cattle supplemented with dietary vitamin E were preferred over controls during display by 91% of surveyed Japanese (n = 10941) (Sanders et al., 1997). Vitamin E affected the length of the MetMb induction period (Sanders et al., 1997) and diminished the adverse effect of temperature abuse (Chan et al., 1995). That effect was dependent on dose (the higher the vitamin E supplement the longer the induction period) and muscle type (LD had longer induction period than the color labile GM). Faustman et al. (1989) and Arnold et al. (1993) reported that 3–3.5 mg vitamin E/kg muscle was needed depending on the muscle. In contrast, other researchers found that vitamin E delayed lipid oxidation but had no effect on color stability in turkey muscles (Mercier et al., 1998) or beef (Yang et al., 2002) and did not change the antioxidant enzymes, catalase, superoxide dismutase, and glutathione reductase (Renner et al., 1999). The role of vitamin E has been reviewed by Liu et al. (1995), Morrissey et al. (1994), and Faustman et al. (1998).

Vitamin B3 (niacin) is another antioxidant, which has shown promising results in increasing the color stability of fresh meat. Depending on the muscle type, niacin supplements can produce a significantly more intense red color than controls for up to 12 days of storage (Naruse et al., 1998). The decrease of NAD nucleosidase and the consequent preservation of NAD may be the direct effect of niacin addition and hence result in the maintenance of meat color (Schwimmer, 1981).

Organic selenium supplements do not affect the susceptibility of beef meat to oxidative processes (O'Grady et al., 2001b). Chan and Decker (1994) suggested that the better stability of white muscle fibers against oxidative processes could be due to the higher concentrations of anserine and carnosine in these muscles compared to red muscles. However, Bekhit et al. (2003, 2004) found carnosine to promote Mb oxidation at normal meat pH (5.5–5.7).

Renner (1999) and Renner et al. (1996, 1999) found that the antioxidant enzyme activities in oxidative (red) muscles were higher than in glycolytic (white) muscles, but it did not appear to offer increased protection against free radicals or improve its color stability. Furthermore, the supplementation with vitamin E did not affect the activity of antioxidant enzymes (Renner et al., 1999).

## Light

Photooxidation normally refers to modification or destruction of amino residues especially the histidyl imidazole; the phenylalanyl and tyrosyl rings; the heterocyclic tryptophyl ring system; and the methionyl and cysteinyl sulfurs (Foote, 1968; Giddings, 1977). Incident light in display cabinets can contribute to meat discoloration and is a major problem in frozen meat. Its impact depends on the wavelength and intensity of the light, temperature, O<sub>2</sub> pressure, meat pH, storage time, and the presence of transition metal ions (Hunt et al., 1975; Owen et al., 1976; Satterlee and Hansmeyer, 1974). Photocatalysis, either indirectly through riboflavin associated compounds or directly through photosensitization of heme or changes in the amino acid in Mb, has been reported (Lynch et al., 1976; Griddings, 1977).

A predisplay dark storage period of 30 days of meat supplemented with vitamin E delayed MetMb accumulation in meat subsequent displayed due to a reduction in the OxyMb autooxidation rate (Lanari et al., 1994). There is now sufficient evidence verifying that meat kept in the dark has better color stability (Taylor, 1981).

MacDougall and Powell (1997) studied the effects of display temperature and light properties on the color stability of fresh and aged meat. They concluded that meat temperature in the display case was the most important factor. If the temperature was low then the illumination level was the factor dictating the case life. The type of light can affect meat color, but illumination level is the most important factor. There are intrinsic differences between muscles (LD and PM) and within muscles (outside and inner side of the SM), which can affect color stability as they respond differently to light.

High rate of discoloration of meat cuts displayed under illumination was due to the heat generated from the light bulbs (Greer and Jeremiah, 1980, 1981) and UV light (Anderson et al., 1989). The energy gained from light can induce the formation of excited ROS, which cause pigment autooxidation (Schwimmer, 1981). Berthelsen and Skibsted (1987) stated that light is the most important factor in frozen meat discoloration. It is worth noting that singlet O<sub>2</sub> has been implicated for Hb oxidation in light (Possani et al., 1970). Since Mb and Hb share a lot of characteristics, Mb may equally be affected by light.

## Microorganisms

Bacterial activity is another factor contributing to pigment changes in raw meat (Faustman et al., 1990). Psychrotrophic bacteria, such as *Pseudomonas* and *Brochothrix thermosphacta*, utilize  $O_2$  act as a prooxidant in low population numbers ( $10^4$  CFU/g) and reduce MetMb at high populations ( $10^8$  CFU/g) (Faustman and Cassens, 1989).

Depending on the conditions of meat packaging, meat discoloration will vary according to the availability of  $O_2$ . Aerobic bacteria in  $O_2$  permeable packages have a high  $O_2$  requirement during the logarithmic phase of growth that leads to low  $O_2$  tension at the surface and, consequently, discoloration. *Pseudomonas geniculata*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Achromobacter faciens* are examples of aerobic bacteria that can cause meat discoloration (Robach and Costilow, 1962). As aerobic bacteria increase in number, the surface of the meat changes from red OxyMb to brown MetMb and then to purple reduced Mb (Cross et al., 1986). Also, Cross et al. (1986) explained the purple color imparted in raw meat by high counts of bacteria that create an anaerobic environment due to blocking the  $O_2$  from entering the meat. This enhanced the reduction of MetMb by the MetMb-reducing system and, consequently, prevented the oxygenation of the newly formed Mb.

Bacteria may also cause greening discoloration. For example, in vacuum packed meat that has pH > 6.0, bacteria such as *Pseudomonas mephitica* and *Alteromonas putrefaciens* can produce hydrogen sulfide that contributes to the formation of the green-colored sulfmyoglobin (Cross et al., 1986). However, on opening the vacuum package, the green color will be converted to oxysulfmyoglobin, which is red, but the meat will have the odor of rotten eggs.

Although bacteria, in general, cause color problems, Arihara et al. (1993) have found that certain species convert MetMb to OxyMb.

## Conclusions

Color of fresh meat and its stability can vary widely among species and cuts from the same animal due to differences in anatomical, physiological function and biochemical processes of the muscles where the meat is obtained from. The color and shelf life are the result of the initial muscle, the changes as the muscle goes into rigor, and how the meat is stored and displayed. Different systems that try to delay oxidative processes (cooling, vacuum and modified packaging, addition of antioxidants) appear to be successful in improving color stability and shelf life. The success of these strategies, however, is not universally successful and should be considered on for individual meat cuts.

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## Meat Colour: Chemistry and Measurement Systems

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### Glossary

**Aging** The process where meat held at refrigerated temperatures becomes more tender as a result of proteolysis.

**Blooming** The oxygenation process responsible for the formation of oxymyoglobin following exposure to air or high concentrations of oxygen.

**CIE LAB or L\* a\* b\*** Representation of colour measurements system recommended by an International Commission on Illumination known as Commission International de l'Eclairage (CIE).

**Colour** Colour is an appearance property attributable to the spectral distribution of light. *Colour is made up of varying proportions of the three additive primary colours red, green and blue.*

**Discoloration** Formation of colours other than the colour desired by consumers.

**Metmyoglobin** Oxidised form of myoglobin.

**Myoglobin** A globular cytoplasmic protein found in muscle that binds oxygen on a haem. It is the main pigment responsible for meat colour.

**Oxidation** The state where atoms in an element lose electrons as a result of reaction and an increase in the valency of the element is correspondingly achieved.

**Oxymyoglobin** The oxygenated form of myoglobin.

### Introduction

Consumers judge the quality of meat at the point of sale on colour, visible fat content and odour (Bickerstaffe, 1996). Colour is perhaps the most important sensory attribute of a food because if the colour is objectionable for the consumer, the food will not be purchased and/or eaten, and consequently, all other sensory attributes lose significance. The consumer often uses colour as an indicator of the freshness and quality of meat (Hood and Riordan, 1973). For example, if the colour of beef is not bright cherry-red, the meat is usually considered undesirable or even spoiled. Fig. 1 shows the discolouration in a plant and in meat. While, discolouration in plant materials is taken as indication for loss of freshness, as in meat, it is also associated with loss of nutrients and other important sensory attributes, which is not considered with old meat. The change in fresh meat colour is a reflection of the net balance between oxidative, antioxidative and reducing systems in meat. Discolouration of fresh meat indicates dominance of oxidative processes that can have great consequences on the digestion and bioavailability of nutrients, but this is yet to be considered in meat science.

The colours associated with good meat quality depend on the type of meat. For Chicken, turkey, veal and pork are considered white meats and a pink-greyish colour is considered to be normal. Fresh lamb and beef products are red meats and a bright red colour is usually desired (Schwimmer, 1981). The colour of meat ultimately depends on the amount and state of pigments in meat, namely, myoglobin, haemoglobin and cytochromes. Myoglobin is the primary pigment associated with meat since most of the haemoglobin is discharged during bleeding after exsanguination. As such, the level of meat discolouration and colour formed are also influenced with the amount of myoglobin present. For example in meat that is rich in myoglobin such as venison, beef and lamb the discoloured meat is brown while in meat that contains small amounts of myoglobin the discoloured meat is grey.

### Meat Pigments and Colour Development

Myoglobin (Mb) is an intracellular protein found in red skeletal muscle and heart. It binds oxygen (O<sub>2</sub>) reversibly and enhances O<sub>2</sub> consumption especially under conditions of low O<sub>2</sub> availability (Bailey et al., 1990) by facilitating the diffusion of O<sub>2</sub> from the extracellular space to the mitochondria (Wittenberg and Wittenberg, 2003). Myoglobin consists of a single polypeptide protein, globin, and a prosthetic group, haem. The haem has a centrally located iron atom with six coordination sites. Four coordination sites are in the plane of, and bound to, the N atoms of four flat porphyrin rings, while the other two coordination sites lie perpendicular to this structure. One of the perpendicular coordination sites is connected to an N atom of the globin protein molecule and the other site is open and available for binding a variety of ligands (Cornforth and Jayasingh, 2004). Each molecule of Mb contains a single iron atom, which may bind to one molecule of O<sub>2</sub> when the iron is in the ferrous state (Mb-Fe<sup>2+</sup>). Mb-Fe<sup>2+</sup> undergoes spontaneous oxidation *in vitro* to the ferric state (Mb-Fe<sup>3+</sup>) known as MetMb, which cannot bind O<sub>2</sub>. The type of molecule attached to the sixth bond and the oxidation state of the iron atom (ferrous or ferric) determines the colour of the



**Figure 1** A representation of oxidative change in plant (A) and meat (B) samples.

meat. Myoglobin is commonly found in three forms; oxymyoglobin (OxyMb), deoxymyoglobin and MetMb. The relative proportions of the various forms of Mb determine the colour of fresh meat. When the animal is alive, the majority of Mb is in the oxygenated form (the sixth coordination site is occupied with  $O_2$  molecule).

Freshly cut meat is purple in colour due to the presence of myoglobin in its native form (deoxymyoglobin). Upon exposure to air, myoglobin is oxygenated to the bright red oxymyoglobin. During storage, myoglobin (Mb), and oxymyoglobin (OxyMb) are oxidised to the brown metmyoglobin (MetMb). The accumulation of MetMb at the meat surface is the major factor leading to the discolouration of fresh meat. Apart from the colour change with oxygenation and oxidation, changes in the haem moiety of Mb of meat can give a variety of discolouration as shown in [Table 1](#). Alterations of the haem structure can cause the greening

**Table 1** Meat discolouration as a result of changes in haem iron state and Mb derivatives

Pigment	Colour	State of iron		Remarks
Myoglobin	Purple	$Fe^{2+}$	$H_2O$	Native protein/reduced form
Oxymyoglobin	Bright Red	$Fe^{2+}$	$O_2$	Oxygenated form
Metmyoglobin	Brown	$Fe^{3+}$	$H_2O$	Oxidised form
Carboxymyoglobin	Bright Red	$Fe^{2+}$	CO	Treated with CO
Nitrosomyoglobin	Bright Red (pink)	$(Fe-NO)^{2+}$		Treated with NO or nitrite
Nitrosometmyoglobin	Green	$Fe^{3+}$	NO	Excess nitrite
Metmyoglobin nitrite	Reddish brown	$Fe^{3+}$	NO	Metmyoglobin with excess nitrite
Globin myohaemichromogen	Dark brown	$Fe^{3+}$	$H_2O$	Cooked meat
Nitric oxide myohaemochromogen	Bright red (pink)	$Fe^{2+}$	NO	Cooked cured
Choleglobin	Green	$Fe^{2+}$ or $Fe^{3+}$	$H_2O$	Interaction with $H_2O_2$
Sulfmyoglobin	Green	$Fe^{3+}$	HS	Interaction with $H_2S$
Oxysulfmyoglobin	Red	$Fe^{3+}$	O-HS	
Nitriheamin	Green	$Fe^{3+}$	NO	Excess nitrite

discolouration of raw meat. There are two derivatives of Mb that may be responsible for the green discolouration, choleglobin and sulfmyoglobin (Faustman and Cassens, 1990a); although other compounds that cause meat green colour were reported (Table 1; Lawrie, 1985). Choleglobin is formed by a reaction between Mb, OxyMb or MetMb with hydrogen peroxide which can be produced by bacteria or the interaction of ascorbic acid with the O<sub>2</sub> molecule of OxyMb or from endogenous muscle reactions (Faustman and Cassens, 1990b). Sulfmyoglobin is formed by hydrogen sulfide and O<sub>2</sub> reacting with reduced Mb (Lawrie, 1985). A relevant pigment in fresh meat is carboxymyoglobin that is formed by inclusion of carbon monoxide (CO) in meat package at level <0.4% to form a bright cherry-red colour that is more stable than oxymyoglobin. The affinity of CO for binding with deoxymyoglobin is stronger than O<sub>2</sub>, but both OxyMb and CarboxyMb are vulnerable to be oxidised via lipid oxidation reactions in the same way (Joseph et al., 2009). Premature browning during cooking is a safety problem with fresh meat treated with CO as consumers use browning as indication of cooking of meat and this can occur at low temperatures in meat treated with CO, giving rise to risk of exposure to viable microorganisms.

## Haemprotein Interactions

Myoglobin and haemoglobin in their native physiological state have the characteristic of being oxygenated (oxygenated form which is bright red in colour) and maintaining the iron atom in the ferrous state. They are capable of reversible oxygenation, where the OxyMb and oxyhaemoglobin (OxyHb) release the O<sub>2</sub> molecule and change to the deoxygenated form, which is purple in colour. At low O<sub>2</sub> partial pressure tension and during aging, Mb and OxyMb are oxidised to MetMb which is brown in colour. MetMb can be reduced enzymatically by MetMb reductase to Mb, subject to the availability of cofactors and substrates, which in turn can be oxygenated to OxyMb (Bekhit and Faustman, 2005; Bekhit et al., 2007). However, in harsh environments (high temperature and UV ray), the globin moiety is denatured which leaves the iron atom unprotected. This leads to a spontaneous oxidation of the iron atom to the ferric state and the occupation of the sixth coordination site with H<sub>2</sub>O.

The ligand attachment is influenced by accessibility to the iron ion and the protein shape, which is determined by its amino acid sequence. Amino acid substitution in Mb from different marine species has resulted in different myoglobin-oxygen affinities and disassociation rates (Carver et al., 1992; Marcinek et al., 2001; Stewart et al., 2004). Suman and Joseph (2013) found the molecular mass of myoglobin from several commercial red and white meat sources ranged between 16,824 and 17,380 Da, reflecting differences in Mb sequences. They also reported high Mb homology of 88.2–98.7 among red meat species (cattle, buffalo, sheep, goat and pig), and lower values (68.6–74.5) when comparing Mb from birds with red meat species.

## Myoglobin Content

The Mb content in meat varies with species, breed, individual animals, age, diet and muscle type. The highest Mb contents in commercial meats can be found horse meat and venison, but the exact values vary in literature depending on the study. Yasui (1956–1957) reported the average Mb contents in fresh pork, horse, sheep and cattle meats to be 1.9, 4.5, 2.1 and 3.3 mg/g fresh weight for *deltoideus*, 0.78, 4.4, 1.1, and 4.0 for *longissimus dorsi*, and 0.9, 3.3.2, 0.4 and 2.6 for *semitendinosus*, respectively. They found very high variation in Mb content among animals from the same species. McKenna et al. (2005) found Mb content in 19 beef muscles varies from 3.6 to 5.62 mg/g fresh weight. Wide range of Mb contents has been reported for different pork cuts that is likely to be due to breed and diet. For example, Topel et al. (1966) reported a range of 2.9–6.4 mg/g in various pig muscles, whereas Kim et al. (2010) found a range of 1.2–2.1 mg/g.

Among game meats, Zebra meat was reported to contain the highest Mb content (7.2 mg/g) (Onyango et al., 1998).

## Colour Models

A colour model is a three dimensional orthogonal coordinate representation of colour. The purpose of a colour model is to allow convenient specification of colours. Several different colour models have been proposed to model the characteristics of a colour. All the colour models use three components to describe a colour. The use of one colour model over another depends on the nature of the problem.

Some of the models used to describe colour are; RGB model (red, green, blue), CMY (cyan, magenta, yellow), YIQ (luminance, inphase, quadrature) and intensity-chromaticity colour spaces (HSB, hue, saturation, brightness; HSI, hue, saturation, intensity; HSV, hue, saturation, value; HSL, hue, saturation, lightness; HVC, hue, value, chroma). The later model colour space represents colour as human tends to perceive and are widely used in food literature. Thus colour measurements utilizing only these models will be reported in this section. The major systems depicting colour are the CIE system, Munsell system and Hunter Lab system.

## Colour Measurement Systems

Colour is an appearance property attributable to the spectral distribution of light. The colour that we perceive is the result of three components; a source of illumination, an object to interact with the light which comes from this source and a human eye to observe the resulted effects. Thus understanding colour requires a consideration of all three components, which brings together concepts arising from three natural sciences disciplines, chemistry, physics and biology. In addition, the determination of colour as seen by the human eye depends on a physical stimulus in the eye followed by an interpretation by the brain. Thus the phenomenon of colour is said to be psychophysical (Kramer and Twigg, 1970).

The colour of muscle foods is critically appraised by consumers and often is their basis for product selection or rejection (Hunt et al., 1994). Colour measurements, whether for fundamental research or product development, description or specification, must be done as carefully as other analytical methods. The colour of fresh meat is most commonly evaluated by visual assessment or direct measurement using a colorimeter or reflectance spectrophotometer.

## Visual Colour Evaluation

While subjective (visual) assessment of meat colour reflect the actual perception of colour by panellists, which will vary within the population, it can be an inaccurate and imprecise method since a number of variables are associated with colour detection (human judgments may not be repeatable from day to day and are influenced by personal preference, lighting, visual deficiencies of the eye, the psychological status and appearance factors other than colour). Various scoring scales have been utilized for panel evaluations (these have been reported in detail in AMSA (2012)). There are two potential types of panels used in subjective colour evaluation, trained or consumer panels. Sensory studies recommend 8–12 for trained panel and 120 panellists for the consumer panel. The information received from these panels is different. Trained panels are used to evaluate colour attributes and colour stability with high precision and minimum biases, whereas consumers' panels are used to evaluate acceptance of and satisfaction with meat samples and possibly willingness to pay. The scales are mainly descriptive and imply averaging the colour over the entire meat surface area or worst-point score for a single or cumulative discoloured area. This is an important structure for evaluation of meat colour since the discolouration process during storage is not uniform and irregular discolouration patches can form sporadically on the surface of meat leading to two-tone colour. This characteristic change of meat can have drastic effect on meat quality as the tolerance of discolouration can vary greatly among consumers depending on the individuals familiarity and knowledge of meat (farmers prefer discoloured meat as they know it has been aged longer and likely to be more tender than a fresh meat cut) as well as the consumer nationality (Japanese consumers are very interested in bright red colour than Egyptian for example).

Another visual way of colour evaluation is the use of colour cards or colour standards (such as the Japanese colour standards).

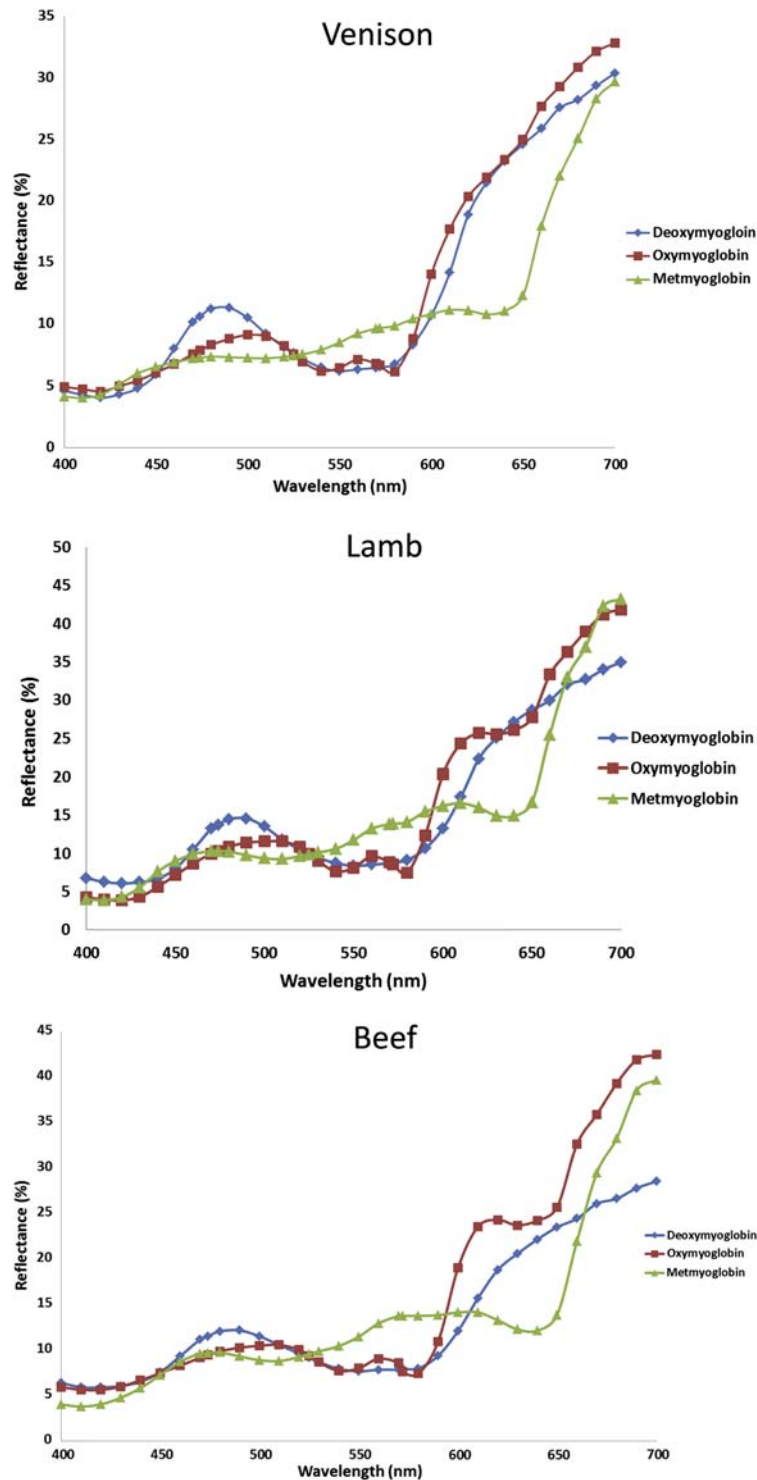
## Instrumental Colour Evaluation

The measurement of colour using instruments that have been standardised for the light source and the detection of colour differences, results in a more accurate evaluation of meat colour. The most widely used instruments for colour measurements are colorimeters and spectrophotometers.

Colorimeters determine the proportions of the primary additive lights that match the colour reflected or transmitted by the sample. Tristimulus colorimeters employ filters to stimulate the response to the human eye. White light from a standard CIE source is shone on the sample. Light reflected at 45° is measured by a photocell after it passes through an X, Y or Z filter.

Spectrophotometers differ from the measurements determined by colorimeters. Reflectance spectrophotometers measure the amount of light reflected by a surface as a function of wavelength to produce a reflectance spectrum (Fig. 2). The reflectance spectrum of a sample can be used, in conjunction with the CIE standard observer function and the relative spectral energy distribution of an illuminant, to calculate the CIE XYZ tristimulus values for that sample under that illuminant. A spectrophotometer illuminates the sample with white light and then calculates the amount of light that is reflected by the sample at each wavelength interval. Typically data are measured for 31 wavelength intervals starting at 400, 410, 420, ..., 700 nm. This is achieved by passing the reflected light through a monochromating device that splits the light into separate wavelength intervals. The instrument is calibrated using white and black tiles whose reflectance at each wavelength is known compared to a perfect diffuse reflecting surface. The reflectance of a sample is either expressed between 0 and 1 (as a fraction) or between 0 and 100 (as a percentage). The reflectance values obtained are relative values and, therefore independent of the quality and quantity of the light used to illuminate the sample. Slight differences in the reflectance of meat from different species (Fig. 2) can exist, but the impact of these differences on estimation of Mb derivatives is not established.

Both systems produce data in the form  $L^*a^*b^*$ , where  $L^*$  indicates lightness and it has a scale of 0–100, 100 = white, 0 = black, and lighter colours will have higher  $L^*$  value,  $a^*$  measures redness when value is positive and indicate green colour if the value is negative, and  $b^*$  measures yellowness  $b^*$  values is positive and indicate blue colour when the value is negative. Using  $a^*$  and  $b^*$  values, the actual colour (Hue angle) can be calculated using the equation  $\text{Hue angle} = \tan^{-1} b^*/a^*$ , where the shifting of the angle



**Figure 2** Reflectance spectra for 100% myoglobin, 100% oxymyoglobin and 100% metmyoglobin in venison, lamb and beef.

from the red colour region to higher angles indicate browning and discolouration. The Chroma “Saturation index” can be calculated using the following equation  $C = (a^2 + b^2)^{1/2}$  and larger C values means the colour is more intense.

Spectrophotometers provide a rapid, non-invasive method of determining the percentage of Mb, OxyMb and MetMb on the surface of the meat. Several equations have been derived based on the reflectance (%) values transformation into K/S values using Kubelka–Munk equation  $[(1-R)^2/2R]$  to take into consideration the variation due to light scatter. To determine the three forms of Mb, 3 references for 100% Mb, OxyMb and MetMb are generated either chemically or physiologically as described in [AMSA \(2012\)](#) and the following equations are used;



$$\% \text{ OxyMb} = \frac{\frac{\frac{K}{S} 610}{\frac{K}{S} 525} \text{ for 100\% MetMb} - \frac{\frac{K}{S} 610}{\frac{K}{S} 525} \text{ for sample}}{\frac{\frac{K}{S} 610}{\frac{K}{S} 525} \text{ for 100\% MetMb} - \frac{\frac{K}{S} 610}{\frac{K}{S} 525} \text{ for 100\% OxyMb}} \times 100$$

$$\% \text{ MetMb} = \frac{\frac{\frac{K}{S} 572}{\frac{K}{S} 525} \text{ for 100\% Mb} - \frac{\frac{K}{S} 572}{\frac{K}{S} 525} \text{ for sample}}{\frac{\frac{K}{S} 572}{\frac{K}{S} 525} \text{ for 100\% Mb} - \frac{\frac{K}{S} 572}{\frac{K}{S} 525} \text{ for 100\% MetMb}} \times 100$$

$$\% \text{ Mb} = \frac{\frac{\frac{K}{S} 474}{\frac{K}{S} 525} \text{ for 100\% OxyMb} - \frac{\frac{K}{S} 474}{\frac{K}{S} 525} \text{ for sample}}{\frac{\frac{K}{S} 474}{\frac{K}{S} 525} \text{ for 100\% OxyMb} - \frac{\frac{K}{S} 474}{\frac{K}{S} 525} \text{ for 100\% Mb}} \times 100$$

## Rapid Systems for Online Colour Measurements

New technologies are promising the ability to measure meat colour during meat processing with the possibility of feedback to producers. Measuring  $L^*a^*b^*$  values from RGB digital images (León et al., 2006) with high accuracy provides the potential of online measurement for rapid evaluation of meat colour as a tool for quality control. Similarly the use of NIR to measure colour and other quality attributes and composition (Mitsumoto et al., 1991; Hildrum et al., 1994; Forrest et al., 2000; Geesink et al., 2003) provides a great opportunity for a comprehensive and non-invasive evaluation of meat. Video image scanning and analysis (VISA) systems (Stanford et al., 1998; Hopkins et al., 2004) can also provide opportunities for evaluation of yield, colour and other important quality attributes that enable fast grading and evaluation of overall quality of meat.

## Conclusions

The colour of meat is very important as it can directly affect the marketing of meat. While consumers use colour as indicator of freshness and discoloured meat is likely to be discriminated against, this is more likely to be based on safety concerns rather than impact on nutrition and digestive consequences of consuming oxidised material that can propagate oxidative processes during digestion. Myoglobin, OxyM and MetMb are the main pigments controlling fresh meat. Several methods are available for the evaluation of meat colour. These methods have advantages and disadvantages in terms of ease to use in different locations (e.g. processing plants and research labs), research questions (subjective or objectives methods) and how fast the answer is required versus cost. Colour measurements can be a useful tool to improve breeding and on farm management system to address any low shelf life fresh meat products.

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# Homeostasis of Plasmalogens in Mammals

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## Glossary

**Alkylglycerol** ether lipids have an alkyl chain linked to the *sn*-1 position of the glycerol backbone by an ether bond.

**Peroxisome** a single-membrane-bounded organelle that contains hundred enzymes involved in essential lipid metabolisms including plasmalogen synthesis.

**Peroxisome biogenesis disorders** fatal human recessive disorders caused by defects of matrix protein import and/or impairment of membrane assembly.

**PEX** gene encoding a protein essential for biogenesis of peroxisome.

**Plasmalogen** ethanolamine-containing alkenyl ether glycerophospholipid.

**RCDP** a rare genetic disease in children who manifest impairment in the synthesis of plasmalogens due to the dysfunction or defect in peroxisomal localization of enzymes responsible for plasmalogen synthesis.

## Nomenclature

**ADAPS** alkyl-dihydroxyacetonephosphate synthase

**ADHAPR** acyl/alkyl-DHAP reductase

**AG** alkylglycerol

**CHO** Chinese hamster ovary

**DHAPAT** dihydroxyacetonephosphate acyltransferase

**ER** endoplasmic reticulum

**Far1** fatty acyl-CoA reductase 1

**PBDs** peroxisome biogenesis disorders

**PE** phosphatidylethanolamine

**PlsCho** choline-plasmalogen

**POPC** palmitoyl-oleyl-phosphatidylcholine

**RBC** red blood cell

**RCDP** rhizomelic chondrodysplasia punctata

**ROS** reactive oxygen species

## Introduction

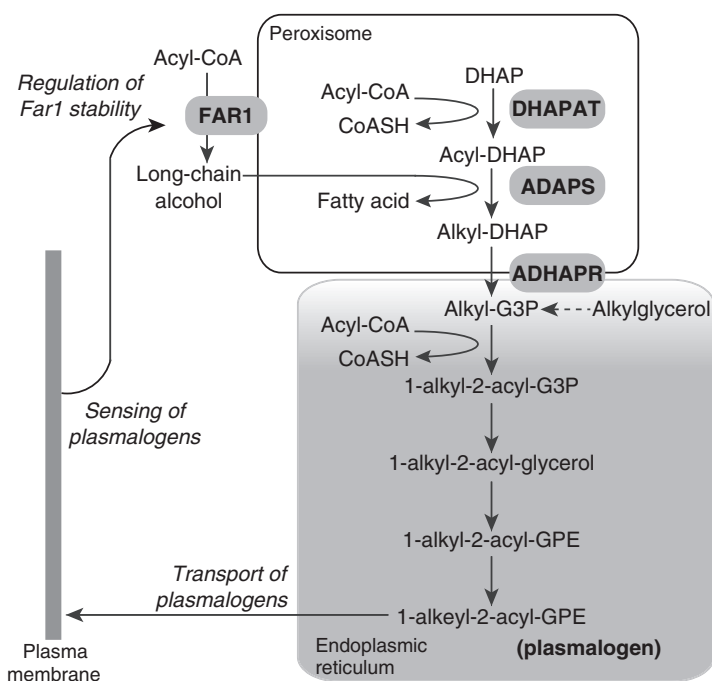
Plasmalogens belong to the class of ether glycerophospholipids characterized by the presence of vinyl-ether bond at the *sn*-1 position of the glycerol backbone. Ethanolamine plasmalogens (hereafter referred as plasmalogens) are major constituents of biological membranes where plasmalogens constitute approximately 5%–20% of the phospholipids in most mammalian cell membranes (Braverman and Moser, 2012), implying that plasmalogens are major phospholipid component of cellular membranes. The presence of vinyl-ether bond makes plasmalogens susceptible to reactive oxygen species (ROS) in cultured cell (Zoeller et al., 1999). However, recent studies reconsider the protective function of plasmalogens as a scavenger of ROS because the increased oxidative stress is not detectable in plasmalogen-deficient mouse and *Caenorhabditis elegans* (Brodde et al., 2012; Drechsler et al., 2016).

By taking into account the relatively higher amount of plasmalogens in the cell membranes, plasmalogens are also thought to potentially effect on the biophysical properties of cell membranes including thickness, rigidity, lateral phase separation, and fusion (Rog and Koivuniemi, 2016; Koivuniemi, 2017). A study on molecular dynamics in simulations of lipid membranes composed of plasmalogens and palmitoyl-oleyl-phosphatidylcholine (POPC) bilayers shows that plasmalogens form more compressed, thicker, and rigid lipid bilayers as compared with bilayers composed of phosphatidylethanolamine (PE) and POPC, which is likely due to a highly ordered state of *sn*-1 chain and a closer packing of the *sn*-1 and *sn*-2 chains in plasmalogens (Rog and Koivuniemi, 2016). However, how plasmalogens effect the biophysical properties of cell membranes containing different lipid species remains unknown. Plasmalogens are enriched in lipid rafts (Pike et al., 2002; Honsho et al., 2008), a cholesterol- and sphingomyelin-enriched domain of the plasma membrane, and myelin, a specialized membrane to insulate axon, in both of which plasmalogens

are mostly located in the inner leaflet (Fellmann et al., 1993; Kirschner and Ganzer, 1982; Honsho et al., 2017). In addition, it has been shown that plasmalogens are abundant in vesicles containing raft-like lipid composition, such as extracellular vesicles (Simbari et al., 2016), and envelope of viruses including HIV and influenza virus (Brugger et al., 2006; Gerl et al., 2012). Based on these observations together with the finding that fluorescence anisotropy showing a lower-order parameters in fibroblasts from patients defective in plasmalogen synthesis such as those with rhizomelic chondrodysplasia punctata (RCDP) and peroxisome biogenesis disorders (PBDs), as compared to those in a healthy control (Hermetter et al., 1989), it seems plausible that vinyl-ether linkage of plasmalogen increases the rigidity of cellular membranes. Moreover, the complete lack of plasmalogens has been reported in several organs of patients defective in peroxisome biogenesis (Heymans et al., 1983) and decline of plasmalogen content in several neurological disorders including Alzheimer's disease, Niemann-Pick type C disease (Ginsberg et al., 1995; Schedin et al., 1997), autism (Yu et al., 2013), and Down syndrome (Murphy et al., 2000), and even during normal aging (Farooqui and Horrocks, 2001; Nagan and Zoeller, 2001; Maeba et al., 2007). However, precise mechanisms concerning how dysregulation of plasmalogen homeostasis impairs cellular functions remain unknown. The finding of the feedback regulation of plasmalogen biosynthesis (Honsho et al., 2010, 2013) infers that regulation of plasmalogen synthesis is important for the accomplishment of the functions of plasmalogens in cellular membranes.

### Physiological Consequence of Plasmalogen Biosynthesis in the Homeostasis of Plasmalogens

The level of plasmalogens in mutant cells defective in plasmalogen synthesis is lower than 10% of that in wild-type cells (Honsho et al., 2008; De Vet et al., 1999; Nagan et al., 1997, 1998). In addition, such a striking reduction of plasmalogen level is also found in several organs including brain, heart, and kidney from patients with PBDs (Heymans et al., 1983). Similarly, severe decline of plasmalogens is reported in several tissues of mouse defective in *PEX7* (Brites et al., 2011), an essential cytosolic receptor for the peroxisomal import of the peroxisome targeting signal type 2-containing matrix proteins such as alkyl-dihydroxyacetonephosphate synthase (ADAPS) (Fig. 1) (Braverman et al., 1997; Motley et al., 1997; Purdue et al., 1997). Together, these studies strongly suggest



**Fig. 1** Biosynthetic pathway of plasmalogens and its regulation. Plasmalogen synthesis is initiated in peroxisomes, in which dihydroxyacetonephosphate acyltransferase (DHAPAT) and alkyl-DHAP synthase (ADAPS) sequentially catalyze the generation of alkyl-DHAP by replacing the acyl chain of acyl-DHAP with a long chain alcohol. Fatty acyl-CoA reductase1 (Far1), a peroxisomal C-tail anchored protein, is the enzyme responsible for the synthesis of long chain alcohols. Alkyl/acyl-DHAP reductase (ADHAPR) reduces alkyl-DHAP to yield 1-alkyl-*sn*-glycero-3-phosphate (G3P), followed by synthesizing 1-alkenyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (GPE), plasmalogen, via the remaining four steps in the endoplasmic reticulum. Plasmalogens are transported to the post-Golgi compartments including endosomes and plasma membranes in a manner dependent on ATP, but not vesicular transport (Honsho et al., 2008), followed by localizing in the inner leaflet of plasma membrane. Plasmalogens can be synthesized from alkylglycerol through its phosphorylation (Watschinger and Werner, 2013) by bypassing the first three steps of plasmalogen synthesis in peroxisome (dashed arrow). The biosynthesis of plasmalogen is regulated in a manner dependent on total cellular level of plasmalogens through a mechanism comprising three steps: sensing plasmalogens in the inner leaflet of plasma membrane, transferring the signal indicating the cellular level of plasmalogens, and modulating the Far1 level on peroxisome.

that *de novo* synthesis of plasmalogens plays a pivotal role in the homeostasis of plasmalogens in cells and tissues. Nevertheless, feeding alkylglycerol (AG), a precursor of plasmalogens, and administration of plasmalogens would be attractive approaches to elevating plasmalogens in animals.

### Potential Approaches to the Elevation of Plasmalogens by Feeding Alkylglycerol or Administration of Plasmalogens

AG restores the impaired synthesis of plasmalogen in plasmalogen-deficient cells by bypassing the first three steps of plasmalogen synthesis (Fig. 1) (Honsho et al., 2008; Nagan and Zoeller, 2001; Nagan et al., 1997; Zoeller et al., 1988). Administration of AG, such as 1-*O*-octadecyl-*sn*-glycerol, to *PEX7*-knockout mouse elevates the level of plasmalogen containing C18:0 fatty alcohol in peripheral tissues to the levels of AG-fed wild-type mice (Brites et al., 2011). Moreover, restoration of plasmalogen levels significantly slows down the progression of the pathology in several organs and improves nerve conduction in peripheral nerves (Brites et al., 2011). Similarly, feeding 1-*O*-heptadecyl-*sn*-glycerol, an uncommon ether lipid, to young rats shows its incorporation to a high extent into plasmalogens of several tissues (Brites et al., 2011; Das et al., 1992; Das and Hajra, 1988). However, total level of plasmalogens in the peripheral tissues in rats fed with 1-*O*-heptadecyl-*sn*-glycerol is not elevated, perhaps due to the concurrent reduction of endogenous plasmalogens harboring C16:0, C18:0, and C18:1 fatty alcohols (Brites et al., 2011; Das et al., 1992). Therefore, plasmalogens in peripheral tissues can be synthesized from AG, giving rise to an elevation of the level of plasmalogens derived from the fed AG by reducing the level of endogenously synthesized plasmalogens. Such alternation of plasmalogen species in organs may contribute potential hazardous effects on metabolism (Zhang et al., 2013).

Plasmalogens are not found in plants (Felde and Spiteller, 1994), whereas considerable amount of plasmalogens are present in mammalian meat, fish, and marine invertebrates (Blank et al., 1992; Yamashita et al., 2016). Interestingly, similar total plasmalogen levels are found in red blood cell (RBC) from humans on western diets or vegan diets (Moser et al., 2011). Therefore, it is tempting to speculate that dietary plasmalogens less contribute the level of plasmalogens in RBC, although oral administration of plasmalogens through foods containing high concentration of plasmalogens increased plasmalogen levels in rat RBC (Mawatari et al., 2012; Yamashita et al., 2017).

Plasmalogen level in brain is severely reduced in *PEX7*-knockout mouse (Brites et al., 2003, 2011) and mice each with brain specific deficiency of *PEX5* (Krysko et al., 2007) and *PEX13* (Müller et al., 2011), essential genes for the biogenesis of peroxisome (Fujiki et al., 2014). Moreover, plasmalogen level in brain of *PEX7*-knockout mouse is not increased by feeding AG despite the elevation of plasmalogen in peripheral tissues (Brites et al., 2011). These studies suggest that plasmalogens required for the function of brain are more likely supplied by locally synthesized plasmalogens in brain rather than the transport of peripherally synthesized plasmalogens by crossing blood brain barrier. Therefore, it is crucial to understand the mechanism underlying the regulation of plasmalogen synthesis, a system highly contributing to the homeostatic regulation of quality and quantity of plasmalogens in brain as well as peripheral tissues.

In the following sections, we will summarize the pathway of plasmalogen synthesis and the mechanism addressing how synthesis of plasmalogens is regulated.

### Biosynthesis of Plasmalogens in Mammalian Cells

Biosynthesis of plasmalogen is initiated in peroxisomes (Fig. 1). The initial enzyme, dihydroxyacetonephosphate acyltransferase (DHAPAT) catalyzes the generation of acyl-DHAP. At the second step, ADAPS replaces the acyl chain of acyl-DHAP with a long chain fatty alcohol. This fatty alcohol is synthesized by fatty acyl-CoA reductase 1 (Far1), the enzyme responsible for the reduction of fatty acyl-CoA to generate C16:0, C18:0, and C18:1 fatty alcohols (Honsho et al., 2010, 2013; Cheng and Russell, 2004). Alkyl-DHAP is further reduced by acyl/alkyl-DHAP reductase (ADHAPR) and plasmalogen is synthesized via the remaining four steps in the endoplasmic reticulum (ER). The biosynthetic pathway of plasmalogen was comprehensively reviewed by several groups of investigators (Braverman and Moser, 2012; Nagan and Zoeller, 2001; Wanders and Waterham, 2006; Malheiro et al., 2015).

### Regulation of Plasmalogen Synthesis in Cells

The elevated activity of Far1, not DHAPAT, ADAPS, and ADHAPR in plasmalogen-deficient cells (De Vet et al., 1999; Nagan et al., 1998; Rizzo et al., 1993; James et al., 1990; Thai et al., 2001) suggests that Far1 is a potential rate-limiting enzyme in plasmalogen synthesis. Indeed, the elevated activity of Far1 in plasmalogen-deficient cells is reduced to normal by restoring cellular level of plasmalogens, concomitant with the reduced level of Far1 without lowering the transcription of *FAR1* (Honsho et al., 2010). In addition, elevation of plasmalogen levels reduces the expression of Far1 in several cultured cell lines (Honsho et al., 2010, 2013). Collectively, these results strongly suggest that synthesis of plasmalogen is regulated by modulating the Far1 level in a manner dependent on total cellular level of plasmalogens (Honsho et al., 2010, 2013), not hexadecanol, the product of Far1

(Bishop and Hajra, 1981). Molecular mechanisms underlying plasmalogen-mediated degradation of Far1 on peroxisomes remain unknown. Far1 is degraded upon restoring of plasmalogens even in *PEX1*-deficient Chinese hamster ovary (CHO) cells defective in the transport of peroxisomal matrix proteins (Honsho et al., 2010), suggesting that peroxisomal matrix proteinases such as trypsin domain-containing 1 (Tysnd1) (Kurochkin et al., 2007; Okumoto et al., 2011) and peroxisomal Lon protease (PsLon) (Okumoto et al., 2011) are less likely involved in the plasmalogen-mediated degradation of Far1. It is tempting to speculate that peroxisomal membrane proteins such as ATPase family AAA domain-containing protein 1 (ATAD1) localized in mitochondria and peroxisomes (Chen et al., 2014; Liu et al., 2016), might be involved in the degradation of Far1.

Plasmalogen sensing is also a crucial step for the regulation of plasmalogen synthesis. Plasmalogen sensing in peroxisomes is less likely because plasmalogens are under the detectable level in peroxisomes (Honsho et al., 2008; Yang et al., 2003; Kuerschner et al., 2005). Recent studies by monitoring the protein level of Far1 suggest that plasmalogens localized in the inner leaflet of plasma membranes are most likely sensed (Honsho et al., 2017). In the plasma membranes, plasmalogens are mostly localized in the inner leaflet (Fellmann et al., 1993; Kirschner and Ganter, 1982; Honsho et al., 2017). This asymmetric distribution of plasmalogens is mediated by flippase belonging to the P4 subfamily of P-type ATPases (P4-ATPases). Eliminating the expression of *CDC50A* encoding a  $\beta$ -subunit of P4-ATPases essential for exiting of most of P4-ATPases from the ER by forming a hetero-oligomer (Lopez-Marques et al., 2014; Van der Velden et al., 2010; Bryde et al., 2010) dislocates plasmalogens to the outer leaflet of plasma membranes (Honsho et al., 2017) and stabilizes Far1 without altering total cellular level of plasmalogens (Honsho et al., 2017). Similarly, Far1 is stabilized in the presence of nystatin (Honsho et al., 2017) by inducing distortion of the structure and function of cholesterol-rich membrane domain thorough sequestering cholesterol (Ros-Baró et al., 2001). In contrast, degradation of Far1 is accelerated by inhibiting endocytosis with two distinct inhibitors, dynasore and myristyl trimethyl ammonium bromide (MitMAB) of dynamin, a GTPase required for membrane scission during endocytosis, and by knockdown of flotillin1, a protein enriched in lipid rafts of the plasma membrane (Glebov et al., 2006; Bickel et al., 1997) without changing the total cellular level of plasmalogens (Honsho et al., 2017). Importantly, the amount of plasmalogens in the Triton X-100-insoluble fraction obtained from flotillin1-knocked down HeLa cells is augmented, perhaps due to the inhibition of the endocytosis of plasmalogens. Collectively, these studies suggest that plasmalogens enriched in the plasma membrane is most likely sensed, followed by accelerating degradation of Far1. Therefore, biosynthesis of plasmalogen is spatiotemporally regulated by sensing plasmalogens in the inner leaflet of plasma membranes, followed by transferring a signal emerged from the sensing of plasmalogen level to peroxisomes and modulating the Far1 level (Fig. 1).

## Regulation of Plasmalogen Synthesis in Organs

Regulation of plasmalogen synthesis in tissues has not been addressed. The finding that total plasmalogen level in rat remains unchanged even by reducing the level of endogenous plasmalogens harboring C16:0, C18:0, and C18:1 fatty alcohols after administration of 1-*O*-heptadecyl-*sn*-glycerol suggests that synthesis of plasmalogens in peripheral tissues is likely downregulated by sensing the elevated level of plasmalogens containing C17:0 fatty alcohol (Das et al., 1992), perhaps via the reduction of Far1 protein. Conversely, Far1 protein level is increased in kidney derived from plasmalogen-deficient mouse as compared to that in wild-type mouse (Wiese et al., 2012), suggesting that plasmalogen synthesis is regulated by modulating the Far1 level in kidney as well, similar to the mechanism found in cultured cells (Honsho et al., 2010).

In humans and mouse, the isozymes, Far1 and Far2, sharing 59% identity and 78% similarity were differentially expressed. Far1 shows a broad distribution in several organs and acts on fatty acids that vary in their size and saturation, whereas Far2 is highly expressed in eyelid of mouse and has narrow substrate specificity (Cheng and Russell, 2004). Unlike Far1, Far2 is not degraded upon restoring the plasmalogen level in plasmalogen-deficient CHO mutant and MCF7 cells (Honsho et al., 2013). Moreover, the severely reduced level of plasmalogens in red blood cells from *FAR1*-defective patients further supports that fatty alcohols required for the synthesis of plasmalogens are mainly provided by Far1 (Buchert et al., 2014). Collectively, Far2 seems to possess more specialized function rather than the reduction of acyl-CoA for the synthesis of plasmalogens.

Choline-plasmalogen (PlsCho), another type of plasmalogen, is highly abundant in heart and muscle where approximately 50% of plasmalogens are PlsCho (Braverman and Moser, 2012). PlsCho is thought to be synthesized from hydrolysis of plasmalogens and a following modification of the resultant alkenylglycerol with phosphocholine catalyzed by choline phosphotransferase (Braverman and Moser, 2012; Nagan and Zoeller, 2001; Brites et al., 2004; Lee, 1998). Regulatory mechanism of PlsCho synthesis remains unknown. Feeding 1-*O*-octadecyl-*sn*-glycerol to wild-type mouse increases the total plasmalogen level in heart with a reduced level of plasmalogens containing C16:0 fatty alcohol. However, reduction of PlsCho containing C16:0 fatty alcohol is not evident despite of the elevation of PlsCho containing C18:0 fatty alcohol (Rasmiena et al., 2015). Given these data, synthesis of plasmalogens and PlsCho is most likely regulated by a mutually distinct mechanism in heart.

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## Future Reading

- Dorninger, F., Fross-Petter, S., Berger, J., 2017. From peroxisome disorders to common neurodegenerative diseases - the role of ether phospholipids in the nervous system. *FEBS Lett.* 591, 2761–2788.

## Relevant Website

The LipidWeb <http://www.lipidhome.co.uk/lipids/complex/ethers/index.htm>.

# Biochemical Reactions During Fresh Meat Storage

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## Glossary

**Calpain** calcium-dependent protease involved in proteolytic degradation of myofibrillar proteins during meat maturation.

**Free radical** molecule which possesses an unpaired electron and is extremely reactive, being capable of attack other molecules thereby generating many more free radicals.

**Phenolic compound** compound with a structure containing an aromatic ring bearing one or more hydroxyl groups.

**Superchilling** refrigeration at temperatures comprised between freezing and chilling, usually between  $-0.5$  and  $-2.8$  °C.

**Vacuum skin packaging** vacuum package that achieves a tight disposition of the film on meat surface, reducing the formation of pockets and consequently, decreasing residual  $O_2$ .

## Nomenclature

ROS Reactive oxygen species

PUFA Polyunsaturated fatty acid

SFA Saturated fatty acid

MAP Modified atmosphere packaging

VP Vacuum packaging

VSP Vacuum skin packaging

AP Active packaging

GRAS Generally Recognised As Safe

TBARS Thiobarbituric acid reactive substances (mg malondialdehyde/kg of meat)

## Introduction

Meat consists entirely of skeletal muscle of animals. Animal death occurs at slaughter; nevertheless, muscles remain alive. All muscle cell constituents and structures, basically proteins, lipids and enzymes, are still functional, so that biochemical reactions may further proceed. Those reactions are no more subjected to hormonal or nervous regulation nor to any extracellular supply, thus they are regulated solely by intracellular factors such as substrate concentration. After rigor mortis onset, due to ATP depletion, carbohydrate metabolism and lactic acid formation, the environment becomes acid, reaching a pH within the range 5.5–5.8. From this time onwards, two type of reactions may chiefly occur: enzymatic degradation of proteins and oxidative reactions focusing on lipids. It must not be overlooked that, besides these, other degradative reactions linked to microbial growth may also occur, mainly at late stages of meat storage but depending on environmental conditions.

## Proteolytic Changes

Protein myofibrillar structures are known to be responsible for muscle function, i.e. contraction and relaxation. Myofibrils no longer work after rigor mortis onset, since they need ATP to fueling the process, though they still render a stable complex structure which results in a tough fibrous texture as meat is chewed. Consumers consider toughness the most negative sensory property of meat, so it is highly negatively evaluated at this post mortem stage (Koochmaraie and Geesink, 2006; Huff-Lonergan et al., 2010).

A number of muscle proteases may be active under the conditions prevailing in meat early post mortem. As meat ageing proceeds they target the myofibrillar proteins leading to structure disruption and finally to meat tenderization. As a consequence, meat becomes progressively less chewy with ageing time, i.e. its sensory valuation steadily increases throughout storage (Koochmaraie and Geesink, 2006). Table 1 summarizes some comprehensive data on the proteases present in muscle tissue, including factors which affect their activity.

Conditions prevailing in meat must be considered in order to come to a conclusion on whether they may be active throughout meat storage. Regarding tissue location, only calpains and the proteasome are available for proteolytic action within the cytosol and the myofibrils (Goll et al., 2003; Houbak et al., 2008); cathepsins must be released from the lysosomes and caspases from apoptosomes, which appears to be unlikely, at least in early phases of meat storage (Sancho et al., 1997; Underwood et al.,

**Table 1** Proteases present in muscle tissue and factors which affect their activity

Protease	Location	Optimum pH	Regulating factors
Calpains $\mu$ -calpain $m$ -calpain calpain 3	Cytosol	7,0–7,5	Calcium ( $\mu\text{mol-mmol}$ ) Calpastatin (specific inhibitor) Phospholipids
Cathepsins	Lysosomes	4,0–6,0	Release from lysosomes Temperature Inhibitors
Proteasome	Cytosol	7,5–8,0	Ubiquitin ATP
Caspases	Apoptosomes	6,5–7,5	Apoptosis Cellular death

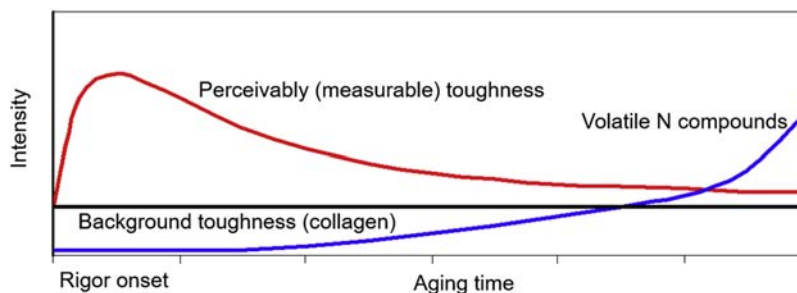
2008). Despite the optimum pH of the former seems to be far from that existing in meat after rigor mortis onset, they are known to show a significant activity at a pH of 5,5–6,0 (Ceña et al., 1992; Goll et al., 2003). Concerning the proteasome, a complex regulation which involves the ubiquitin system based on the energy provided by ATP is needed for activity (Lamare et al., 2002; Houbak et al., 2008). Therefore, only calpains appear to be actually available to afford to accomplish the proteolytic degradation of myofibrillar proteins (Roncalés et al., 1995; Koohmaraie and Geesink, 2006).

Calpains need calcium for its activation though very low concentrations ( $\mu\text{molar}$ ) of this cation are sufficient, which are indeed existing in meat at that level. Furthermore, the  $m$ -calpain and calpain-3 forms act as a reservoir since they may transform into  $\mu$ -calpain as this latter inactivates itself after exerting its proteolytic action (Goll et al., 2003; Huff-Lonergan et al., 2010). A further concern on calpain activity raised when the existence of its specific inhibitor calpastatin was reported; nevertheless, it has been demonstrated that calpain may overcome calpastatin inhibition under normal conditions of meat storage (Goll et al., 2003; Koohmaraie and Geesink, 2006). Besides this, the presence of phospholipids is known to enhance calpain activation. In summary, the cysteine-protease calpain is likely to be the sole responsible for myofibrillar proteins degradation throughout meat storage and therefore for meat tenderization, though at a low rate due to low pH and temperature (Ceña et al., 1992; Goll et al., 2003; Huff-Lonergan et al., 2010).

Not all the myofibrillar proteins are targeted by calpain, in fact only a few of them appear to be hydrolyzed by this endoprotease, mainly the giant protein titin, desmin, nebulin and  $\alpha$ -actinin (Koohmaraie and Geesink, 2006; Huff-Lonergan et al., 2010). These are essential components of some crucial myofibril structures, respectively, elastic filaments, transversal filaments, N filaments and Z lines. It is noteworthy to highlight that the most abundant myofibrillar proteins myosin and actin, essential components of thick and thin filaments, respectively, are not affected by calpain action (Sancho et al., 1997; Huff-Lonergan et al., 2010). Electrophoretic patterns of meat throughout storage closely fit with this outlook and demonstrate the unique involvement of calpain on meat aging (Roncalés et al., 1995; Koohmaraie and Geesink, 2006). It must be also emphasized that collagen, the main protein of the tough connective tissue of muscle, is neither hydrolyzed by calpain (Purslow, 2005).

Hydrolysis of those target proteins onto large polypeptides causes the cleavage of critical ultrastructural filaments and lines and this leads to myofibril disruption and progressive breakup. Ultimately, the result is that meat exerts a lower resistance to mastication and therefore becomes more tender (Goll et al., 2003; Koohmaraie and Geesink, 2006). However, as collagen is not degraded, tenderization occurs within the limits of the connective tissue contents and characteristics of any particular meat cut.

Fig. 1 depicts the changes occurring along meat aging related to meat texture. Perceived (measurable) toughness reaches a maximum at rigor mortis onset and decreases thereafter in a hyperbolic manner tending asymptotically to the lower limit, which is determined by the collagen content (Purslow, 2005). This process may go on for weeks depending on meat characteristics and environmental conditions. This is of course a schematic approach to how tenderisation proceeds though it reflects the actual behavior of meat texture, which steadily passes from tough to increasingly tender.


**Figure 1** Changes of texture and volatile nitrogen compounds occurring throughout meat aging.

According to this view meat might be aged as long as possible in order to reach the most tender texture. But a limit for aging arises from the increasing amount of volatile nitrogen molecules, which results in odor enhancement and finally in putrid smell. Those compounds are produced by microbial metabolism, so that their concentration depends on microbe counts. In fact, a significant upsurge of the nitrogen compounds concentration is closely related to the phase of microbial exponential growth (Casaburi et al., 2015). As a consequence, any factor delaying microbial growth, such as low temperature, low pH, oxygen absence or inhibitors presence, will result in an extended aging period.

## Oxidation During Meat Storage

Oxidation is a biochemical process involving a transfer of oxygen atoms, hydrogen atoms or electrons between two coupled compounds maintaining a net chemical charge. In the biological systems the donor is called pro-oxidant and the acceptor antioxidant. Antioxidant defences, which protect the body against oxidative damage *in vivo*, are rapidly depleted after slaughter, thus leaving meat exposed to oxidation during storage (Morrissey et al., 1998).

### Lipid Oxidation

Lipid oxidation consists of the oxidative degradation of lipids through complex linked reactions which result in a wide range of compounds that involve a lessening of nutritional and sensory value of meat and even may compromise its safety.

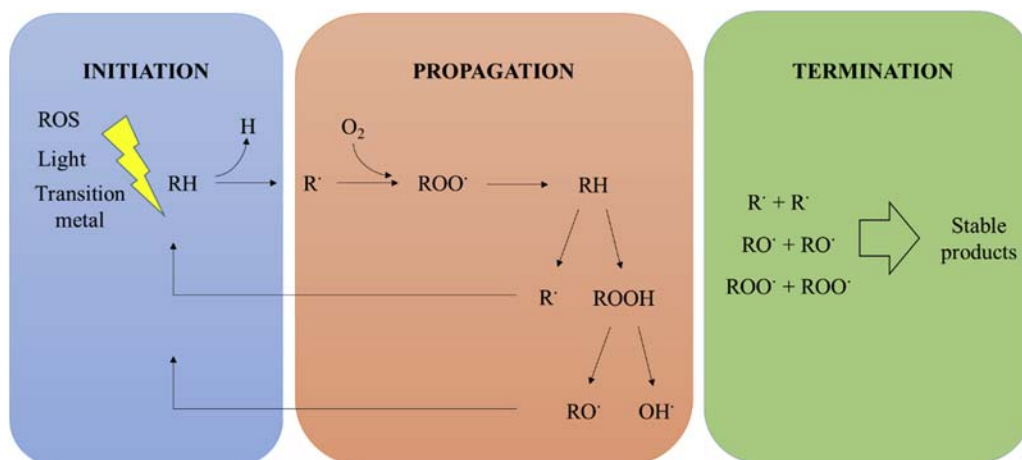
#### Mechanism of Action

Lipids can be oxidized by enzymatic or non-enzymatic reactions; however, autoxidation is the main mechanism of lipid oxidation in fresh meat. It starts at the phospholipid membrane of cell followed by a free-radical chain reaction commonly divided into three major steps: initiation, propagation and termination (Cheng, 2016). Fig. 2 shows the main reactions involved in lipid oxidation.

An initiator such as light, transition metals or oxygen reactive substances (ROS) is required to spring the process. Initiation starts by the attack of any reactive substance to abstract a highly reactive hydrogen atom from a fatty acid, thereby generating a lipid radical. The higher reactivity of hydrogen atoms of a methylene group bound to 2 carbon-carbon double bonds make them more prone to become oxidized (Blanksby and Ellison, 2003). The latter lipid radical reacts with oxygen to form a peroxy radical ( $\text{ROO}\cdot$ ), which in a subsequent reaction attacks a new unsaturated fatty acid and thus arising two different compounds: a new alkyl radical ( $\text{R}\cdot$ ) and a hydroperoxide ( $\text{ROOH}$ ). At this stage, a cascade of chain reactions spreads, in which each new molecule boosts the reaction speed (Min and Ahn, 2005). The reaction of a radical with a fatty acid substrate always generates another radical, thereby the oxidation process continues. The termination step takes place when two radicals are combined between them forming non-reactive compounds. Commonly, termination starts when there are enough radical species for favoring the contact of two radicals (Min and Ahn, 2005).

#### Effect of Lipid Oxidation on Meat Quality

Hydroperoxides are tasteless and odorless compounds with no impact on meat sensory attributes. Nonetheless, they are highly unstable and tend to disintegrate in hundreds of secondary lipid oxidation-by-products that concern severe changes in meat quality. In the presence of nonheme iron ( $\text{Fe}^{2+}$ ) there is a breakdown of hydroperoxides which generates hydroxyl ( $\cdot\text{OH}$ ) and alkoxy ( $\text{RO}\cdot$ ) radicals, followed by the scission of the fatty-acid chain next to the alkoxy radical. Thereby, a huge variety of volatile and non-volatile compounds are generated, which are responsible of oxidation-related modifications of meat sensory properties. The



**Figure 2** Overview of lipid autoxidation steps.

kind of compounds produced depends on both the alkyl chain of the hydroperoxide and the position where cleavage occurs (Papuc et al., 2017; Resconi et al., 2013).

Nutritionists recommend increasing the consumption of polyunsaturated fatty acids (PUFA) instead of saturated fatty acids (SFA). On the other hand, a high ratio of n-6:n-3 PUFA is also a risk factor in cancer and coronary heart disease (Enser et al., 2001). Unfortunately, both PUFA and especially n-3 PUFA are more prone to oxidation and tend to degrade during meat storage, hence decreasing meat nutritional value (Álvarez et al., 2009; Bellés et al., 2018). Furthermore, lipid-derived free radicals and hydroperoxides give rise to the conversion of amino acids into Strecker aldehydes,  $\alpha$ -keto acids, and amines, thus reducing their bioavailability (Hidalgo and Zamora, 2016). Otherwise, lipid autooxidation also exacerbates the depletion of valuable micronutrients such as vitamins A, C, E and flavonoids.

Lipids contribute both to desirable and undesirable meat flavors during storage. In fact, the characteristic flavor of the different meat species depends on its particular fatty acid profile. On the other hand, the oxidation of lipids during storage results in hundreds of volatile compounds directly responsible of the unpleasant odor and flavor of oxidized meat. Among all of them, aldehydes are generally believed to be the chief contributors to rancidity, since they are abundant in oxidized meat and possess low olfaction thresholds. Other oxidation-by-products like ketones, alcohols and furans may play a significant role in rancidity too (Resconi et al., 2013). The accumulation of unpleasant odors and flavors arisen from lipid autooxidation can overpower meat desirable flavors, thus resulting in a decrease of meat quality. Moreover, some studies suggested that secondary by-products of lipid peroxidation, which are bioavailable, would be likely to have cytotoxic and genotoxic properties and might be related to inflammatory diseases, cancer, atherosclerosis etc (Vieira et al., 2017).

## Protein Oxidation

Proteins become oxidized by different chemical pathways. ROS can attack directly multiple side-chain and backbone sites of a protein, thereby exerting modifications in its conformation structure. On the other hand, products of lipid oxidation, mainly secondary compounds, can interact with the amino acid residues of proteins modifying their structure and function. Furthermore, both lipid and protein oxidation can exacerbate each other (Zhang et al., 2013).

The oxidation of a protein may lead to increased side-chain hydrophilicity, side-chain and backbone fragmentation, aggregation and altered conformation. All these modifications would compromise its proper function thereby resulting in meat modifications such as reduced water holding capacity, discoloration, toughness and increased lipid oxidation (Zhang et al., 2013).

## Factors Affecting Meat Oxidation

### Meat Composition

Oxidative stability is a balance between meat antioxidants and pro-oxidants, the rate of oxidative reactions in meat matrix is actually highly related with the availability and abundance of several compounds as well as the interaction among them. The content of fat in muscle is critical to its stability against oxidation: the more fat, the faster development of oxidation through display (Estévez et al., 2005; Park et al., 2008). Unsaturated fatty acids are particularly prone to oxidation. A higher content of PUFA in muscle may favor the initiation step, thus increasing the susceptibility of meat to oxidize. In this regard, non-ruminant meat presents an intense lipid autooxidation because of its PUFA/SFA ratio. Similarly, several studies evidenced that meat enrichment with PUFA and particularly n-3 PUFA exacerbated lipid oxidation due to their greater tendency to be oxidized during storage (Wood et al., 1999; Díaz et al., 2011). Otherwise, red meat is rich in heme and nonheme iron which may catalyse heme protein-mediated lipid oxidation (Rhee and Ziprin, 1987).

Grass feeding favors the deposition of natural antioxidants in muscle thereby increasing the antioxidant defences of meat after slaughter. As a consequence, meat from grass fed animals shows more stability against oxidation than that providing from concentrate fed (Mercier et al., 2004). Differences among muscles are also related with the proportion of different muscle fibers. Muscles with greater relative proportions of type I muscle fibers have a higher content of myoglobin and oxygen consumption rate, hence enhancing myoglobin oxidation and consequently discoloration (Jeong et al., 2009).

### pH

It is generally accepted that low pH environments enhance both lipid and protein oxidation. At low pH, heme protein is exposed to external environments which compromise its stability. On the other hand, a lower pH environment exacerbates the protonation of bound oxygen and stimulates the formation of superoxide anion (Yin and Faustman, 1993).

### Atmosphere Composition

Oxygen plays a key role in promoting meat oxidation, increasing its rate with the concentration of oxygen in the surrounding atmosphere. In fact, oxidative processes usually involve reactive oxygen species. Carbon dioxide may act as pro-oxidant in concentrations above 30%, owing to the decrease of meat pH. Carbon monoxide (CO) is not likely to affect lipid oxidation but it has been very effective in preventing myoglobin from oxidation by promoting the formation of carboxymyoglobin. In contrast, nitrogen (N<sub>2</sub>) and argon (Ar) do not seem to be involved in oxidative processes (Martínez et al., 2005; Bellés et al., 2017a).



### Light

Light may initiate lipid and myoglobin oxidation. It can excite either unsaturated fatty acids or oxygen thereby favoring the formation of hydroperoxides. Photo-oxidation depends both on the wavelength and the intensity of light. White fluorescent lighting is more oxidizer than incandescent lighting while the incorporation of UV-light filters may significantly protect meat from photo-oxidation during display (Whang and Peng, 1988; Martínez et al., 2007). On the other hand, new light emitting diode (LED) seems to reduce discoloration but it may slightly enhance lipid oxidation (Cooper et al., 2016; Steele et al., 2016).

### Temperature

Both protein and lipid oxidation reactions are not completely inhibited at chilling temperatures but progress at a reduced rate. Temperature has been described to be the main factor for protecting meat from discoloration and lipid oxidation during display (Jakobsen and Bertelsen, 2000; Yin and Faustman, 1993). The effect of temperature against oxidation is still more pronounced at freezing temperatures, at which lipid oxidation is almost inhibited (Bellés et al., 2018). Nevertheless, there is a range between refrigeration and freezing in which a decrease of temperature may exacerbate oxidative processes. At superchilling temperatures (usually between  $-0.5$  and  $-2.8$  °C) meat could suffer mechanical damages which result in the release of pro-oxidant compounds, thereby increasing meat oxidation (Bellés et al., 2017b).

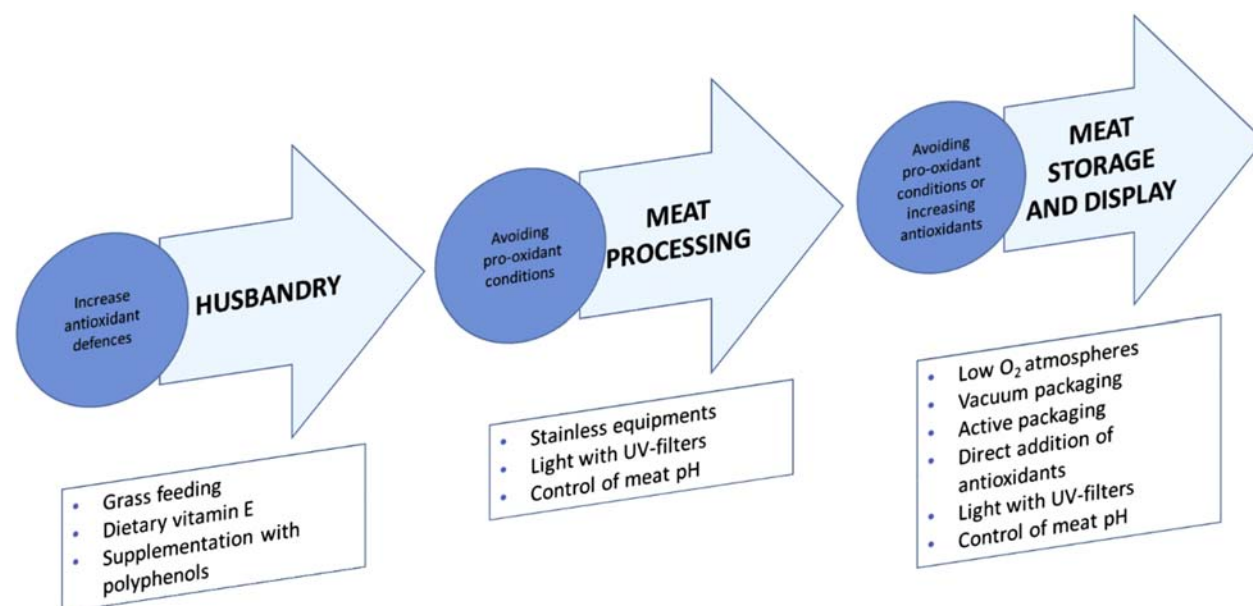
## Strategies to Inhibit Oxidative Reactions

Fig. 3 summarises the most common strategies used for reducing meat oxidation from farm to fork.

### Packaging

With the development of vacuum packaging (VP) and modified atmosphere packaging (MAP), meat industry started modifying the atmosphere surrounding the meat in order to change the atmospheric composition for a more profitable mixture of gases. Red meat is commonly packed in oxygen-enriched atmospheres (70%–80% O<sub>2</sub>) with the aim of producing optimum color by promoting oxymyoglobin formation. However, high proportions of oxygen in the package headspace have been described to favor lipid and ultimately protein oxidation leading to rancidity development, PUFA degradation and protein oxidation. In fact, oxidation is one of the main causes of deterioration of aerobic packaged meat (Bellés et al., 2017a). In contrast, the removal of oxygen from the package provides an anoxic atmosphere which minimize oxidative reactions. Despite low amounts of oxygen are enough to promote oxidation, vacuum skin packaging (VSP) seems to be able to avoid the formation of wrinkles which minimize residual oxygen in the package thus improving the antioxidant effect of traditional vacuum packaging (Bellés et al., 2017b). Table 2 presents some atmospheres commonly used for meat preservation.

The development of active packaging (AP) represents a new concept in meat packaging. Packaging becomes active when it plays some desirable role in food preservation besides providing an inert barrier to external conditions. AP incorporates additives (loose within the pack, attached to the inside of packaging materials or incorporated within the packaging materials) with preservative properties that enhance primary functions of the package (Hutton, 2003). Some interesting mechanisms such as scavengers of oxygen and ROS or emitting of natural antioxidants have shown interesting effects on inhibiting meat oxidation.



**Figure 3** Common strategies used to reduce lipid oxidation in meat from farm to fork.



**Table 2** Common atmospheres used for fresh meat preservation

<i>Specie</i>	<i>MAP composition</i>	<i>References</i>
Beef	30% O <sub>2</sub> -70% CO <sub>2</sub>	Esmer et al., 2011
Beef	50% O <sub>2</sub> -50% CO <sub>2</sub>	Esmer et al., 2011
Beef	70% O <sub>2</sub> -30% CO <sub>2</sub>	Esmer et al., 2011
Beef	50% O <sub>2</sub> -30% CO <sub>2</sub> -20% N <sub>2</sub>	Esmer et al., 2011
Beef	30% O <sub>2</sub> -30% CO <sub>2</sub> -40% N <sub>2</sub>	Esmer et al., 2011
Beef	60% O <sub>2</sub> -40% CO <sub>2</sub>	Djenane et al., 2003
Beef	80% O <sub>2</sub> -20% CO <sub>2</sub>	Camo et al., 2011
Beef	70% O <sub>2</sub> -20% CO <sub>2</sub> -10% N <sub>2</sub>	Djenane et al., 2001
Lamb	70% O <sub>2</sub> -20% CO <sub>2</sub> -10% N <sub>2</sub>	Camo et al., 2008
Lamb	50% O <sub>2</sub> -30% CO <sub>2</sub> -20% Ar	Camo et al., 2008
Lamb	80% O <sub>2</sub> -20% CO <sub>2</sub>	Kennedy et al., 2004
Lamb	60% O <sub>2</sub> -20% CO <sub>2</sub> -20%N <sub>2</sub>	Kennedy et al., 2004
Lamb	60% O <sub>2</sub> -40% CO <sub>2</sub>	Kennedy et al., 2004
Lamb	70% O <sub>2</sub> -30% CO <sub>2</sub>	Lauzurica et al., 2005
Lamb	70% O <sub>2</sub> -30% CO <sub>2</sub>	Bellés et al., 2018
Lamb	40% O <sub>2</sub> -30% CO <sub>2</sub> -30% Ar	Bellés et al., 2017c
Chicken	30% CO <sub>2</sub> -70%N <sub>2</sub>	Chouliara et al., 2007
Chicken	70% CO <sub>2</sub> -30%N <sub>2</sub>	Chouliara et al., 2007
Chicken	30% CO <sub>2</sub> -70%N <sub>2</sub>	Patsias et al., 2008
Chicken	30% CO <sub>2</sub> -70%N <sub>2</sub>	Meredith et al., 2014
Chicken	70% CO <sub>2</sub> -30%N <sub>2</sub>	Meredith et al., 2014
Chicken	50% CO <sub>2</sub> -50%N <sub>2</sub>	Meredith et al., 2014
Pork	70% O <sub>2</sub> -30% CO <sub>2</sub>	Lund et al., 2007
Pork	70% O <sub>2</sub> -20% CO <sub>2</sub> -10% N <sub>2</sub>	Rossi et al., 2017
Pork	80% O <sub>2</sub> -20% CO <sub>2</sub>	Alonso et al., 2012

### Herb Extracts and Essential Oils

Synthetic compounds have been commonly used in food industry to make a profit of their powerful antioxidant properties (Moure et al., 2001). Nevertheless, increasing demand of cleaner foods have immersed chemical additives in the eye of the storm. In contrast to synthetic additives, natural antioxidants are included in the Generally Recognised As Safe (GRAS) list of the American Food and Drug Administration. Essential oils and herb extracts contain substances with antioxidant and inhibitory effects against numerous pathogenic and spoilage bacteria. As can be seen in Table 3, potential sources of antioxidant compounds have been searched in different plant materials such as fruits, leaves, oilseeds, cereal crops, spices and herbs.

Polyphenols are the plant compounds with antioxidant activity mostly used for meat preservation. Phenolic compounds have an aromatic ring attached to one or more hydroxyl groups and their structure range from a simple molecule to a complex high-molecular weight polymer. The antioxidant activity of phenolic compounds, which depends on the structure, consists of their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations (Balasundram et al., 2006). Natural antioxidants have been employed for meat preservation obtaining a significant delay of lipid oxidation, discoloration as well as microbial growth, thus extending meat shelf life.

### Nutritional Strategies

The improvement of the balance between muscle pro-oxidants and antioxidants *in vivo* leads to an increase of antioxidants defences of meat after slaughter. A positive balance may be achieved by including supranutritional doses of natural antioxidant in animals feed, thereby favoring their depot accumulation in muscle cells.

Vitamin E is the main fat soluble antioxidant in mammalian cells, preventing oxidative reactions at the start site by a potent chain-breaking antioxidant. Vitamin E compounds possess a hydroxylic group which is able to transfer a hydrogen atom to a peroxy radical, which becomes a lipid hydroperoxide, while vitamin E becomes a low activity radical, the  $\alpha$ -tocopheroxyl radical. This radical, which does not take part in propagation reactions, can be reduced to tocopherol by other antioxidants like vitamin C, showing again antioxidant activity (Fennema et al., 2004). Tocopherols can inactivate different kinds of high reactive molecules such as singlet oxygen, alkoxyl radicals, peroxyxynitrite, nitrogen dioxide, ozone and superoxide. A large number of studies have observed that the administration of supranutritional doses of vitamin E before slaughter favors the deposition of tocopherol in the muscle increasing the stability of meat against oxidation (Bellés et al., 2017d, 2018; Muela et al., 2014; Jose et al., 2016).

Otherwise, feeding with plants rich in phenolic compounds or the supplementation with purified phenolic compounds and essential oils may improve the antioxidant status of meat (Vasta and Luciano, 2011).

**Table 3** Natural antioxidant used for meat preservation

Source	Obtention	Species	Notes on experimental set-up	References
Clove ( <i>Syzygium aromaticum</i> )	Plant extract	Beef	Direct application	Krishnan et al., 2014
Cinnamon ( <i>Cinnamomum</i> )	Plant extract		Direct application	Krishnan et al., 2014
Oregano ( <i>Origanum vulgare</i> )	Plant extract		Direct application	Krishnan et al., 2014
Savory ( <i>Satureja hortensis</i> )	Leave extract		Direct application	Aksu and Ozer, 2013
Rosemary ( <i>Rosmarinus officinalis</i> )	Essential oil		Alginate-based edible coating	Vital et al., 2016
Oregano ( <i>Origanum vulgare</i> )	Essential oil		Alginate-based edible coating	Vital et al., 2016
Rosemary ( <i>Rosmarinus officinalis</i> )	Essential oil		AP	Sirocchi et al., 2017
Mentha ( <i>Mentha piperita</i> )	Essential oil		Direct application	Smaoui et al., 2016
<i>Zataria multiflora</i>	Essential oil		Chitosan loaded with the antioxidant	Bazargani-Gilani et al., 2015
Clove ( <i>Syzygium aromaticum</i> )	Plant extract	Chicken	Direct application	Krishnan et al., 2014
Oregano ( <i>Origanum vulgare</i> )	Plant extract		Direct application	Krishnan et al., 2014
Cinnamon ( <i>Cinnamomum verum</i> )	Plant extract		Direct application	Krishnan et al., 2014
Mustard ( <i>Brassica nigra</i> )	Plant extract		Direct application	Krishnan et al., 2014
Thyme ( <i>Thymus</i> )	Essential oil		Agar solution loaded with the antioxidant	Fratianni et al., 2010
Balm ( <i>Melissa officinalis</i> )	Essential oil		Agar solution loaded with the antioxidant	Fratianni et al., 2010
Rosemary ( <i>Rosmarinus officinalis</i> )	nd		AP	Bolumar et al., 2011
Tea catechins	nd		Direct application + MAP	Mitsumoto et al., 2005
Oregano ( <i>Origanum vulgare</i> )	Essential oil	Foal	AP	Lorenzo et al., 2014a
Green Tea ( <i>Camellia sinensis</i> )	Leave extract		AP	Lorenzo et al., 2014a
Thyme ( <i>Thymus</i> )	Essential oil		Direct application + MAP	Karabagias et al., 2011
Oregano ( <i>Origanum vulgare</i> )	Essential oil		Direct application + MAP	Karabagias et al., 2011
Borage ( <i>Borago officinalis</i> )	Seed extract		Direct application + MAP	Bellés et al., 2017a–d
Green Tea ( <i>Camellia sinensis</i> )	Leave extract		Direct application + MAP	Bellés et al., 2017a–d
Rosemary ( <i>Rosmarinus officinalis</i> )	nd		AP	Camo et al., 2008
Oregano ( <i>Origanum vulgare</i> )	nd		AP	Camo et al., 2008
Oregano ( <i>Origanum vulgare</i> )	Leave extract		Direct application + MAP	Fernandes et al., 2016
Pu-erh tea ( <i>Camellia sinensis</i> )	Leave extract		Direct application	Hu et al., 2010
Cinnamon ( <i>Cinnamomum verum</i> )	Essential oil	Pork	Chitosan nanoparticles loaded with the antioxidant	Hu et al., 2015
Cinnamon ( <i>Cinnamomum verum</i> )	Essential oil		AP	Wang et al., 2017
Ginger ( <i>Zingiber officinale</i> )	Essential oil		AP	Wang et al., 2017
Green Tea ( <i>Camellia sinensis</i> )	Leave extract		Direct application + MAP	Lorenzo et al., 2014b
Grape ( <i>Vitis vinifera</i> )	Seed extract		Direct application + MAP	Lorenzo et al., 2014b
Chestnut ( <i>Castanea</i> )	Leave extract		Direct application + MAP	Lorenzo et al., 2014b
Seaweed ( <i>Algae</i> )	Dried algae		Direct application + MAP	Lorenzo et al., 2014b

nd: not defined; AP: active packaging; MAP: modified atmosphere packaging.

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## Nonenzymatic Browning Reactions: Overview

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### Glossary

**Caramelization** Caramelization is the degradation of sugar in the absence of an amino group where the initial sugar enolization proceeds with a series of dehydration, fragmentation and oxidation followed by polymerization of reaction intermediates and reactants. Caramelization usually occurs in the range of temperatures from 120 to 200 °C.

**Lipid oxidation** Lipid oxidation is a major deterioration reaction during degradation leading to the loss of nutrients, alteration of colors and generation of off-flavors.

**Maillard reaction** Maillard reaction is a term used to describe the reaction of proteins with sugars under application of heat.

**Melanoidins** Melanoidins are polymerized and colored products resulting from the advanced stages of the Maillard reaction. Melanoidins can be formed in wide variety of food products during domestic and industrial food processing.

**Strecker degradation** Strecker degradation is the conversion of  $\alpha$ -dicarbonyls into  $\alpha$ -aminocarbonyls. The reaction involves the formation of a Schiff base of an  $\alpha$ -dicarbonyl with an amino acid. Following the rearrangement, decarboxylation and hydrolysis, an  $\alpha$ -aminocarbonyl and the Strecker aldehyde are formed.

### Abbreviations

AGEs Advanced glycation end-products  
AMRP Advanced Maillard reaction product  
BHA Butylated hydroxyanisole  
BHT Butylated hydroxytoluene  
CEA Carboxyethylarginine  
CMA Carboxymethylarginine  
GLUT Glucose transporter  
Glarg Glyoxal-derived hydroimidazolone  
GOLD Glyoxal–lysine dimers  
HAA Heterocyclic aromatic amines  
4-HHE 4-Hydroxyhexenal  
HMF Hydroxymethylfurfural  
4-HNE 4-Hydroxynonenal  
ALEs Lipoxidation end-products  
MRPs Maillard reaction products  
MDA Malondialdehyde  
MG-H<sub>n</sub> Methylglyoxal-derived hydroimidazolones  
MOLD Methylglyoxal–lysine dimer  
CEL N<sup>ε</sup>-carboxyethyllysine  
CML N<sup>ε</sup>-carboxymethyllysine  
ONE 4-Oxo-2-nonenal  
PUFA Polyunsaturated fatty acids  
RTE Ready-to-eat  
TBARS Thiobarbituric acid-reactive substances  
UHT Ultrahigh-temperature

### Introduction

The majority of food items consumed daily are subjected to heat treatment to develop certain organoleptic characteristics. The main reasons for these changes in taste, flavor, texture, and color involve nonenzymatic browning reactions (Kanzler et al., 2017). With increased consumption of processed foods globally, food processors are becoming more interested in understanding the ways in which browning reactions can be controlled to better achieve more functional and healthful foods.



There are three general types of nonenzymatic, so-called “browning,” reactions recognized in food technology. The most common reaction, the carbonyl-amine reaction, was named after its discoverer French biochemist Louis Camille Maillard, who was the first to report that aqueous solutions consisting of amino acids and reducing sugars turn progressively brown during heating (Maillard, 1912). It includes the reactions of aldehydes, ketones, and reducing sugars with amines, amino acids, peptides or proteins (Hodge, 1953). The Maillard reaction occurs effectively at temperatures  $>50^{\circ}\text{C}$  and is favored at pH 4–7 (Kroh, 1994). A second reaction type, called “caramelization,” occurs when polyhydroxycarbonyl compounds, including sugars and polyhydroxycarboxylic acids, are heated above their melting points ( $>120^{\circ}\text{C}$ ) under acidic (pH 3) or alkaline (pH 9) conditions in absence of nitrogen-containing compounds. This type of browning usually requires more energy than the Maillard reaction to get started. The Maillard reaction and caramelization are independent of the presence of oxygen (Hodge, 1953). On the other hand, another type of browning important to the food processor is the group of oxidative reactions. Here, for example, ascorbic acid and polyphenols are converted into di- or polycarbonyl compounds that can then participate in the Maillard reaction itself. With enzymatic oxidation, such as polyphenoloxidase oxidation of dietary polyphenols, a catechol structure is formed, *o*-quinone, representing a source of carbonyls in the Maillard reaction. Secondary reactions of the *o*-quinones include the Strecker degradation of amino acids to form flavor-important volatile aldehydes (Rizzi, 2006). Ascorbic acid can also react with proteins (Meucci et al., 1991; Pischetsrieder et al., 1995). It is well-established that ascorbic acid can be oxidized to dehydroascorbic acid, which is then hydrolyzed irreversibly to 2,3-diketogluconic acid. The latter undergoes amine-catalyzed degradation following both the  $\beta$ -dicarbonyl fragmentation and the oxidative  $\alpha$ -dicarbonyl cleavage routes (Smuda and Glomb, 2013). The oxidation of lipids also produces carbonyl compounds, which readily react with free amino groups to produce browning (Gillatt and Rossell, 1992). For instance, the decomposition of lipid hydroperoxides generates aldehydes and ketones which then catalyze browning. All types of browning are caused by the formation of unsaturated, colored polymers of varying composition. Since compounds that produce browning usually contain carbonyl groups, polyhydroxy compounds and sugars in which the carbonyl function is blocked do not give rise to browning (Hodge, 1953).

## The Pathways of Browning Reactions

### The Major Steps in the Development of the Maillard Reaction

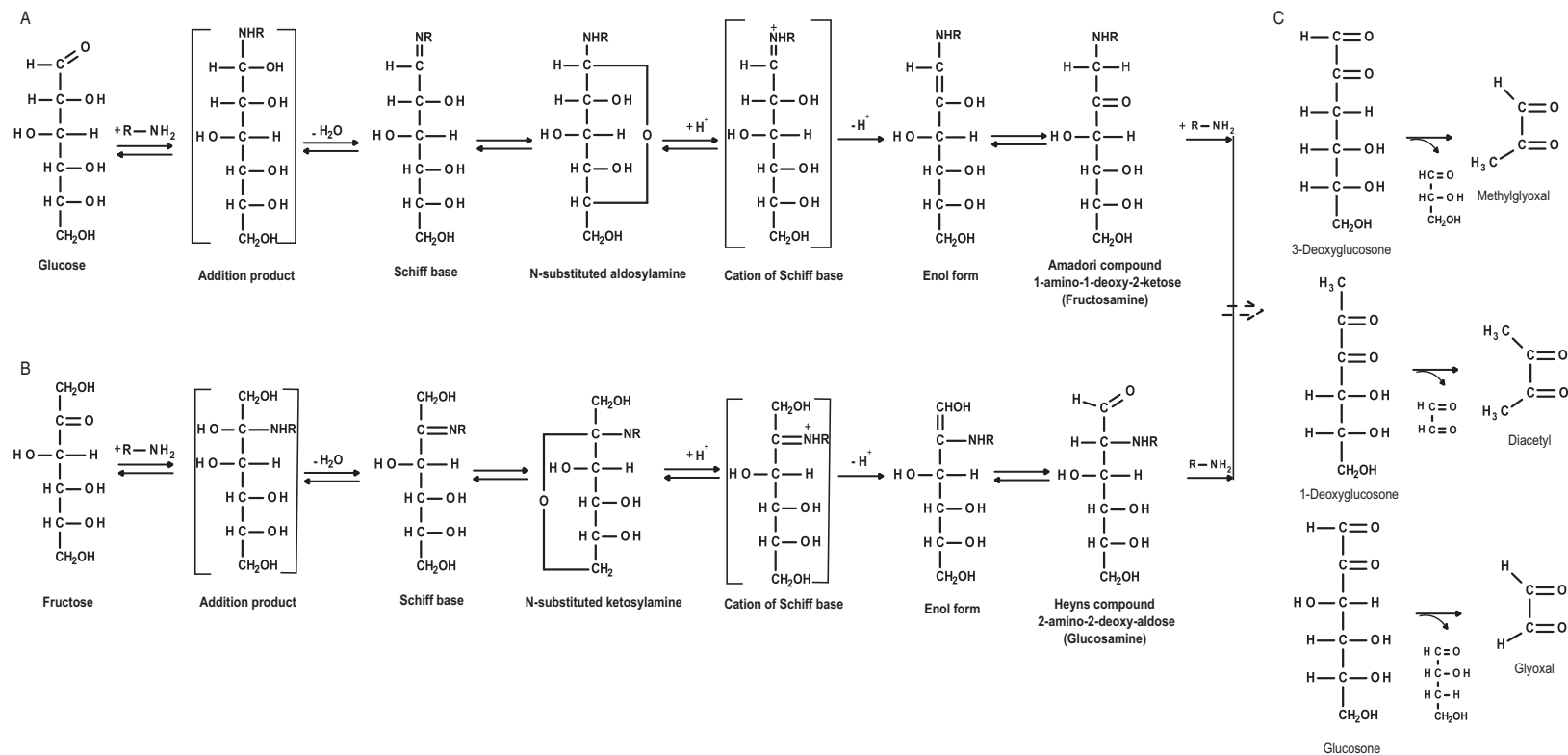
Maillard reaction is a sequence of reactions, starting with the formation of covalent bond between the amine groups and carbonyl compounds. In 1953 John E. Hodge proposed the reaction to be divided into “early,” “advanced” and “final” stages, where each step is dependent on the reaction conditions and distinguished by the characteristic reaction product (Silván et al., 2011; Hellwig and Henle, 2014). The main participants of the Maillard reaction are biomolecules with free amine groups including proteins, nucleotides and some phospholipids. Amines have several properties that are responsible for their reactivity, where major is their ability to act as nucleophiles by possessing a lone pair of electrons on the nitrogen atom. The properties of the carbonyl group are primarily those of the carbon-oxygen double bond. Its reactivity is mainly due to the difference in electronegativity between carbon and oxygen, leading to a dipolar resonance form (Feeney et al., 1975).

The initial stage of the Maillard reaction involves the condensation of a carbonyl group, for instance from a reducing sugar such as glucose or fructose, with a free amino group, typically the  $\epsilon$ -amino group of a lysine residues within proteins, to form an addition product. The addition product dehydrates to form the unstable imine known as a Schiff base, that spontaneously cyclizes to form a more stable *N*-glycosylamine (Fig. 1). Glycosylamines are hydrolyzable and the free amino acid and sugar are usually re-generated and therefore nutritionally available (Dills, 1993). *N*-glycosylamine from aldose sugars further rearranges to 1-amino-1-deoxy-2-fructose (ketoamine), which is known as the Amadori product (Fig. 1A) after the Italian scientist Mario Amadori. Derivatives from amino acids and ketoses (ketosylamines), similarly to the Amadori rearrangement, undergo the Heyns rearrangement (Fig. 1B) (Davidek and Davidek, 2003) named after Kurt Heyns, a chemist at the Chemical Institute of the University of Hamburg (Hellwig and Henle, 2014), who reported the formation of D-glucosamine in the reaction of fructose with ammonia (Heyns and Koch, 1952). Typically, no major color changes are observed at this stage of reaction (Damodaran, 2008), yet the nutritional value can be reduced due to irreversible modification of lysine, arginine, tryptophan, and isoleucine residues (Dworschak and Carpenter, 1980). Ketoses are more reactive in the sugar degradation reaction than their aldose isomers (Brands and van Boekel, 2001).

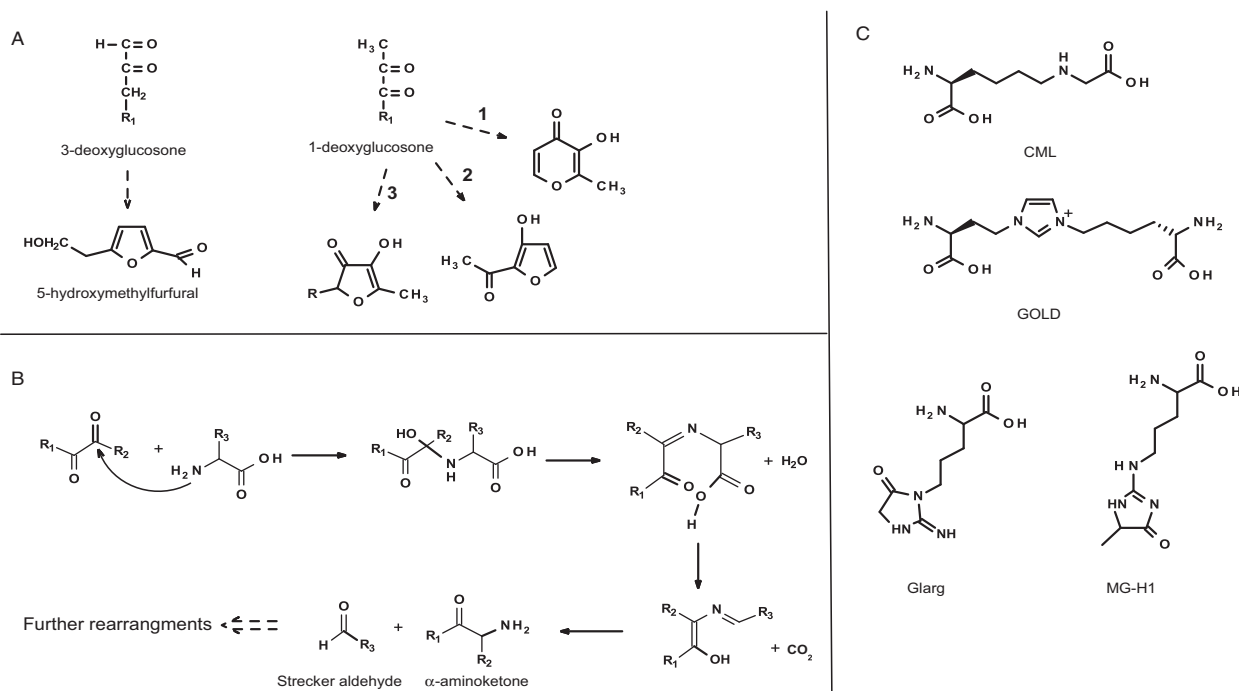
Intermediate stage of the Maillard reaction is more complicated in its pathways, reactants and products. It often shown as separate modules or pathways, however a myriad of possible interactions between unstable intermediates from variety of the pathways needs to be taken into account (Hoskin and Dimick, 1995). In any case, this stage involves the degradation of Amadori and Heyns rearrangement products to deoxydicarbonyl sugars (deoxyosones), which is pH-dependent (Fig. 1C). These deoxyosones contain two vicinal carbonyl groups and are therefore also named  $\alpha$ -dicarbonyl compounds. At lower pH, 1,2-enolization occurs resulting in formation of 3-deoxyglucosone, whereas neutral and alkaline pHs favor 2,3-enolization generating 1-deoxyglucosone. The other important  $\alpha$ -dicarbonyl compounds include methylglyoxal, glyoxal and diacetyl (Henle et al., 1996). The enolization pathways and formation of  $\alpha$ -dicarbonyls from Amadori and Heyns compounds is described in details by Davidek et al. (2002) and Hrynets et al. (2015), respectively.

Following cyclization and elimination of water, 3-deoxyglucosone forms hydroxymethylfurfural (HMF), whereas 1-deoxyglucosone produces isomaltol, maltol and furanones (Fig. 2A). These compounds are important aroma compounds which also have antioxidative properties (Coghe et al., 2004).





**Figure 1** The early stage of the Maillard reaction leading to the formation of (A) Amadori or (B) Heyns compounds; (C) Structures of the major deoxyosones produced from Amadori and Heyns rearrangement products. The structures are adapted from Nursten, H., 2005. The chemistry of nonenzymic browning. In: Nursten, H. (Ed.), *The Maillard Reaction: Chemistry, Biochemistry and Implications*, first ed. Royal Society of Chemistry, Cambridge, UK, pp. 5–30. Adapted with permission from The Royal Society of Chemistry.



**Figure 2** (A) Cyclization of 3-deoxyglucosone into 5-hydroxymethylfurfural; and 1-deoxyglucosone cyclization into (1) maltol (2) isomaltol and (3) furanones (R=H, CH<sub>3</sub> or CH<sub>2</sub>OH); (B) Mechanism of Strecker degradation; (C) Structures of glyoxal- and methylglyoxal-derived AGEs modification of lysine (CML and GOLD) or arginine (Glarg and MG-H1) residues. (A) Adapted from Coghe, S., Derdelinckx, G., Delvaux, F.R., 2004. Effect of nonenzymatic browning on flavor, color and antioxidative activity of dark specialty malts—a review. *Monatsschr. Brauwiss* 57, 25–38. (B and C) Adapted with permission from Frolov, A., Schmidt, R., Spiller, S., Greifenhagen, U., Hoffmann, R., 2014. Arginine-derived advanced glycation end products generated in peptide–glucose mixtures during boiling. *J. Agric. Food Chem.* 62, 3626–3635; Scalone, G.L.L., Cucu, T., De Kimpe, N., De Meulenaer, B., 2015. Influence of free amino acids, oligopeptides, and polypeptides on the formation of pyrazines in Maillard model systems. *J. Agric. Food Chem.* 63, 5364–5372; Kaur, H., Kamalov, M., Brimble, M.A., 2016. Chemical synthesis of peptides containing site-specific advanced glycation endproducts. *Acc. Chem. Res.* 49, 2199–2208. Copyright 2014, 2015, 2016 American Chemical Society.

Apart from cyclization Strecker degradation can occur in which α-dicarbonyls act as oxidizing agents to start decarboxylation of amino acids which is usually followed by hydrolysis of the resulting imine to produce free ammonia or a primary amine (α-aminoketone) and an aldehyde, known as a Strecker aldehyde (Fig. 2B) (Yaylayan, 2003). A comprehensive review on the pathways of the Strecker degradation is published by Yaylayan (2003). The Strecker degradation is an important for the generation of flavor-active compounds during the Maillard reaction. Strecker aldehydes of certain amino acids, in particular leucine, phenylalanine, or methionine, belong to key aroma components of many fresh and processed foods. They are important for flavor development in bread, coffee, cocoa, roasted meat, and dark beer (Vanderhaegen et al., 2006); however, can be perceived as off-flavors in ultrahigh-temperature (UHT) processed milk (Jansson et al., 2014a,b).

Alpha-dicarbonyls, glyoxal and methylglyoxal in particular, readily react with both lysyl and arginyl residues yielding advanced glycation end-products (AGEs) (Fig. 2C). Among the AGEs formed, N<sup>ε</sup>-carboxymethyllysine (CML), N<sup>ε</sup>-carboxyethyllysine (CEL), pyrrolidine, methylglyoxal–lysine dimer (MOLD), glyoxal–lysine dimers (GOLD) are the major lysine-related products. Glyoxal-derived hydroimidazolone (Glarg), carboxymethylarginine (CMA), methylglyoxal-derived hydroimidazolones (MG-H1, MG-H2 and MG-H3) and carboxyethylarginine (CEA) are arginine-related AGEs (Frolov et al., 2014; Gruber and Hofmann, 2005). For a thorough review on different AGEs formed refer to Arena et al. (2014).

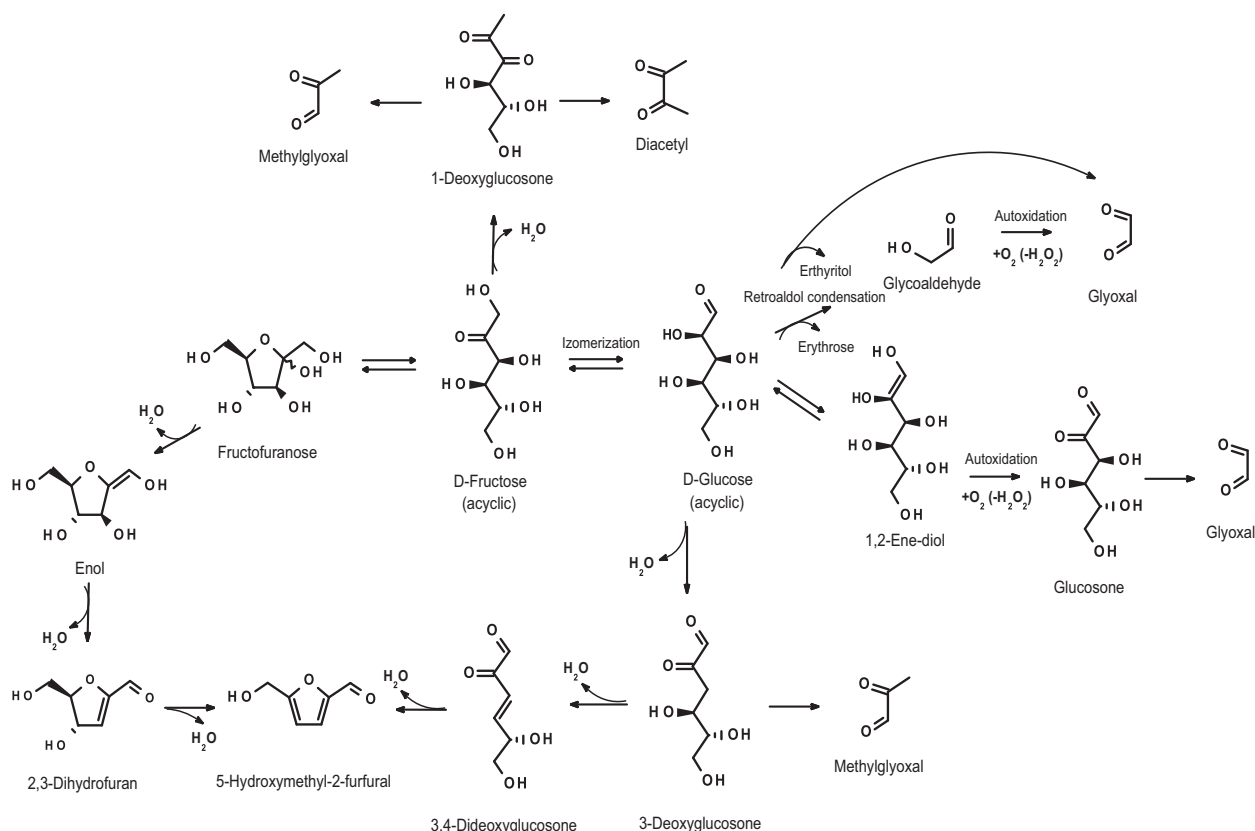
Further rearrangements occur later in the final stage, including cyclization, dehydration, retroaldolization, isomerizations, rearrangements and condensation of low molecular weight Maillard reaction products (MRPs). This results in the formation of a large group of high molecular weight, brown-colored and nitrogen-containing polymerized products, known collectively as melanoidins (Henle et al., 1996; Fogliano and Morales, 2011). These products are mainly responsible for specific color and typical appearance of processed foods (Mesias and Delgado-Andrade, 2017). Though, the definite structure of variety of melanoidins unidentified due to the variety of the reactants and reaction conditions, several mechanisms of their formation were proposed, described as (1) cross-linking of low-molecular weight colored substances to free amino groups of lysine or arginine of protein (Hofmann, 1998); (2) furan or pyrrole unit which upon polycondensation form repeating units of melanoidins (Heyns and Hauber, 1970; Tressl et al., 1998); (3) a skeleton made up from proteins cross-linked by MR reaction products (i.e. melanoproteins) (Hofmann et al., 1999). For more detailed review on the structural properties, the mechanisms of formation and biological activity of melanoidins refer to Wang et al. (2011). Additional information about the biological activity of melanoidins and melanoproteins in the gastro-

intestinal can be found in review from Tagliazucchi and Bellesia (2015). For the strategies to control the Maillard reaction in foods refer to the review of Lund and Ray (2017).

### The Chemistry of Caramelization Reaction

Caramelization is a complex process in which sugar reaction products condense and form brown no-nitrogen containing polymers. The reaction can occur under both acidic and alkaline conditions. Under acidic conditions the dehydration is promoted and yield furfural derivatives. Under alkaline conditions, isomerization and fragmentation are favored, although it can occur to some extent in weakly acidic solutions (Kroh, 1994). In aqueous alkaline medium monosaccharides undergo both reversible and irreversible transformations. The reversible reactions include (1) ionization, to give an equilibrium of neutral and ionized monosaccharides; (2) mutarotation, to give an equilibrium of the different cyclic hemiacetal structures of monosaccharides, and (3) enolization, causing the transformation of interconvertible monosaccharides. The isomerization via the enolization is accompanied by (4) irreversible transformation of the monosaccharides into carboxylic acids, generally known as the alkaline degradation reaction (De Bruin, 1986) (Fig. 3).

Alpha-dicarbonyl compounds are key intermediates which can form not only during the Maillard reaction, but also from sugar itself. Dehydration of hexose sugars produces mainly 1-deoxyglucosone, 3-deoxyglucosone, and 3,4-dideoxyglucosone, whereas oxidation of hexoses produces glucosone (Fig. 3). Fragmentation of these  $\alpha$ -dicarbonyl compounds produces shorter chain glyoxal, methylglyoxal, and diacetyl. Removal of three molecules of water from a hexose sugar forms 5-hydroxymethyl-2-furfural (De Bruin, 1986) (Fig. 3). Alpha-dicarbonyl compounds not only lead to the formation of caramel color but give rise to the important volatile products which are typical of caramel flavor (Kroh, 1994). Further reactions include intramolecular cyclization and dehydration giving rise to variety of heterocyclic compounds of furan and pyran types (Kroh, 1994). For instance hydroxymethylfurfural (HMF) (Fig. 3) contributes to desired taste, aroma and color of caramel and its amount is used as a quality indicator for certain foods (i.e. caramel products, dried fruits, coffee, honey) since it is related to the heat load applied during processing of carbohydrate-rich foods (Zappala et al., 2005). These heterocyclic compounds further undergo a series of polymerization reactions.



**Figure 3** Selected degradation mechanisms of glucose and fructose showing the formation of  $\alpha$ -dicarbonyl compounds, hydroxymethylfurfural and carboxylic acids. Adapted from Kocadağlı, T., Gökmen, V., 2016. Effect of sodium chloride on  $\alpha$ -dicarbonyl compound and 5-hydroxymethyl-2-furfural formations from glucose under caramelization conditions: a multiresponse kinetic modeling approach. J. Agric. Food Chem. 64, 6333–6342. Copyright 2016 American Chemical Society.

Browning of reducing mono- and disaccharides typically occurs more rapidly, as compared to glycosidically bound carbohydrates, since oligomers first have to be converted into more reactive, lower molecular weight intermediates (Kramhöller et al., 1993). Fructose and xylose solutions brown much more rapidly than those of maltose, glucose, lactose or sucrose (Buera et al., 1987). For instance, the initial reaction of hydrothermolysis of short-chain glucan like maltotriose generates maltose and D-glucose. Further rearrangements include the production of 1,6-anhydro- $\beta$ -D-glucose. Increase in degree of polymerization ( $>3$ ) of carbohydrates changes the kinetic of thermolysis for oligomeric glucans. If maltotriose degrades in part the same way as maltose and D-glucose, increase in polymerization degree to 4–5 residues result in degradation behavior comparable to that of polymeric glucans (Kroh et al., 1996). Kroh et al. (1996) showed that the thermolysis/hydrothermolysis of maltodextrins and soluble starch is initiated by the scission of a glycosidic linkages leading to the formation of D-glucose and 1,4-linked maltooligosaccharides, which act as reaction intermediates. These further undergo hydrothermolysis, transglycosylation, dehydration, and isomerization. As a result of these reactions non-volatile degradation products with sugar structure such as monomeric sugars (D-glucose), anhydrosaccharides, fructose containing sugars and short chain, partly branched, maltodextrins are formed. Consequently two parallel processes are taking place in the overall thermolysis, such as the simultaneous degradation of the oligo- or polymeric carbohydrate and new formation of oligomeric carbohydrate structures (Schulz et al., 2007). In some foods the caramelization and the Maillard reaction occur simultaneously and the reaction products of caramelization are at the same time starting material for the Maillard reaction resulting in a production of volatile (flavor) and non-volatile (pigments) compounds. For more details on the mechanisms of caramelization and resulting products refer to the Kroh (1994) and Kroh et al. (1996). The Maillard reaction of dextrins and starch was investigated by Kramhöller et al. (1993).

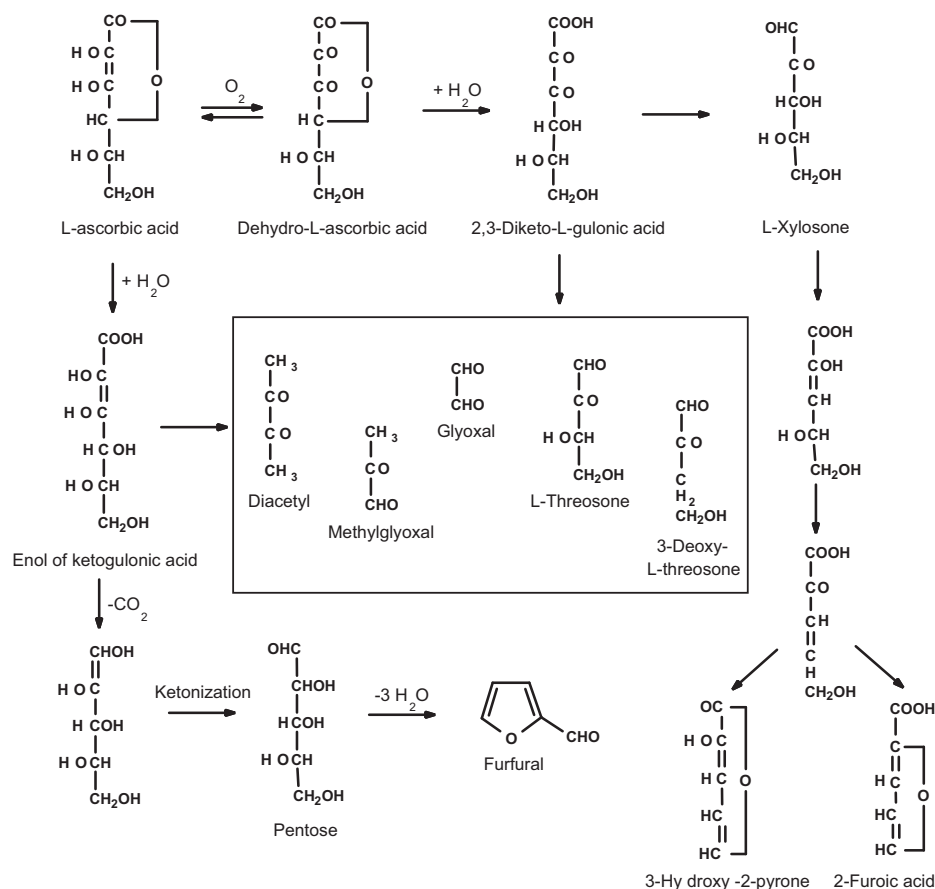
### Browning of Ascorbic Acid

L-ascorbic acid (further referred to as ascorbic acid) is a well-known natural antioxidant, so-called vitamin C, which occurs naturally in many food materials, especially citrus fruits and green vegetables. Physiologically this compound is attributed to the class of essential vitamins. Chemically, ascorbic acid belongs to the family of carbohydrates, classified as a sugar acid ( $\gamma$ -lactone) and an enediol (Schulz et al., 2007; Vernin et al., 1997). Ascorbic acid is widely utilized as food additive, cosmetic ingredient and pharmaceutical agent. Its degradation is among the common mechanisms of nonenzymatic browning during storage and processing of foods. Upon common practices in food processing, including heat, oxygen, and exposure to transition metals of ascorbic acid-containing foods, it behaves similarly to reducing sugars in the Maillard reaction (Vernin et al., 1997). Its degradation products react with amino acids, peptides, and lipids (or their degradation products) and give rise to a large number of aromatic and polymeric products via nonenzymatic browning reactions.

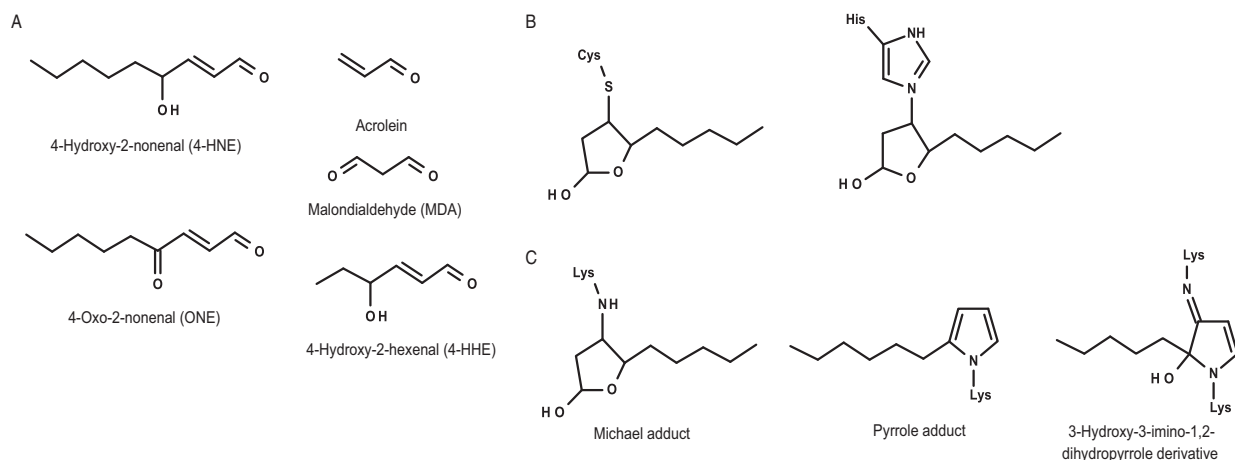
Several of the mechanisms of nonenzymatic browning of ascorbic acid are summarized in Fig. 4. Decomposition of ascorbic acid is rapid in an alkaline medium and is much slower at a pH less than 7 and in the absence of metal catalysts. The oxidation of ascorbic acid starts with the formation of the intermediates dehydroascorbic acid and diketogulonic acid. The latter can form L-xylosone and further degrade to various five-carbon compounds (Kimoto et al., 1993; Sawamura et al., 1994; Yuan and Chen, 1998) including 2-furoic acid and 3-hydroxy-2-pyrone. Ascorbic acid can also be readily converted into pentoses, which upon dehydration form cyclic products (i.e. furfural). Important intermediates,  $\alpha$ -dicarbonyls, are also generated in the course of the nonenzymatic browning initiated by thermal treatment of ascorbic acid, including glyoxal, methylglyoxal, diacetyl, 3-deoxy-L-pentosone, and L-threosone (Schulz et al., 2007). The carbonyl compounds resulting from decomposition of ascorbic acid can subsequently react with amines in the Maillard reaction. For more information on degradation of ascorbic acid refer to Schulz et al. (2007), Vernin et al. (1997), Kimoto et al. (1993), Sawamura et al. (1994), Yuan and Chen (1998), and Nursten (2005a).

### Interplay Between Lipid Oxidation and Nonenzymatic Browning

The role of lipids in the nonenzymatic browning reactions is their ability to be oxidized. Lipids in food can be oxidized by both enzymatic and nonenzymatic reactions, however autoxidation is the central reaction involved in the oxidative deterioration (Zamora and Hidalgo, 2005). Lipid autoxidation involves free radical mechanisms, with hydroperoxides being the initial products. Since hydroperoxides are relatively unstable, they further degrade in the second step of lipid oxidation generating volatile and nonvolatile monomers. For instance, glyoxal and methylglyoxal are formed not only during the Maillard and caramelization reactions, but also are the products of lipid peroxidation (i.e. polyunsaturated fatty acids (PUFA)) (Negre-Salvayre et al., 2008). Among the reactive carbonyl compounds, 4-hydroxynonenal (4-HNE), 4-hydroxyhexenal (4-HHE), 4-oxo-2-nonenal (ONE), malondialdehyde (MDA) and acrolein are the most characteristic products of lipid peroxidation (Fig. 5A). MDA is one of the most common aldehydes, resulting from peroxidation of arachidonic, eicosapentaenoic and docosahexaenoic acid (Esterbauer et al., 1991). MDA determination by thiobarbituric acid-reactive substances (TBARS) assay is one of the most frequent assays in lipid peroxidation studies (Uchida, 2003). These compounds can further react resulting in the generation of colored polymers by aldol condensations and/or carbonyl-amine polymerization (Zamora and Hidalgo, 2005). 4-HNE can react with histidine (His), cysteine (Cys) or lysine (Lys) residues of proteins (Fig. 5B). Upon reaction with lysine, HNE forms multiple products, including the HNE-lysine Michael adduct, pyrrole-type adduct, and fluorescent cross-linking type adduct (3-hydroxy-3-imino-1,2-dihydropyrrole) (Fig. 5C) (Uchida, 2003). MDA also reacts with Lys residues by forming Schiff bases (Esterbauer, 1993).



**Figure 4** General scheme of ascorbic acid degradation pathways. The structures and mechanisms are combined and modified from Schulz, A., Trage, C., Schwarz, H., Kroh, L.W., 2007. Electrospray ionization mass spectrometric investigations of  $\alpha$ -dicarbonyl compounds-probing intermediates formed in the course of the nonenzymatic browning reaction of L-ascorbic acid. *Int. J. Mass Spectrom.* 262, 169–173; Vernin, G., Chakib, S., Rogacheva, S.M., Obretenov, T.D., Párkányi, C., 1997. Thermal decomposition of ascorbic acid. *Carbohydr. Res.* 305, 1–15. Kimoto, E., Tanaka, H., Ohmoto, T., Choami, M., 1993. Analysis of the transformation products of dehydro-L-ascorbic acid by ion-pairing high-performance liquid chromatography. *Anal. Biochem.* 214, 38–4; Sawamura, M., Takemoto, K., Matsuzaki, Y., Ukeda, H., Kusunose, H., 1994. Identification of two degradation products from aqueous dehydroascorbic acid. *J. Agric. Food Chem.* 42, 1200–1203; Yuan, J.P., Chen, F., 1998. Degradation of ascorbic acid in aqueous solution. *J. Agric. Food Chem.* 46, 5078–5082; Nursten, H., 2005a. Nonenzymic browning mainly due to ascorbic acid. In: *The Maillard Reaction*, pp. 146–149; Zamora, R., Hidalgo, F.J., 2005. Coordinate contribution of lipid oxidation and Maillard reaction to the nonenzymatic food browning. *Crit. Rev. Food Sci. Nutr.* 45, 49–59.



**Figure 5** (A) General structures of lipid peroxidation-specific aldehydes; (B) Formation of HNE-adducts with cysteine or histidine; and (C) HNE-adducts of lysine. The structures are reproduced from Uchida, K., 2003. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Prog. Lipid. Res.* 42, 318–343. License number 4198450851446.

As mentioned in the section about [The Major Steps in the Development of the Maillard Reaction](#), CML ([Fig. 2C](#)) and CEL are AGEs formed during the Maillard reaction. However, both CML and CEL are also detected as products of lipid peroxidation, where they formed directly from glyoxal or methylglyoxal, or from other precursors, during the metal-catalyzed oxidation of PUFA in the presence of protein ([Nursten, 2005b](#)). The formation of CML and CEL, along with protein adducts of MDA and HNE during lipid peroxidation is inhibited by AGEs inhibitors (i.e. aminoguanidine and pyridoxamine) ([Brownlee et al., 1986](#); [Booth et al., 1997](#)).

Owing to the similarity of some reaction products of the Maillard, caramelization and lipid oxidation reactions it was proposed to be more suitable to use the term advanced Maillard reaction product (AMRP) to describe the broad range of products formed by carbonyl-amine chemistry during the Maillard reaction *in vivo* ([Nursten, 2005b](#)). In this scenario, AMRPs may be divided into AGEs and advanced lipoxidation end-products (ALEs), as well as into a mixed group. While the formation of AGEs may or may not require oxygen, the formation of ALEs is an oxygen-dependent process ([Nursten, 2005b](#)). To learn more on the contribution of lipid oxidation to the Maillard reaction and vice versa refer to [Zamora and Hidalgo \(2005\)](#), [Negre-Salvayre et al. \(2008\)](#), [Uchida \(2003\)](#), and [Hidalgo and Zamora \(2000, 2005\)](#).

## Role of MRP and Their Applications

Maillard reaction and its products are one of the most important during food processing and storage. As noted in the previous section due to some level of the structural similarity between the reaction products derived from the Maillard reaction, caramelization and ascorbic acid degradation collective term “Maillard reaction end products” (MRPs) can be applied. Due to the complexity of the food matrixes, major types of browning reactions can occur simultaneously resulting in a great variety of MRPs.

The role of the MRPs in food is diverse and at the same time controversial. It is well-known that Maillard reaction affects multiple qualities of food, including color, organoleptic properties and protein functionality. While it contributes to the development of pleasant aromas of baked goods, roasted meats and coffee, its advancement to the later stages yields bitter and burnt flavors and reduced protein functionality. The effect of Maillard reaction on the nutritional qualities of foods is also debatable, where some studies report the beneficial side of MRPs, while the others show the adverse effects. These conflicting findings once again confirm the versatility of MRPs and thus tremendous possibilities in food and pharmaceutical biotechnology.

## Nonenzymatic Browning in Fruits and Vegetables

MRPs have an important role in fruit/vegetable processing and cooking. They mostly contribute to flavor and aroma via nonenzymatic browning and caramelization reactions during high heat processing. Depending on cooking pattern beneficial or toxic MRP are produced. [Wellner et al. \(2011\)](#) showed that low temperature treatments during vegetable processing can induce pro-oxidant compounds, while higher temperatures induce MRP formation and thus contributes towards antioxidant property. [Billaud et al. \(2005\)](#) reported MRPs can significantly inhibit initial step of enzymatic browning caused by polyphenol oxidase and thus help to maintain food quality. This finding demonstrated protective role of MRPs against polyphenol oxidases and tyrosinases-mediated oxidative degradation of phenolic compounds produced in fruits and vegetables. Along with these properties anti-allergenic property of MRPs were also reported by [Gruber et al. \(2004\)](#). Along with beneficial properties of MRPs there are several reports ([Hofmann, 1998](#)) which demonstrate pro-oxidant and mutagenic property ([Nagao et al., 1977](#); [Gazzani et al., 1987](#); [Kim et al., 1991](#); [Kitts et al., 1993](#)) of these compounds. [Nagao et al. \(1977\)](#) discovered several heterocyclic amines which act as multi-site tumor inducer and it was further demonstrated that some of the metabolized products of these amine compound can make adduct with DNA which causes further toxicity ([Felton and Knize, 1998](#)).

## The Effect of Nonenzymatic Browning on Milk and Milk Products

Milk is required to undergo thermal treatment during production to guarantee its safety and to prolong the shelf life ([Troise et al., 2014](#)). In addition to lactose some other reducing species that could participate in the Maillard reaction can be found in milk-based products, including infant formulas or PUFA- and vitamin-enriched liquid milk ([Morales and Jiménez-Pérez, 2000](#)). For instance, other reducing sugars, vitamin C, and other low molecular weight compounds can be added or generated from other reactions (i.e. lipid oxidation, sugar molecule degradation) ([Labuza and Schmidl, 1986](#)). These compounds can react with proteins causing protein polymerization, development of brown color due to the formation of melanoidins and formation of undesired off-flavors ([Morales and Jiménez-Pérez, 2000](#)).

MRPs in milk products and their concentrations increases according to thermal treatment and water activity, where pasteurization > UHT > sterilization > milk powders ([Leclère and Birlouez-Aragon, 2001](#)). Negative flavor changes (i.e. “cooked” and bitter) in UHT milk develop during heat treatment and ambient storage are associated with the Maillard reaction and lipid oxidation ([Simon et al., 2001](#)). [Finot \(1983\)](#) discussed this issue of MRP formation during dried milk storage. Previously [Isaacs and Coulson \(1996\)](#) also reported that spray-dried whey contains significant amounts of lactose and protein thus conventional high temperature processing easily leads to MRPs formation. MRPs formation and its subsequent correlation with nutritional value of milk showed that production of lactulosyllisine significantly changes the protein bioavailability ([Evangelisti et al., 1994](#); [Pizzoferrato et al., 1998](#)). Hydroxymethylfurfural is another important MRP which can be obtained during milk processing and quantitatively this compound is assessed to determine the quality of processed milk ([Albalá-Hurtado et al., 1999](#)).



Cattle are ruminants and their digestive system is specialized for feed with large amounts of roughage. To increase the production of milk of dairy cows, modern feeding practices includes feed with a greater energy density and more nitrogen compared to its natural food sources (Schwarzenbolz et al., 2016). Hence, the addition of molasses, coarse colza, and soy, rapeseed and sugar beet are used in conventional farming with the consequence of elevated MRP levels in modern cow's nutrition (Schwarzenbolz et al., 2016; Allen, 2000). Schwarzenbolz et al. (2016) compared commercial milk originating from "organic" or "conventional" farming and reported great variations in the amounts of the individual MRPs (i.e. CML, pyrroline, MG-H, fructosyllysine), likely due the nutritional uptake of glycated proteins. For instance, significant differences in the content of free pyrroline (organic milk, 20–300 pmol mL<sup>-1</sup>; conventional milk, 400–1000 pmol mL<sup>-1</sup>) were reported. These signifies not only the effect of processing conditions (i.e. temperature of thermal treatment), but also importance of dairy animals feeding practices.

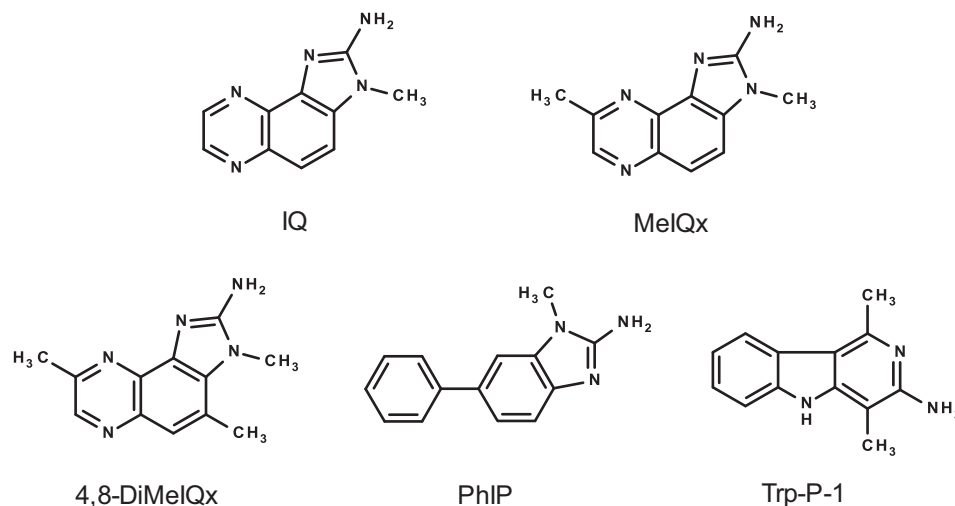
### Nonenzymatic Browning in Meat

Among the MRPs found in cooked meat, heterocyclic aromatic amines are among the most studied due to reports of increased incidences of cancer in individuals who frequently consume grilled meats, in particular well-done meats (Lang et al., 1994; Zheng et al., 2001). However, the relationship between HAA consumption and a risk of cancer is not conclusive (Barrett et al., 2003). The structures of some of the common HAAs found in cooked meats are shown in Fig. 6 (Pais et al., 1999). For a comprehensive review on the HAAs formation and risk evaluation refer to the reviews of Skog (1993) and Skog et al. (1998). Generation of HAAs in cooked meats is dependent on the type of meat, temperature and duration of cooking, resulting in high variability of HAAs concentration by more than 100-fold (Guy et al., 2000). Typically HAAs are formed at normal temperatures of cooking, however their concentration increases with higher temperatures and longer cooking time. For instance, Hatch et al. (1988), reported that meat cooked at lower than 150 °C to rare or medium-rare doneness showed lower content of HAAs compared to well-done meat cooked at higher than 150 °C.

Sinha et al. (1998) evaluated the HAAs content of different pork products and reported that HAA type and level varied substantially by product type (pork chops, ham slices, bacon, hot dogs, sausages or patties), cooking method (pan-frying, oven-broiling, grilling/barbecue or boiling) and degree of doneness/surface browning (just until done, well done or very well done). Puangsombat et al. (2011) ranked ready-to-eat (RTE) meat products in the following order of increasing total HAAs content: pepperoni < hot dogs and deli meat products < fully cooked bacon < rotisserie chicken meat < rotisserie chicken skin. They suggested that consumption of RTE meat products contributes very little to HAA intake.

### Bioactivities of the Maillard Reaction Products

MRPs have different bioactivities which can be used in food and pharmaceutical industries. Antioxidant properties of MRPs were first reported as early as in 1950s (Franzke and Iwainsky, 1954). Later studies reported comparable activity between well-known synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) and xylose-glucose derived MRPs. Casein-glucose heated mixtures produced MRPs reported to increase the shelf life of full-cream products (McGookin and Augustin, 1997) and decrease the oxidation of deoxyribose in a Fenton reaction system (Jing and Kitts, 2002). Though, the mechanism of antioxidant action depends on the type of MRPs, midstage or late stages, it can be generalized to the following abilities (1) strong



**Figure 6** Structure formulas of representative heterocyclic aromatic amines formed in cooked meats: 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1). The structures are adapted from Pais, P., Salmon, C.P., Knize, M.G., Felton, J.S., 1999. Formation of mutagenic/carcinogenic heterocyclic amines in dry-heated model systems, meats, and meat drippings. *J. Agric. Food Chem.* 47, 1098–1108. Copyright 1999 American Chemical Society.

reducing power (2) ability to scavenge free radicals and reactive oxygen species (ROS) (Yoshimura et al., 1997), (3) capacity to chelate potential metal prooxidants (Dittrich et al., 2003) and (4) the ability to inhibit hexanal oxidation and lipid peroxidation reaction (Chawla et al., 2007). In contrast, there are studies which found the prooxidant and genotoxic activities of MRPs in the presence of iron and copper (Wijewickreme and Kitts, 1997; Deguine et al., 1998). More on toxicology antioxidant activities of MRPs can be found in the review of Lee and Shibamoto (2002).

Beside antioxidant powers MRPs also possess antimicrobial properties (Hiramoto et al., 2004; Rufián-Henares and Morales, 2007). Some early stage MRPs (i.e. aminoreductones) are recognized for their antimicrobial potential (Knerr et al., 2001; Pischetsrieder et al., 1998; Trang et al., 2013). Trang et al. (2009) and Trang et al. (2011) demonstrated that aminoreductones significantly inhibit the growth of different *Helicobacter pylori* and *Staphylococcus aureus* species. Gobbo et al. (2002) demonstrated addition of sugar to a peptide can strongly influence their antimicrobial activity. Hrynets et al. (2016) and Bhattacharjee et al. (2016) demonstrated the antimicrobial activity of fructosazine, glucosamine (Heyns compound) self-condensation product, and glucosamine-derived  $\alpha$ -dicarbonyls against a heat resistant strain of *Escherichia coli*. To contribute significantly towards human health MRPs need to be bioavailable. There are a few studies that have demonstrated fructose and lysine-derived MRPs have poor bioavailability (Erbersdobler et al., 1981; Hellwig et al., 2011; Grunwald et al., 2006). However Hellwig et al. (2009) also demonstrated peptide-bound MRP (pyrraline) can be transported across a human intestinal cell line. The uptake of advanced glycation end-products (AGEs) like alanylpyrraline and pyrralylalanine were also reported by the human proton-coupled peptide transporter hPEPT1 (Geissler et al., 2010). These studies indicate that the transport of MRPs widely varies depending on their structure, however it is still not clear how these MRPs can pass across the intestinal cell barrier. Feng and Betti (2017) reported that chemically synthesized glycopeptides can pass across the Caco-2 cell membrane by using peptide transporter PEPT-1 and glucose transporters (GLUT). This study was further supported by Bhattacharjee et al. (2017), where the role of hexose transporters in transport of fructosazine across human intestinal cell line was shown. Further studies are required to access the MRP transport across human cell line to measure bioavailability and establish their merit and relevance to human health.

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## Relevant Website

<https://chrismasterjohnphd.com/2011/10/07/where-do-most-ages-come-from-o/> – Chrismaster John.

# Pulsed Electric Fields Processing of Plant-Based Foods: An Overview

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## Introduction

Pulsed Electric Fields (PEF) processing involves the application of short electric pulses (typically in the range of micro-to milliseconds) of high voltage across a food product placed between two conducting electrodes. As a consequence, the integrity of cell structure could be disrupted leading to cell leakage (Fincan and Dejmek, 2002). PEF applications are based on the “cell electroporation” principle (Weaver and Chizmadzhev, 1996) that induce local structural changes and breakdown of the cellular membranes (Zimmermann et al., 1974). The process of electroporation in a biological cell takes place in 4 consecutive steps: (i) formation of a transmembrane potential across the cytoplasmic membrane to destabilise the lipid bilayer and proteins of the membrane, (ii) formation of small metastable hydrophilic pores at the membrane when the transmembrane potential has built up, (iii) increment in size and number of pores, and (iv) post-treatment stage involving the formation of reversible pores (transient pores in which pore shrinkage and disappearance is taking place) or irreversible pores (permanent pores in which leakage of intracellular compounds occurs) after the applied electric field strength is removed. Depending on the level of electric field strength applied, reversible electroporation is commonly used for biotechnological applications for stress induction (Ye et al., 2004) or gene electrotransfer (Guo et al., 2014), whereas killing microorganisms (Castro et al., 1993) and mass transfer improvement (Donsì et al., 2010b) require irreversible electroporation.

In general, the required PEF processing parameters to perform specific food applications or to obtain desired quality improvement in the final product can be typically classified into low intensity ( $E < 2$  kV/cm) and high intensity ( $E > 15$  kV/cm) electric field strengths (Soliva-Fortuny et al., 2009). High intensity PEF processing ( $E > 15$  kV/cm) can be very effective for microorganism inactivation in liquid foods, leading to production of safe and stable food products (Jermann et al., 2015). On the other hand, low intensity PEF processing ( $E < 2$  kV/cm) would be sufficient to cause structural changes in the cell membrane of plant tissues, leading to improvement in mass transfer process, extractability of valuable phytochemicals, texture modification and to increase the efficiency of many existing food processes (Oey et al., 2016).

The aim of the present work is to provide introductory knowledge on the different technological applications that PEF can potentially offer to improve the process efficiency and the final quality of plant-based foods. The critical processing parameters of PEF recommended in the literature to achieve certain technological applications are summarised and specific examples of PEF-assisted processing of plant tissues from selected technological applications are discussed.

## Critical Processing Parameters of PEF to Achieve Cell Electroporation Effects on Plant Tissues

Plant tissues are highly heterogeneous matrices, which is a challenge to deliver evenly high voltage with very short electric pulses to affect the targeted plant tissue. The effectiveness of PEF processing to achieve cell electroporation varies depending on the processing parameters applied as well as the physical characteristics of plant materials (e.g. electrical conductivity and resistivity, particle size, size and type of cells, chemical composition, pH, temperature, etc.) placed between the conducting electrodes (Barsotti and Cheftel, 1999). Key processing parameters of PEF to achieve specific food applications or to obtain desired quality improvement in the final product include electric field strength ( $E$ ), specific energy input ( $W$ ), pulse duration or pulse width ( $\tau$ ), number of pulses ( $N$ ), treatment time ( $t$ ), pulse repetition rate or pulse frequency ( $f$ ), and pulse shape or pulse polarity (Barsotti et al., 1999). Many of these processing parameters are interdependent; for example, the specific energy input varies according to the applied electric field strength or the treatment time, whereas the effective treatment time depends on the pulse width and the number of pulses.

### Electric Field Strength ( $E$ )

Electric field strength is the most critical processing parameter of PEF processing affecting the degree of cell membrane electroporation. The intensity of electric field strength (units in kV/cm) is determined by the amount of voltage discharged (in kV) into the chamber and the distance (in cm) between the two electrodes, where the plant tissue is placed. Other factors including the electrode configuration (parallel, collinear, coaxial, etc.), treatment chamber design and presence of gas bubbles in the plant material would affect the homogenous distribution of electric field strength inside the treatment chamber (Puértolas et al., 2012).

When plant tissue is exposed to an external electric field strength, its transmembrane potential increases because of the charging process at the cell membrane by PEF. Application of a low electric field strength ( $E < 0.2$  kV/cm) with short pulse width ( $\tau < 10$   $\mu$ s) is usually below the critical value ( $E_c$ ) of the transmembrane potential difference of plant cell. Thus, transient or reversible pore formation occurs spontaneously and the cell membrane reseals when the external field strength is removed (Weaver and Chizmadzhev, 1996). However, electric field strengths applied between 0.5 and 2 kV/cm are generally effective to rupture permanently the cell membrane of plant tissue through establishment of high transmembrane potential difference, in which the induced membrane potential exceeds the critical value (Weaver and Chizmadzhev, 1996). Once the electric field strength applied is beyond the critical



**Table 1** Summary of critical electric field strengths for different plant tissues

Plant tissue	Critical electric field strength (kV/cm)	Other PEF processing parameters involved	References
Apple	0.20–0.40	$N = 1\text{--}1000$ , $t = 0.01\text{--}1$ , $P = \text{n.d.}$ , $f = \text{n.d.}$ , $\tau = \text{n.d.}$ , $W = \text{n.d.}$ , batch	Bazhal et al. (2003)
Aubergine	0.50–0.60	$N = 1\text{--}1000$ , $t = 0.01\text{--}1$ , $P = \text{n.d.}$ , $f = \text{n.d.}$ , $\tau = \text{n.d.}$ , $W = \text{n.d.}$ , batch	Bazhal et al. (2003)
Banana	0.90–1.10	$N = 1\text{--}1000$ , $t = 0.01\text{--}1$ , $P = \text{n.d.}$ , $f = \text{n.d.}$ , $\tau = \text{n.d.}$ , $W = \text{n.d.}$ , batch	Bazhal et al. (2003)
Carrot	0.20–0.40	$N = 1\text{--}1000$ , $t = 0.01\text{--}1$ , $P = \text{n.d.}$ , $f = \text{n.d.}$ , $\tau = \text{n.d.}$ , $W = \text{n.d.}$ , batch	Bazhal et al. (2003)
Cucumber	0.20–0.40	$N = 1\text{--}1000$ , $t = 0.01\text{--}1$ , $P = \text{n.d.}$ , $f = \text{n.d.}$ , $\tau = \text{n.d.}$ , $W = \text{n.d.}$ , batch	Bazhal et al. (2003)
Onion	0.33	$N = 100$ , $t = \text{n.d.}$ , $P = \text{square}$ , monopolar, $f = 1$ , $\tau = 100$ , $W = \text{n.d.}$ , batch	Ersus and Barrett (2010)
Pear	0.90–1.10	$N = 1\text{--}1000$ , $t = 0.01\text{--}1$ , $P = \text{n.d.}$ , $f = \text{n.d.}$ , $\tau = \text{n.d.}$ , $W = \text{n.d.}$ , batch	Bazhal et al. (2003)
Potato	0.18	$N = \text{n.d.}$ , $t = 0.9\text{--}25$ , $P = \text{exponential decay}$ , $f = \text{n.d.}$ , $\tau = \text{n.d.}$ , $W = \text{n.d.}$ , batch	Angersbach et al. (2000)

Abbreviation for PEF processing parameters:  $N$ , pulse number;  $t$ , treatment time (ms);  $P$ , pulse shape (exponential decay or square waveform) and polarity (mono- or bi-polar);  $f$ , pulse frequency (Hz);  $\tau$ , pulse width ( $\mu\text{s}$ );  $W$ , specific energy input (kJ/kg); batch or continuous mode PEF treatment. n.d., no data available.

value of cell ( $E \gg E_c$ ), permanent pores formed at cell membrane and plant tissues lose their cell permeability. **Table 1** summarises the optimum range of critical electric field strengths necessary to initiate drastic membrane rupture for different plant tissues with variation in physical and biological characteristics. Application of electric field strength between 0.4 and 0.8 kV/cm has been recommended to cause a significant cell electroporation for plant cells with size averaged between 50 and 120  $\mu\text{m}$  (Angersbach et al., 2000).

### Pulse Frequency ( $f$ )

Pulse frequency is defined as the number of pulses discharged per second (units in Hz) (Puértolas et al., 2012). The effectiveness of PEF to induce a greater cell electroporation effect on plant tissue is highly influenced by the pulse frequency. It has been demonstrated that apple tissue treated with low pulse frequencies between 0.02 and 100 Hz resulted in more tissue damage, while increasing the pulse frequency beyond 100 Hz would not lead to a higher cell membrane electroporation (Lebovka et al., 2001). Likewise, Asavasanti et al. (2011) reported that lower pulse frequencies ( $f < 1$  Hz) caused more damage to the integrity of onion tissue than higher pulse frequencies ( $f = 1$  to 5000 Hz). It appeared that high pulse frequency permits only a very short pause between two consecutive pulses, which has prevented the cell membrane to return to the pre-pulse state. Consequently, the cell membrane showed less resistance towards the electric pulses and exerted no remarkable cell electroporation effect. Overall, the application of low pulse frequency between 0.5 and 10 Hz would be sufficient to cause cell electroporation in plant tissue. However, increasing the pulse frequency is preferable to achieve higher enzyme inactivation (Terefe et al., 2013).

### Pulse Shape and Polarity

Pulse shape influences the way voltage is introduced across the food placed within two conducting electrodes. Exponential decay and square waveform are the most commonly applied pulse shapes for PEF processing, with square waveform considered as an ideal pulse shape because intensity of the applied electric field strength will remain constant during the predefined pulse duration/width. Additionally, square waveform could reduce the absorption energy by the treated plant material, which will be eventually transformed into heat (Knorr et al., 1994). Exponential decay pulses are less effective for cell electroporation because the applied voltage may diminish exponentially, or decrease at least 37% of the peak value, after reaching the peak pulse voltage.

Pulses can be modulated in different polarities, namely unipolar (retains positive charge and involves no reversal of polarity in the electric field) and bipolar (instant reversal of charges which rapidly reverse the electric field orientation) pulses. Compared to unipolar pulses, plant tissues exposed to bipolar pulses are expected to experience a greater structural fatigue at the cell membrane and became more susceptible towards cell membrane breakdown under PEF exposure. Moreover, bipolar pulses have been found to be more effective to result in a higher level of enzyme activity reduction (Terefe et al., 2013) and microbial inactivation (Evrendilek and Zhang, 2005).



### Pulse Width ( $\tau$ ) and Treatment Time

Pulse width (or pulse duration) is the amount of time a pulse is held at an effective voltage. Pulse duration is determined depending on pulse shape applied. For square waveform, effective pulse width is measured at 50% of peak voltage since an ideal/perfect square pulse shape is hardly achieved. For exponential decay pulses, the effective pulse width is defined as the time until the electric field decreases to 37% of peak voltage (Reberšek, 2017).

Treatment time of PEF is defined as a function of the pulse width ( $\tau$ ) and the number of pulses ( $N$ ) applied. This indicates that a longer effective treatment time of PEF at which the plant cells are exposed to the applied electric field strength can be achieved by increasing the pulse width. The work of De Vito et al. (2008) has reported that the efficiency of apple tissue electroporation by PEF improved with increasing pulse width. The reason for this could be due to the long pulse width that allows longer membrane charging time. As plant cells are generally larger in size (10–200  $\mu\text{m}$ ) compared to microbial cells (1–10  $\mu\text{m}$ ), it is expected that effective cell membrane electroporation and leakage of cell contents from plant tissue induced by PEF requires more time to charge up the membrane, leading to dramatic cellular damage.

### Specific Energy Input ( $W$ )

Specific energy input is an integrated processing parameter of PEF that takes into account the voltage applied, treatment time and resistance of the treatment chamber (Heinz et al., 2001). The resistance parameter depends on the cross-sectional area of the treatment chamber as well as the electrical properties (or conductivity) of plant material to be treated. Electrical properties of plant tissue can affect the ability of electrical current to pass through it. Calculation of specific energy input is important to allow a comparison on the treatment efficiency of PEF with other technologies and provides an estimation on the energy consumption and processing cost. This would be valuable information for the food industry when deciding for the implementation of PEF processing at a commercial level.

## Technological Applications of PEF for the Production of Plant-Based Foods

### Improvement in Juice Extraction and Clarification

It is always a challenge to the juice and beverage industries in finding effective methods to enhance the rate of mass transfer that can ease the release of natural intracellular liquid, i.e. juice contained in fruit and vegetable matrix, without compromising their inherent organoleptic attributes. Being a gentle cell membrane rupturing technique, PEF pre-treatment applied at moderate field strengths between 0.4 and 3 kV/cm has been shown to improve the juice yield (increase at least 2.5% to 45%) from various fruit and vegetables (e.g. apple, carrot, sugar beet, potato, fennel, pepper, alfalfa, and chicory) (Donsì et al., 2010b). Therefore, PEF has been proposed as an alternative to conventional enzyme-assisted and thermal-assisted juice extraction. For better juice extraction, this technology can also be applied either as a pre-treatment before juice pressing or intermediate treatment during juice pressing (Praporscić et al., 2007). A study by Grimi et al. (2009) has shown that the application of PEF treatment on grapes, at an electric field strength of 0.4 kV/cm for 10 square-wave bipolar pulses, during pressing (pressure increased from 0 to 1 bar for 60 min) can lead to at least 8% increase in juice yield. It is clear that PEF can assist the pressing process of plant material and provides a more efficient mass transfer phenomenon that considerably accelerates the juice extraction kinetics. The reason behind a higher juice yield is due to the increased permeability of the cellular membrane after PEF processing.

Apart from gaining a higher juice yield, juice extracted from PEF-treated plant tissue has been demonstrated to possess better qualitative characteristics, such as higher purity, less turbidity, and can be more easily purified (Loginov et al., 2011; Mhemdi et al., 2012). The increase in juice clarity and purity after a PEF treatment application is most likely because of the selective permeability of cell membranes by PEF. Therefore, lesser amounts of high molecular weight impurity compounds (proteins, pectin and colloids) can be detected in the extracted juice after PEF, which prevents the sedimentation of suspended particles and cloudiness in juice over storage time.

### Improvement in the Extraction of Phytochemicals

Nutritious phytochemicals such as anthocyanins, carotenoids and vitamin C are either bound to cell walls or enclosed inside the vacuole and other organelles of plant cells. Therefore, the degradation of the semi-permeable cell membrane is a fundamental step to reduce the resistance to mass transfer processes and promote the release or the extraction of phytochemicals from their localisation in the plant cell. PEF technology increases the cell permeability of plant tissue that leads to mass transfer enhancement of cell contents or phytochemical compounds from semi-solid plant-based foods. With the aim of enhancing mass transfer by electroporation, extraction of nutritious phytochemicals can be achieved at moderate electric field strengths of 0.5 to 5 kV/cm and treatment times in the range of 0.1 and 10 ms (Vorobiev and Lebovka, 2006).

#### Anthocyanins

Anthocyanins are water-soluble red pigments located in the cell vacuoles of plant cells, and hence they are extracted easily under the influence of PEF. The effect of PEF treatment ( $E = 2.5$  kV/cm,  $W = 16$  kJ/kg,  $\tau = 15$   $\mu\text{s}$ ,  $f = 1$  Hz and  $N = 50$  exponential decay

pulses) on red cabbage mash has been shown to extract at least 2.15-fold greater amount of total anthocyanins (Gachovska et al., 2010). On the other hand, PEF applied at an electric field strength of 13.3 kV/cm was able to release a 6-fold higher amount of total anthocyanin from blackberry fruits (Barba et al., 2015). It is clear that once the electric field strength applied to plant tissue is sufficient to create electroporation at the cell membranes, this occurrence favours mass transfer process allowing more anthocyanins to release immediately after PEF. While mass transfer is a time-dependent process, it should be expected that the release of anthocyanins from PEF-treated plant material would increase progressively after PEF. The release of anthocyanins in red raspberry fruit was found to reach its maximum level (more than 50% from untreated fruits) within 3 h after PEF treatment ( $E = 3$  kV/cm,  $\tau = 0.5$ – $1.5$   $\mu$ s,  $f = 1$  Hz and  $N = 420$  exponential decay pulses) (Luo et al., 2008). Similarly, PEF-treated cherries were also found to release a substantial amount of cyanidin-glucoside within 24 h after PEF ( $E = 2.5$  kV/cm,  $\tau = 20$   $\mu$ s,  $f = 100$  Hz and  $W = 45$  kJ/kg) (Sotelo et al., 2018).

### Carotenoids

Carotenoids are hydrophobic and lipophilic plant molecules due to their long unsaturated aliphatic chains. Moreover, the physical state and localisation of carotenoids in plants have limited their extractability. Despite this, PEF applied at low electric field strength ( $E < 2$  kV/cm) has been found adequate in inducing a cell electroporation effect that fosters an increase in the extractability of carotenoids from the carrot matrix (Roohinejad et al., 2014) and even helps to improve the carotenoids extractable yield from yellow coloured carrot cultivar with low initial amount of carotenoids (Leong et al., 2016c). Another study on tomato demonstrated that PEF treatment ( $E = 1.2$  kV/cm,  $\tau = 4$   $\mu$ s,  $f = 0.1$  Hz and  $N = 5$  monopolar square wave pulses) on intact tomato fruits can lead to substantial increase in the relative lycopene content (+32%) and total carotenoid content (+38%) (Vallverdú-Queralt et al., 2013). The PEF-treated tomato fruits were also found to contain higher concentrations of *trans*-lycopene (27%),  $\beta$ -carotene (28%), 15-*cis*-lycopene (33%), 9-*cis*  $\beta$ -carotene (44%),  $\alpha$ -carotene (93%), 9-*cis*-lycopene (94%), and 13-*cis*-lycopene (140%).

### Vitamin C

There is a huge interest in using PEF technology to improve the retention of vitamin C in plant-based foods since vitamin C is known to be the most heat-labile vitamin. Studies on oranges (Elez-Martínez and Martín-Belloso, 2007), tomatoes (Odrizola-Serrano et al., 2007), and watermelon (Oms-Oliu et al., 2009) have shown that PEF, applied at high electric field strengths ( $E = 25$ – $40$  kV/cm) with the aim to assure microbial inactivation for safe consumption, can minimise vitamin C degradation of up to 90% retention of the initial content. The effect of PEF, applied at a milder PEF processing condition (by lowering the electric field strength, the treatment time, the pulse frequency or the pulse width), on the vitamin C content of plant tissues whilst facilitating the mass transfer of other important phytochemicals (e.g. anthocyanins and carotenoids) has also been studied. Red bell peppers exposed to PEF ( $E = 2$  kV/cm,  $W = 0.32$  kJ/kg,  $\tau = 400$   $\mu$ s and  $N = 50$  exponential decay wave pulses) were found to experience minimal loss of vitamin C (<10%) (Ade-Omowaye et al., 2003). Likewise, the vitamin C content was better preserved in extracts obtained from fennel slices treated by PEF ( $E = 0.4$  kV/cm and  $N = 450$  monopolar square wave pulses) (El-Belghiti et al., 2008). One plausible explanation for the better retention of vitamin C content in PEF-treated plant foods is the inactivation of vitamin C degrading enzyme namely ascorbic acid oxidase (AAO). Recent work showed that AAO extracted from carrots was rather susceptible to PEF treatment (Leong et al., 2015).

### Raw Material Pre-treatment to Improve Existing Processing Lines

Plant tissues exposed to PEF can lead to disintegration of cellular tissues through electroporation that physically changes the membrane components and structural properties of the tissue. Consequently, PEF can be implemented as a pre-treatment or processing aid to help transforming plant tissue to a state that suits the subsequent processing unit operations. Examples of PEF application as raw material pre-treatment to improve existing potato frying, production of dried, canned and frozen products and the winemaking process will be discussed.

#### Potato Frying

Potato tubers are one of the produce types with a very firm texture and hence they require considerable mechanical force or energy to cut them for further processing. PEF technology induces electroporation leading to increased cell membrane permeability, which also affects tissue structure in a way that softens the potato tissues, resulting in better cutting quality and accuracy (Ignat et al., 2015). Such changes in the potato texture is promising for French fries or potato chips/crisps production to solve product quality issues due to cutting. The improved cutting behaviour offered by PEF-treated potatoes is expected to produce a smoother cut surface, reduce surface feathering, minimise breakage/fracture during cutting, and produce longer fries (Botero-Urbe et al., 2017).

Production of deep-fried potato products (French fries or chips) comprises a series of sequential processes, namely washing and sorting, steam peeling, cutting, blanching, pre-drying, par-frying and freezing (exclusive for French fries making), and frying (Botero-Urbe et al., 2017). While PEF treatment is recommended to apply on potatoes before cutting to produce softer potato that is much easier to cut, changes in the tuber structure by PEF can have implications on how the subsequent unit operations in the production of French fries or potato chips affect the final quality of the fried potato products. Evidences of process and final quality improvements for fried potato products as result of structural modifications by a PEF pre-treatment are summarised as follows:

- PEF-treated potatoes ( $E = 0.75$  kV/cm,  $W = 18.9$  kJ/kg,  $\tau = 20$   $\mu$ s,  $f = 250$  Hz,  $N = 9000$  bipolar square wave pulses) contained lesser amount of fat after frying ( $190^\circ\text{C}$ ) since a smooth and even cut surface after PEF can contribute towards decreased oil uptake during frying and allow better oil drainage after frying (Ignat et al., 2015). The probable reason is that PEF treated potato cells might experience increased cell liquid (or water) diffusion towards the potato surface, which result in a higher vapour pressure difference between surface water and oil and hence formation of thicker water vapour layer may act as a barrier against the oil that prevents oil uptake during frying.
- Electroporated potato cells ( $E = 1.5$ – $2.5$  kV/cm,  $f = 2$  Hz and  $N = 20$  exponential decay pulses) were found to release more intracellular compounds such as reducing sugars and low-molecular substrates involved in the Maillard reaction and hence reduce the tendency of fried potatoes to brown due to acrylamide formation (Janositz et al., 2011).
- Higher release of cell liquid (e.g. free water) from PEF-treated potatoes ( $E = 1.5$ – $2.5$  kV/cm,  $f = 2$  Hz and  $N = 20$  exponential decay pulses) has been reported (Janositz et al., 2011), which increases the dry matter of potato and absorbs less oil during frying, hence producing low-fat French fries or potato chips with mealy texture.

Indeed, there is strong evidence highlighting the practical use of PEF in the potato industry for reducing cutting forces, increasing mass transfer during frying, lowering oil uptake and hence providing consumers with a healthier option (i.e. less oil or low-fat) of potato chips and French fries. Such technological advances achieved in the potato industry through PEF technology should be considered for other underutilised crops such as sweet potato, taro, parsnip, cassava, carrot and beetroot to enable value adding and creating more product variation (e.g. vege chips/fries).

### Drying of Fruits and Vegetables

Drying of fruits and vegetables at high temperatures, for a long time, with constant exposure to oxygen can cause oxidative degradation and heat damage of many nutrients (Sablani, 2006). However, these degradations can be reduced by pre-treating the plant tissues with PEF, leading to formation of permanent “micro-holes/channels” in cell membranes and increasing cell permeability (Ade-Omowaye et al., 2001). Consequently, electroporated cell membranes exert a lower resistance to mass transfer. When mass transfer rates are enhanced after PEF, free water over a large surface area in the electroporated plant tissue can be released or be evaporated quickly; thus increasing the drying rate and reducing the overall drying time needed (a least 20% shorter duration depending on the fruit types) while achieving better retention of nutrients.

Although osmotic dehydration (OD) has been a popular technique to prevent oxygen penetrating into the tissues during drying that could affect the food quality negatively, OD is still considered a very slow drying process (Torreggiani and Bertolo, 2001). Interestingly, a PEF pre-treatment ( $E = 2$  kV/cm and  $\tau = 400$   $\mu$ s) of red bell peppers was found to be effective in enhancing the efficiency of the subsequent OD process by promoting a faster rate of water loss and minimal loss of vitamin C (reduced from 24% to 11%) during convective air drying (Ade-Omowaye et al., 2003). The cell electroporation effect of PEF ( $E = 1.8$  kV/cm,  $\tau = 15$   $\mu$ s,  $f = 300$  Hz,  $W = 42.3$  kJ/kg and  $N = 250$  pulses) has also been reported to effectively facilitate the infusion of hypertonic sugar or salt solutions of high osmotic pressure/low water activity and consequently speed up the osmotic dehydration process of kiwifruit slices (Dermesonlouoglou et al., 2016). Overall, there is strong evidence supporting the potential use of PEF as a pre-treatment to facilitate enhanced water loss during drying.

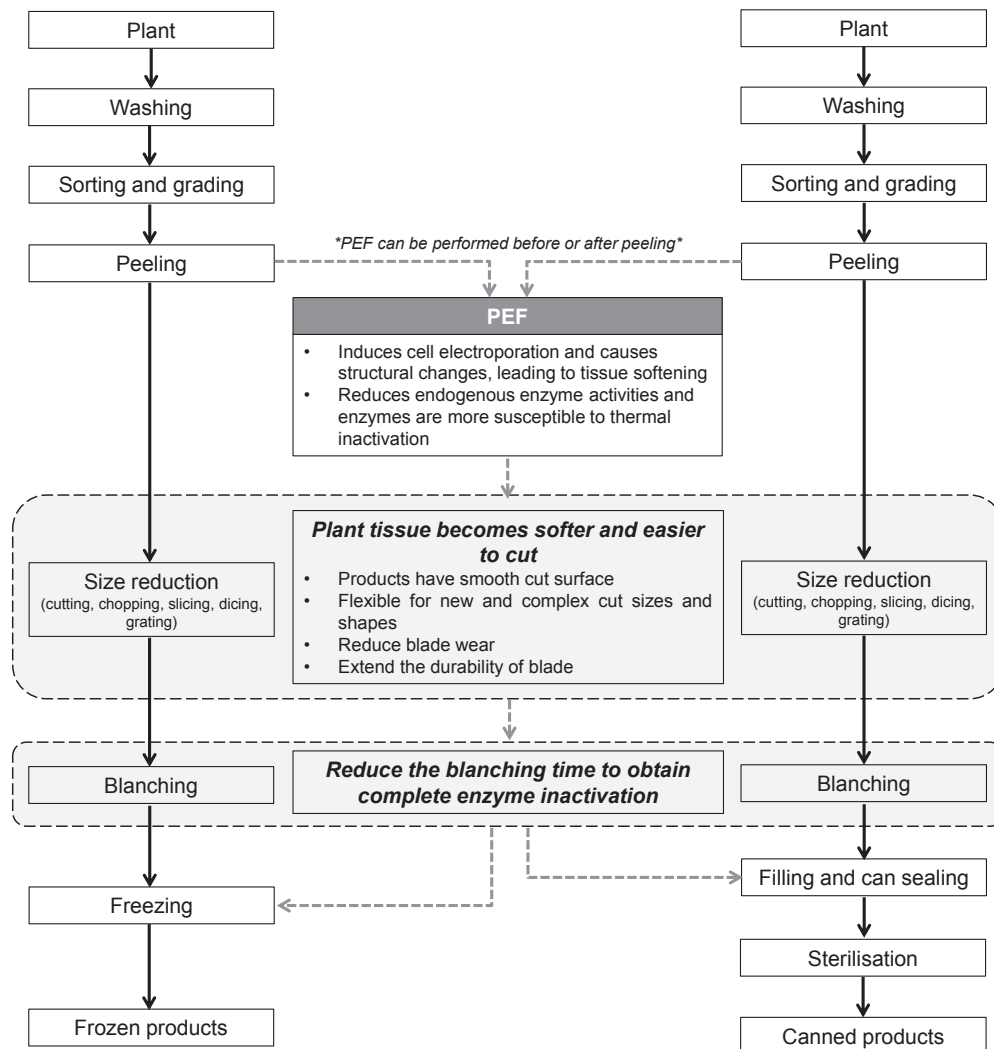
### Production of Canned and Frozen Fruit and Vegetable Products

Fruit and vegetables are highly perishable after harvest and can undergo a progressive loss of quality and nutrients deterioration. Most fruits and vegetables can be processed or preserved, in which the processing techniques involved have the ability to inactivate enzymes causing undesirable quality-related changes and to eliminate microorganisms and pathogens causing spoilage. Fresh produces are often pre-blanching (heating at high temperature for short time) to inactivate undesirable quality-related enzymes before they are cut (or diced, sliced, shredded, grated, etc.) and processed into canned or frozen products. Blanching however may have negative implications on the final products including unwanted changes in colour, excessive texture softening, flavour loss and degradation of heat-sensitive nutrients. PEF processing on plant tissues is very effective in reducing the activity of undesirable quality-related enzymes such as ascorbic acid oxidase (AAO) and peroxidase, without negatively affecting the temperature sensitive components (Leong et al., 2014). A recent investigation by Leong et al. (2015) also showed a major advantage of PEF as an upstream treatment in better controlling the sensitivity of vitamin C degrading enzyme (i.e. AAO) of carrots towards thermal inactivation and hence increase the overall process efficiency. While the initial thermal stability of AAO varied greatly between different carrot cultivars, the application of PEF at similar intensity ( $E = 0.8$  kV/cm,  $W = 35$  kJ/kg,  $\tau = 20$   $\mu$ s and square wave pulses) can reduce such variability and consequently narrow the window of heat processing temperature and time combinations to achieve the same degree of AAO inactivation after PEF. Such findings could benefit fruit and vegetable processing plants adopting multiple streams of fruit and/or vegetable with variable enzyme activities, as PEF appeared to make endogenous enzymes, from a range of raw materials, to respond similarly during the subsequent thermal inactivation treatments and hence more temperature sensitive for inactivation (Leong et al., 2015). It is then recommended that the intensity (temperature–time combination) of blanching to achieve complete enzyme inactivation could be reduced for plant tissues that have been pre-treated with PEF.

Apart from minimising the heat load required for complete enzyme inactivation during blanching, the cell electroporation effect of PEF can induce a certain degree of structural and textural changes of the plant tissues. This would benefit the subsequent size reduction unit operation by producing smaller sizes of fruits and vegetables with significantly reduced mechanical or cutting force

and improved cost-efficiency. Previous studies showed that the hardness of produce with high cellular integrity such as apple (Lebovka et al., 2004), carrot (Leong et al., 2014) and sugar beet (Mhemdi et al., 2013) reduced after PEF. The tissue softening was particularly prominent when combined with mild temperature conditioning (preheating between 20 and 60 °C) prior to or during PEF treatment. Moreover, the study of Leong et al. (2014) showed that PEF-treated carrots have improved smoothness at the cutting surface and a better cutting accuracy. Therefore, a PEF pre-treatment on plant could be an optimal solution to reduce blade wear and increase cutting knife durability since the electroporated plant tissues are softer and flexible and hence provide lesser resistance towards the cutting action. PEF could also offer possibilities in using industrial cutting knives and blades for fruit and vegetable processing with complex edges such as zigzag blade and figured blade (circular, straight, curved, convex, toothed or scalloped) in order to achieve final products with variation in sizes and shapes.

Overall, PEF processing is evidenced to be effective for enzyme inactivation as well as improvement in tissue softening for reduced cutting force and better cutting accuracy. Therefore, PEF can be easily implemented in the canned and frozen fruit and vegetable industries without any major modification of the existing processing line (Fig. 1). Within canned products processing line, PEF treatment can be applied to reduce the heat load required to inactivate enzymes during the sequential blanching process before performing aseptic can filling unit operation. For frozen products processing line, PEF treatment can be combined with mild temperature conditioning to shorten the subsequent blanching time for enzyme inactivation and will require a smaller mechanical force for size reduction. Another interesting feature of PEF for cell electroporation that has yet to be exploited in the freezing operation is to facilitate the infusion of a cryoprotectant such as trehalose across the electroporated cell membranes ( $E = 0.58 \text{ kV/cm}$ ,  $\tau = 25 \text{ }\mu\text{s}$  and square wave pulses) to improve the freezing tolerance of spinach leaves (Phoon et al., 2008).



**Figure 1** Simplified flowchart for the possible integration of PEF treatment in the production of canned and frozen fruit and vegetable products. Authors own elaboration.

### Red Winemaking

One of the essential prerequisites for red winemaking is to allow extraction or release of adequate anthocyanins and phenolics from grape skin cells. Strategies such as extending maceration duration, increasing maceration temperature (thermo-vinification) and addition of pectinolytic enzymes are common practice in wineries (Sacchi et al., 2005). However, extending the maceration duration and increasing the maceration temperature will add processing cost and also it is limited by the capacity and availability of maceration-fermentation tanks especially when they need to handle large volumes of grapes within a short harvest time. Therefore, a gentle extraction technique on grape mash that has the capability to reduce the maceration duration and does not require a high maceration temperature whilst extracting a greater amount of anthocyanins and phenolics is desired. In this context, PEF technology is used as a novel low-energy consumption extraction technique to reach these objectives.

It is recommended to process the grapes immediately after harvest. The grapes are destemmed to produce grape must/mash (consisting of seeds, skins and pulps) before the initiation of maceration-fermentation step. Previous study has reported that PEF-assisted extraction ( $E = 4\text{--}5\text{ kV/cm}$ ) of Cabernet Sauvignon grape mashes accelerated the release of anthocyanins, thus reducing the overall maceration duration (from 144 h to 96 h,  $25^\circ\text{C}$ ) and the finished red wines contained a higher anthocyanin content as compared to the control wines produced from grapes that were macerated for a longer time (Puértolas et al., 2010). Moreover, a PEF pre-treatment ( $E = 1.5\text{ kV/cm}$ ,  $W = 70\text{ kJ/kg}$ ,  $\tau = 20\text{ }\mu\text{s}$ ,  $f = 50\text{ Hz}$  and square wave pulses) on Merlot and Pinot Noir grapes prior to maceration has also shown that the overall maceration duration can be reduced to 4 and 8 days (vs. 14 days with control) respectively to achieve similar level of anthocyanins content and colour characteristics (Leong et al., 2016a, 2016b).

While winemakers have used pectinolytic enzymes to degrade the cell wall material in order to enhance the release of polyphenols and anthocyanins from the skin cells, the use of PEF for red winemaking can solve the problems arising from the use of pectinolytic enzymes, such as incorrect enzyme types, dosage, hydrolysis time, and temperature that can have consequences on the quality of the final wines. The cell electroporation effect of PEF treatment ( $E = 1.5\text{ kV/cm}$ ,  $f = 1\text{ Hz}$ ,  $W = 25\text{ kJ/kg}$ ,  $\tau = 10\text{ }\mu\text{s}$  and  $N = 2500$  square wave pulses) to Aglianico grapes before maceration was more effective in anthocyanins extraction than the addition of pectinolytic enzymes, in which the total anthocyanin content of the finished wine from PEF-treated grapes was higher than enzymatically-treated grapes by at least 20% (Donsi, Ferrari, Fruilo and Pataro, 2010a). Likewise, Puértolas et al. (2009) showed that the finished wine of Cabernet Sauvignon grapes pre-treated with PEF ( $E = 5\text{ kV/cm}$ ,  $W = 2.1\text{ kJ/kg}$ ,  $f = 1\text{ Hz}$  and  $N = 50$  exponential decay wave pulses) contained higher content of anthocyanins (+12%) and better colour characteristics when compared to the wine produced from enzymatically-treated grapes.

It is clear that PEF can facilitate more anthocyanin release from grape mash during maceration and this enhancement effect has been reported to be maintained during fermentation and after a certain period of ageing. The anthocyanin composition of wines was found to be qualitatively similar but quantitatively higher to the control wines after aging for the similar period of time (Delsart et al., 2014; López et al., 2009). Findings from previous studies have successfully pinpointed the potential implementation of this non-thermal technology in the commercial winery settings. However, before implementing this technology to the wineries, it is important to note that grape variety remains a significant factor in determining the effectiveness of the applied PEF processing parameters to improve the extraction of anthocyanins and phenolic compounds since differences in the grape skin structure (i.e. cell thickness, cell wall material composition) can limit their susceptibility towards the cell electroporation of PEF.

### Food Preservation, Safety and Shelf-Life Extension

Apart from inducing the formation of permanent holes at the cell membrane that cause leakage of cell contents from plant tissues, PEF is able to convey the same cell electroporation impact on microorganisms. Consequently, microorganisms will lose their cell viability and lead to cell death. This important feature of PEF can destroy microorganisms, as well as preserving the content and stability of nutritional components and organoleptic qualities of the food product since undesirable quality-related enzymes are rather prone to inactivation under PEF exposure, too. It is important to note that food preservation by PEF technology is only achievable at high intensity electric field strength ( $E > 15\text{ kV/cm}$ ) and microbial inactivation where PEF is more effective for liquid or pump-able food products such as juices, milk and smoothies. Implementation of PEF at industrial scale has already been considered to replace the conventional thermal pasteurisation process, in which the United States Food and Drug Administration (FDA) approved the first commercial application of PEF for fruit juice processing in 2006. To date, Netherlands-based companies such as Hoogesteger, Fruity King and Juicy Line have already adopted PEF technology for different types of juice production.

### Microbial Inactivation

To obtain microbiological-safe and shelf-stable food products, employing PEF at high electric field strength, typically between 30 and 50 kV/cm, is necessary. As a result of PEF treatment applied at high electric field strength, food pathogens and spoilage microorganisms, yeast and mould are reported to be susceptible towards inactivation. Based on previous studies, it was observed that 3.25- to 5-log reduction for vegetative yeast cells and the ascospores of *Zygosaccharomyces bailii* in cranberry and grape juice (Raso et al., 1998), a 6-log reduction for *Escherichia coli* in cranberry juice (Sen Gupta et al., 2005), and 2- to 4-log reduction for *Kloeckera apiculata*, *Saccharomyces cerevisiae*, *Lactobacillus plantarum*, *Lactobacillus hilgardii* and *Gluconobacter oxydans* in grape juice (Marsellés-Fontanet et al., 2009), can be achieved with the use of high intensity PEF processing ( $E > 15\text{ kV/cm}$ ).

While PEF has the capability to stabilise berry juice microbiologically, there is evidence showing no vast change in the nutritional and organoleptic qualities of the juices after PEF exposure. A higher retention of vitamin C, total phenolics, anthocyanins, carotenoids



and lycopene and total antioxidant capacity has been found in blueberry juice (Barba et al., 2012), grape juice (Marsellés-Fontanet et al., 2013), strawberry juice (Odriozola-Serrano et al., 2008) and tomato juice (Odriozola-Serrano et al., 2007) when compared to their thermally-treated counterparts. Moreover, PEF-treated juices were described to retain the characteristic fresh fruit volatiles (Jin and Zhang, 1999), have better colour retention and where non-enzymatic browning was minimised (Aguiló-Aguayo et al., 2009a).

### Enzyme Inactivation

The presence of food quality related enzymes in either raw or processed plant-based foods is critical as their residual enzyme activity may lead to food quality losses during storage (Adams, 1991). Therefore, enzyme inactivation through food processing is often desirable to preserve the quality (texture, colour, flavour, and nutritional quality) of foods. PEF processing is well recognised to provide new opportunities to meet specific quality targets in foods without employing excessive heat load when compared with conventional heat treatment. In this regard, extensive research in the past years has shown that PEF is a promising preservation technology resulting in substantial inactivation (up to 90%) of most enzymes in juices originating from various plant sources (Table 2). However, this goal is usually achieved with the requirement of intense PEF processing parameters, for example application of electric field strength at least greater than 15 kV/cm, high specific energy input, high pulse frequency, long treatment time or delivery of pulses in bipolar mode. Factors such as origin (species and types) of the enzyme, the composition of treatment medium and PEF processing parameters affect the degree of enzyme inactivation. The mechanisms involved in enzyme inactivation by PEF are not yet fully understood although both electrochemical effects and Ohmic heating occurring under the influence of these intense processing parameters are said to contribute to the observed inactivation (Terefe et al., 2013).

### Conclusion

In the last decades, the main area of PEF application has been concentrated on inactivation of microorganisms. However, current research has clearly indicated that PEF can offer numerous potential applications for plant-based food products and can be easily adopted by food industries or integrated into existing processing lines. In fact, there is an emerging interest among the food processors concerning the use of PEF as an efficient, low energy upstream process, e.g. pre-treatment of plant tissue for juice expression, phytochemicals extraction, potato frying and to improve the process efficiency of drying, canning, freezing and winemaking. Forthcoming research is still needed to understand whether changes in the plant material induced by PEF might affect the nutritional, health-promoting and sensorial attributes as well as the storage/shelf life of the final products. It is also important to note that plants are a highly variable material and hence testing the effectiveness of a certain PEF processing parameter to achieve certain food application on a wide range of plant from different species, cultivars, tissue types and arrangement, cell size and structures should be considered for successful industrial implementation of PEF.

**Table 2** Summary of representative studies on the effect of PEF processing on quality-related enzymes from different plant sources

Enzymes	Matrices	PEF processing parameters	Residual enzyme activity (%)	References
<b>I. Texture-related enzyme</b>				
Pectin methylesterase	Tomato juice	$E = 35$ , $N = \text{n.d.}$ , $t = 1$ , $P = \text{square}$ , bipolar, $f = 250$ , $\tau = 7$ , $W = 5512$ kJ/L, continuous, 60 mL/min flow rate	8	Aguiló-Aguayo et al. (2009b)
	Orange juice	$E = 35$ , $N = \text{n.d.}$ , $t = \text{n.d.}$ , $P = \text{square}$ , monopolar, $f = 600$ , $\tau = 1.4$ , $W = \text{n.d.}$ , continuous, 1633.33 mL/min flow rate	10	Yeom et al. (2000)
<b>II. Colour-related enzyme</b>				
Polyphenoloxidase	Apple extract	$E = 24.6$ , $N = \text{n.d.}$ , $t = 6$ , $P = \text{exponential decay}$ , bipolar, $f = \text{n.d.}$ , $\tau = 20$ , $W = 114$ kJ/kg	3	Giner et al. (2001)
<b>III. Flavour- and colour- related enzyme</b>				
Peroxidase	Orange juice	$E = 35$ , $N = \text{n.d.}$ , $t = 1.5$ , $P = \text{square}$ , bipolar, $f = 200$ , $\tau = 4$ , $W = \text{n.d.}$ , continuous, 60 mL/min	0	Elez-Martínez et al. (2006)

Abbreviation for PEF processing parameters:  $E$ , electric field strength (kV/cm);  $N$ , pulse number;  $t$ , treatment time (ms);  $P$ , pulse shape (exponential decay or square waveform) and polarity (mono- or bi-polar);  $f$ , pulse frequency (Hz);  $\tau$ , pulse width ( $\mu\text{s}$ );  $W$ , specific energy input; batch or continuous mode PEF treatment. n.d., no data available.



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# Oleogels in Food

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## Introduction

Solid fat provides functionality, flavour, mouthfeel and texture to foods, playing a distinct and essential role in a large variety of food products. These fats contain a large proportion of high melting triacylglycerols (TAGs) that crystallize at room temperature into a network that confines the lower melting liquid TAGs within it. In general, the high melting TAGs are made up of a combination of saturated and/or *trans* fatty acids, while low melting TAGs are commonly unsaturated fatty acids (Marangoni and Garti, 2011). While lipids are an important component of the diet, it is recommended to limit consumption of saturated and *trans* fatty acids due to their well-documented association with adverse effects on cardiovascular health (Judd et al., 1994; Mensink and Katan, 1990; Mensink et al., 2003; Mozaffarian et al., 2006). In contrast, there are recognized beneficial health effects from increasing unsaturated fatty acid consumption (Lunn and Theobald, 2006). For this reason, the concept of replacing saturated and *trans* fatty acids with unsaturated fatty acids in foods is gaining popularity. However, direct replacement of solid fat with oils is usually not an option due to differences in their physical and sensory properties.

The use of oleogels has the potential for creating foods with desirable physical and sensory properties, in addition to satisfying health concerns. There are many different oleogelators capable of structuring edible oils, and each gelator will produce gels with unique properties. Different foods require the functionality of solid fats possessing different physical properties, including thermal behaviour, mechanical strength and rheology. Oleogels must therefore be produced to match the functionality of the fat source they are intended to replace, in order to achieve all the characteristic or desired properties for the specific product. Outside of solid fat replacement, the concept of oleogelation can also be used to address oil migration in foods, or to protect and deliver hydrophobic molecules. There is also work to determine if oleogels on their own can be used as spreadable alternatives to margarine or butter.

## Types of Oleogels

### Ethylcellulose Oleogels

Ethylcellulose (EC) is one polymer capable of structuring oils into solid gel networks, and is currently the only known food-grade polymer capable of structuring oil. EC is a semi-crystalline cellulose polymer derivative, consisting of a cellulose backbone with ethoxyl substitutions at hydroxyl groups (Atalla and Isogai, 1998; Davidovich-Pinhas et al., 2015a; Roy et al., 2009; Stortz et al., 2012). The degree of substitution (DS), or ethoxy content, is used to characterize the final EC, where lipophilicity is achieved with a DS of 2.4–2.5 (Koch, 1937). EC gels are prepared by heating an EC-oil mixture above the glass transition temperature of the EC, approximately 130–140 °C (dependent on the MW of the EC), at which point the EC will dissolve fully in the oil (Davidovich-Pinhas et al., 2014; Davidovich-Pinhas et al., 2015a; Stortz et al., 2012). Dissolution of EC into the oil above this glass transition temperature takes place due to the melting of the glassy regions of the polymer melt, resulting in exposure of the ethoxy groups to the solvent.

Many EC products are commercially available for use as a food additive, and are differentiated according to the viscosity in centipoise (cP) of the solution that they will create when dispersed in 80% toluene and 20% ethanol. Commonly used EC ranges from, 10, 20 or 45 cP, which is based on the average molecular weight (MW) of polymers present, where a higher MW corresponds to a greater viscosity (Dow Cellulosics, 2005). Increasing the viscosity, and MW, of the EC used results in increasing gel strength, making the selection of EC by viscosity (in cP) or MW can be a simple method for controlling oleogel hardness (Bemer et al., 2016; Davidovich-Pinhas et al., 2015a; Dey et al., 2011). Several studies have also shown that the gel strength can be manipulated by changing the type of oil used, EC concentration or by the addition of surfactants (Davidovich-Pinhas et al., 2015c; Davidovich-Pinhas et al., 2015b; Gravelle et al., 2014; Zetzel et al., 2012, 2014). More recent efforts have focused on enhancing the plasticity of EC oleogels through the addition of stearic acid and stearyl alcohol (Gravelle et al., 2017a,b).

### Wax Oleogels

Wax based oleogels are a large category of oleogels, where waxes form a continuous crystal network capable of structuring liquid oil within. The list of waxes used in this application is long, many of which are approved for use in foods. Studied wax oleogelators include rice bran wax, sunflower wax, carnauba wax, candelilla wax, beeswax, fruit wax and shellac wax. Interest in wax oleogels is due to the low concentration of wax required to form a gel (as low as 1%–4% w/w), the stability of the gels that they create, the wide range of properties the gels can attain, and natural origin of the wax gelators. Waxes are composed of a mixture of wax esters, free fatty acids, free fatty alcohols and hydrocarbons, each of varying carbon chain length, as well as a variety of minor components. It is the content wax esters that is generally considered responsible for the gelation behaviour of natural waxes (Blake et al., 2014; Hwang et al., 2012; Patel et al., 2015).

Properties of wax oleogels depend on many critical variables. Each type of wax takes on a characteristic morphology and crystal size. The compositional diversity between waxes causes these differences, which subsequently affects the overall properties of the three-dimensional crystal network that is formed. These differences also cause variation in solubility in different types of oil, making the choice of oil type significant. In addition, the presence of impurities or surfactants, can impact the physical properties of a gel (Chopin-Doroteo et al., 2011). There have also been studies published which demonstrate the use of different cooling rates or the use of shear during cooling as a means of modifying gel functionality (Blake and Marangoni, 2015b,c,d; Morales-Rueda et al., 2009; Toro-Vazquez et al., 2007). Modification of these parameters produces changes in the nucleation and crystal growth rates, which are critical to the microstructure formation and physical properties of the gels.

### Monoglyceride Oleogels

With hydrophobic tails, and hydrophilic heads, monoglycerides (MAGs) are commonly used in foods as emulsifiers. Because of this structure, saturated MAGs form structured w/o emulsions with water through self-assembly into a three-dimensional network of bilayers (Co and Marangoni, 2012). They can also structure liquid oils through formation of inverse bilayers that grow into platelets and form a continuous network (Valoppi et al., 2017b). The use of MAGs in this application is economical, and therefore presents an encouraging oleogelation strategy. The physical properties of MAG gels can be altered by MAG concentration, type of oil used, addition of surfactants, cooling rate and the application of shear (Lopez-Martinez et al., 2014; Ojijo et al., 2004; Valoppi et al., 2017b). It is common for MAGs to be used in combination with other oleogelators, including EC, waxes or phytosterols, due to MAG gel polymorphic instability over time and the desire to improve the viscoelasticity of the gels (Lopez-Martinez et al., 2015; Sintang et al., 2017; Toro-Vazquez et al., 2013).

### Phytosterol Oleogels

Phytosterols are substances naturally occurring in plants, and some, in combination with the sterol ester  $\gamma$ -oryzanol, can be used to structure edible oils at levels as low as 2%–4% w/w. The mixture commonly contains roughly equal molar proportions of sterol to sterol ester, where no gelation can occur in the absence of the other. Together, the components self-assemble uniquely into nano-scale tubules, made possible by distinct elements in their structure (Bot and Agterof, 2006). The tubules will interact and aggregate to form a solid network that can entrap liquid oil. The most common phytosterol used is  $\beta$ -sitosterol, but dihydrocholesterol, cholesterol and stigmasterol can also be used. Gel properties are affected by the type of sterol used, the amount of structurant used, the relative proportions of sterol to sterol ester, cooling rate and application of shear.

### Other Types of Oleogels

While there are many oleogelators that have been investigated, only a small number have actually been used in foods. This section will briefly cover some of those that have the potential for use, but have not yet been extensively studied for the specific uses in foods. First, saturated fatty acids and saturated fatty alcohols can structure oil by crystallizing at ambient temperatures. Both the mechanical properties of these gels, as well as the oil retention properties depend on the carbon chain length, where longer chain lengths (C18–C22) show more efficient gelation properties (Valoppi et al., 2017a). The required concentration for gelation varies greatly based on the fatty acid or alcohol chosen, where those with longer chain lengths induce gelation at lower concentrations (Gandolfo et al., 2004). Additionally, a synergistic effect is observed when using a combination of fatty acids and fatty alcohols, resulting in harder, more elastic gels. One extensively studied combination with a high structuring capacity is stearyl alcohol and stearic acid, commonly referred to together as SOSA. The resultant gels' mechanical properties depend on the SO:SA ratios, and the greatest mechanical strength has been found to result from ratios of 7:3 or 8:2 (Blach et al., 2016). Hydroxylated fatty acids, long chain saturated compounds where substitution of a hydroxyl group has occurred, are very efficient oleogelators, requiring as low as 1% w/w. Commonly used examples are 12-hydroxystearic acid (12-HSA) and ricinelaidic acid, which are obtained from fully or partially hydrogenated castor oil, respectively (Co and Marangoni, 2012; Rogers et al., 2008). Although, hydroxylated fatty acids are not currently approved for use in foods. Finally, Ceramide sphingolipids, formed through fatty acid amidation onto the amine group of sphingosine, will crystallize into bilayers capable of gelling oil. Gelation by ceramides is affected by the fatty acid chain length, where, contrary to fatty acids and alcohols, shorter chain lengths are associated with greater gelation efficiency (Rogers et al., 2009).

### Health Implications of Oleogel Consumption

Replacement of solid fat with novel ingredients in food products must take into account the digestibility of the new component, however extensive research in this area is lacking. A study by Duffy et al. (2009) concluded that the structure of an oleogel can partially limit interactions between lipases and oil, determined by *in vitro* digestion of phytosterol gelled oleogels. However, contradictory results were reported by Hughes et al. after an investigation into consumption of 12-HSA oleogels found the post-prandial increase in serum lipids resultant from digestion of the oleogel was not significantly different from that of liquid oil (Hughes et al., 2009). Yu et al. (2012) also determined that there was no significant difference in the extent of lipolysis between gelled and liquid oil systems after a 30 minute *in vitro* digestion experiment with medium TAG systems gelled by monoglycerides and containing

sorbitan monolaurate. Investigation by O'Sullivan et al. found through *in vitro* methods that the hardness of an EC oleogel is a significant factor with regards to digestion of the oleogel, as hardness influences mastication breakdown, the particle size swallowed, and the eventual breakdown in the body by lipases (Guo et al., 2013, 2016; O'Sullivan et al., 2016). Harder EC gels showed a significantly lower extent of lipolysis and appeared to be more resistant to breakdown when compared to un-gelled oil control samples and weak oleogels. This leads to the conclusion that, while weaker oleogels retain the digestibility of edible oils, digestion of harder oleogels is altered. Tan et al. (2017) found similar results during *in vivo* digestion of EC oleogels after feeding coconut oil or an EC coconut oil oleogel with carbohydrate rich meals. Harder oleogels experienced delays in digestion, and the monitored postprandial glucose and insulin levels were more similar to meals containing no fat than meals containing ungelled coconut oil. However, it must be noted that this study was not conducted on a representative group of individuals and only indirectly measured lipid digestibility by postprandial TAG levels. Together, these studies demonstrate that the digestibility of the oleogels depends on the type of oleogelator used. Therefore, oleogel digestion cannot be summarized into one general trend; instead, each must be treated individually. Additionally, more *in vivo* studies are required before strong conclusions can be made.

## Oleogels in Food Systems

The use of oleogels in food systems must consider many variables. This includes the approval of the gelator as a food additive, the processing conditions required in both the food and oleogel production, and the impact of oleogels on the food's physical properties. Significant research has been done to determine the different types of foods that can best incorporate oleogels, and which types of oleogels are best for each system. A summary of the documented uses of different types of oleogels in specific food applications can be found in Table 1.

### Processing Conditions

Oleogels are strongly influenced by the processing conditions involved in their production and during incorporation into food products. Important conditions include the cooling rate used and the application of shear. Because of their structured nature, most oleogels will break and leak oil if sheared after gelation, therefore successful integration of gels into food matrices can require strategic planning. Another consideration is heating, where temperatures as high as 80–140 °C are required for oleogelation. Some foods are capable of withstanding these high temperatures, therefore certain food applications may be able to employ direct dispersion of EC into an oil containing food matrix. In most cases, this temperature exceeds that which the food materials can withstand, and gels must be prepared prior to incorporation. The high temperatures during oleogel production must also be monitored closely, as oil held for excessive time at this temperature will oxidize and the oxidation products that develop create off-flavors. Gravelle et al. (2016) demonstrated that after just 20 minutes of holding at 140 °C, canola oil will contain over 10 meq peroxide/kg canola

**Table 1** Applications of oleogels in food systems

Oleogel type	Food system	Oleogel function	References
Ethylcellulose	Cream cheese	Saturated fat reduction	Bemer et al. (2016)
	Frankfurters/comminuted meats	Saturated fat reduction	Barbut et al. (2016b) and Zetzi et al. (2012)
	Breakfast sausages	Saturated fat reduction	Barbut et al. (2016a)
	Cookies and cream fillings	Reduction of oil migration	Stortz et al. (2012)
Waxes	Spreadable fat alternatives	Saturated fat reduction	Patel et al. (2014) and Yilmaz and Ögütcü (2015)
	Ice cream	Saturated fat reduction	Zulim Botega et al. (2013)
	Cookies	Saturated fat reduction	Hwang et al. (2016), Jang et al. (2015), Mert and Demirkisen (2016), and Yölmaz and Ögütcü (2015)
	Deep frying	Decreased oil absorption; saturated fat reduction	Lim et al. (2017)
	Chocolate paste	Replacement of oil binder; partial replacement of palm oil	Patel et al. (2014)
	Cream cheese	Saturated fat reduction	Bemer et al. (2016)
	Cake shortening	Saturated fat reduction	Patel et al. (2014)
	Sweet bread	Saturated fat reduction	Calligaris et al. (2013)
Monoglycerides	Pastry and sweet bread	Saturated fat reduction	Manzocco et al. (2012a,b)
Phytosterols	Frankfurters	Saturated fat reduction	Panagiotopoulou et al. (2016)
Monoglycerides + phytosterols	Frankfurters	Saturated fat reduction	Kouzounis et al. (2017)
	Flavor delivery system	Controlled volatile release	Yang et al. (2017)
Waxes + monoglycerides	Spreadable margarine alternative	Saturated fat reduction	Ögütcü and Yölmaz (2014)
	Laminating shortening	Saturated fat reduction	Blake and Marangoni (2015a)



oil, and can no longer be termed “fresh” oil. Adding food-grade antioxidants can reduce the extent of oil oxidation during heating, added in concentrations ranging from 100 to 500 ppm by weight of oil (Ergun et al., 2016). Barbut et al. (2016b) cite the use of butylated hydroxytoluene (BHT) in combination with rosemary extract, added to oil prior to heating, to reduce the off flavors associated with oil oxidation in beef frankfurters. It is also possible to limit oxidation by heating oil under inert atmospheric conditions, such as under vacuum or 100% nitrogen (Ergun et al., 2016).

### Impact on Texture

Oils and fats contained in food are distributed differently, attributing different characteristics to the food product. Generally, oil globules are distributed evenly and are micrometers in size, while solid fats exist as larger globules varying in size, with a minimum around 100  $\mu\text{m}$  (Youssef and Barbut, 2009). This difference prevents the direct substitution of solid fat with oil as large changes in texture and rheological properties will result. Studies have shown that presence of very small non-gelled oil globules cause a harder, rubbery texture in cream cheese and frankfurters, likely observed due to the increased fat globule surface area, resulting in the ability of proteins to form a stronger network (Bemer et al., 2016; Zetzl et al., 2012). These studies also found that oleogels (of either wax or EC) created statistically similar microstructures and fat globule size distributions to solid fat in cream cheese and comminuted meat systems, leading to comparable hardness by instrumental testing. Breakfast sausages containing harder EC oleogels with added sorbitan monostearate (SMS), a common surfactant, were also found to have comparable instrumental hardness to pork fat controls (Barbut et al., 2016a). Contrasting previous investigations, pork fat sausages in this study were the hardest of the samples, while samples containing liquid oil or softer oleogels were significantly softer. Despite comparable hardness values by instrumental analysis in the harder oleogel samples, sensory analysis revealed that differences in hardness and texture are still perceivable by consumers. All oleogel samples received notably lower hardness scores from panelists when compared to pork fat. These studies all emphasize the need to tailor the physical properties of oleogels for the application, as well as the need for sensory analysis.

Textural implications resulting from the incorporation of oleogels into bakery products have been documented in many studies. Replacement of solid fat with liquid oils in baked products causes problems due to the loss of functionality – namely, the ability of solid fat to stabilize air bubbles throughout the batter or dough – as well as an increase in greasiness or oiliness. Since bakery products are often high in saturated or *trans* fat, many studies have explored the use of oleogels, finding it extremely intriguing and promising. The use of oleogels in cakes or sweet breads was shown to increase the density, decrease height and cause a crumbly texture, however, oleogel based structured emulsions had much greater success (Manzocco et al., 2012a; Patel et al., 2014). In general, the use of oleogels in cookies has been shown to effect hardness and dough spreadability during baking, with the exact magnitude depending on the gelator used (Blake and Marangoni, 2015a; Hwang et al., 2016; Jang et al., 2015; Mert and Demirkesen, 2016; Yölmaz and Ögütçü, 2015). Though, one sensory study found that the differences observed were not beyond what panelists deemed acceptable, and cookies containing wax oleogels were still found to have acceptable flavor, texture, smell and appearance (Yölmaz and Ögütçü, 2015).

Another consideration is the texture or consistency of the food when in the mouth, because the melting temperature of oleogels is greater than traditional solid fats. This higher melting temperature eliminates the common and desirable “melt in your mouth” sensation, especially when considering replacement of butter with oleogels. However, when melting is a key product attribute, it is possible that beginning with only partial replacement could produce products that are more comparable.

### Added Functionality

From a food and nutritional perspective, certain oleogels have more advantages than others do. Phytosterol and sterol ester oleogels have the inherent benefit that phytosterols themselves are bioactive molecules. Studies surrounding the consumption of plant phytosterols have found many beneficial health effects, including lowering LDL cholesterol levels, and therefore decreasing risk of cardiovascular disease, as well as decreasing the risk of certain cancers, including breast, colon and prostate (Awad and Fink, 2000; Bradford and Awad, 2007; Moghadasian and Frohlich, 1999). Though, the bioactivity of phytosterols contained within sterol and sterol ester oleogels has not been investigated, and it is unknown whether the digestibility of the oleogels would affect the deliverability and bioactivity of these molecules.

In the case of EC oleogels, it appears oleogel strength or hardness can impact the residence time of the material in the digestive tract (McClements et al., 2008; O’Sullivan et al., 2016; Tan et al., 2017). This has potential benefits, where harder oleogels could act, for example, as carriers for highly lipophilic bioactive molecules protected by the undigested oleogel structures. An example would be carotenoids, more specifically  $\beta$ -carotene, known to be potent antioxidants, provide some dietary vitamin A, and are also associated with reducing the risk of chronic diseases, such as cardiovascular diseases and cancer (Cooper et al., 1999; Krinsky and Johnson, 2005; Rao and Rao, 2007). Oleogels of sufficient hardness could be capable of providing protection to  $\beta$ -carotene, and therefore have potential controlled release delivery applications (O’Sullivan et al., 2017).

### Conclusions

The use of oleogels can improve the nutritional properties of high saturated and *trans* fat foods through solid fat replacement with unsaturated sources. Additionally, manipulation of known variables allows oleogels to be produced having specific mechanical



properties, similar to that of the solid fat that they will replace and specific to the particular food application. Many oleogelators have been successfully incorporated into different food products, including ground or comminuted meats, soft cheeses, and high fat bakery products. However, there is currently a lack of sensory analysis on oleogel containing foods, knowledge for how oleogels are digested, and the feasibility of large-scale oleogel production. Additionally, taste considerations must be acknowledged to ensure that the gelators themselves, or products of oil oxidization as a result of heating, do not create overwhelming off flavors. Further investigation into aspects inherent in industrial scale production of oleogel containing products, including storage conditions and shelf life, should also be considered.

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# Oxidative Rancidity

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## Introduction

In foods, lipids are mainly found in the form of triacylglycerols (triacylglycerides) (TAG), which make up to 99% of lipids of plant and animal origin (Fennema, 1996). Phospholipids (PL) are important structural lipids in foods and cell membranes. Most of the PL are removed by degumming in the refining of vegetable and fish oils (Frankel, 2005) and these oils therefore mainly consist of triacylglycerols, but in foods such as egg, meat and fish, phospholipids can constitute a larger part of the lipids (e.g. cod phospholipids constitute 87% of the total lipids). Oxidation of the lipids in foods is one of the most important factors limiting their shelf life. PL are more susceptible to lipid oxidation than TAG, partly because they are more unsaturated. Lipid oxidation gives rise to the formation of unhealthy compounds such as free radicals and reactive aldehydes and it reduces the nutritional value of lipids. The negative health effects of lipid oxidation is not acute and therefore the consumer will not be affected immediately. However, lipid oxidation will also result in significant changes in the sensory properties including odor, flavor, color and texture. These changes can be detected by the consumer and may thus determine the shelf life of the product. The term rancid is often used to describe the sensory changes caused by lipid oxidation. Oxidative rancidity is in this case the oxidative deterioration of oils/fats or foods containing oils/fats during food processing and storage. However, rancidity can also occur due to lipid hydrolysis. In this case, we use the term hydrolytic rancidity to describe the chemical deterioration of the food lipids. As an example, both hydrolytic and oxidative rancidity are important reactions in non-pasteurized milk, where oxidation of milk lipids will lead to oxidative rancidity and lipolytic enzymes will give rise to hydrolytic rancidity. In products containing both lipids and protein such as milk, meat and fish, lipid oxidation will go hand in hand with oxidation of the proteins. This chapter will, however, not discuss protein oxidation any further.

## Lipid Oxidation Processes

Unsaturated fatty acids bound in the lipid molecules (TAG or PL) or as free fatty acids are the basic substrate of lipid oxidation. The direct oxidation of unsaturated fatty acids by low energy, ground state oxygen (triplet oxygen  $^3\text{O}_2$ ) is spin forbidden, but this barrier can be overcome in the presence of initiators that can produce radicals or by other means. Thus, three different types of lipid oxidation reactions can occur: 1) Enzymatic lipid oxidation, 2) Autoxidation, which is a reaction between free lipid radicals with oxygen, and 3) Photooxidation resulting from exposure of lipids to light in the presence of photosensitizers. In case of autoxidation (see Fig. 1), the presence of initiators (e.g. metal ions, heat, protein or already existing lipid radicals) causes unsaturated fatty acids (LH) to form alkyl radicals ( $\text{L}\cdot$ ). These radicals react fast with oxygen to form peroxy radicals ( $\text{LOO}\cdot$ ). The peroxy radical reacts with a new unsaturated fatty acid to form hydroperoxides ( $\text{LOOH}$ ) and a new lipid radical, which will subsequently propagate the chain reactions. Lipid hydroperoxides are the primary products of autoxidation. Because of their low volatility they are taste- and odorless. The free radical chain reaction propagates until two free radicals join and form a non-radical product, which will terminate the chain reaction.

The lipid hydroperoxides can be decomposed to alkoxy and peroxy radical intermediates ( $\text{LO}\cdot$  and  $\text{LOO}\cdot$ ) by thermal dissociation or by the presence of trace metals such as iron and copper. Both radicals can effectively propagate the free radical chain

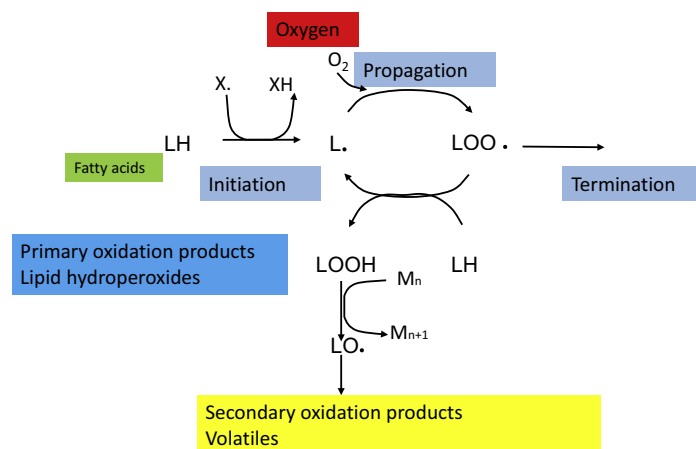


Figure 1 Lipid autoxidation process.

to form new hydroperoxides (Frankel, 1991). Lipids consist of a mixture of different fatty acids, which will result in a complex mixture of lipid hydroperoxides, differing in chain length, the position of the HOO-group and the geometrical structure of the double bonds. In muscle food, decomposition of lipid hydroperoxides is primarily catalysed by heme iron. Both myoglobin (Mb) and hemoglobin (Hb) appear to control the onset of oxidation via several different complex mechanisms as reviewed by Undeland (2016). Hb is a tetrameric molecule with allosteric O<sub>2</sub>-binding properties while Mb is a monomer without allosterism. The ratio between Hb and Mb is approximately equal in dark muscle while Hb dominate in light fish muscle (O'Brien et al., 1992). In general, the ratio between Mb and Hb is lower in fish than in beef (Livingston and Brown, 1981; Matsuura and Hashimoto, 1954).

In the presence of light and photosensitizers (e.g. riboflavin in milk, chlorophyll in unrefined oils) photooxidation of unsaturated fatty acids can occur. The photosensitizers will be activated by absorbing visible or near-UV light. There are two types of photosensitizers with different reaction mechanisms. Type I sensitizers react with the lipids whereby lipid radicals are formed and they subsequently react with oxygen in a similar way as described for autooxidation. Type II sensitizers react directly with triplet oxygen, transforming it into the short-lived but highly reactive singlet oxygen, <sup>1</sup>O<sub>2</sub> (Fig. 2). The double bond of the unsaturated fatty acids then reacts with singlet oxygen to form hydroperoxides (ROOH) (Frankel, 2005). This is a non-free-radical process and will generate other types of lipid hydroperoxides (non-conjugated hydroperoxides) in addition to those formed through the free radical oxidation process (conjugated hydroperoxides) (Fig. 3).

As mentioned earlier alkoxyl radicals are readily formed from decomposition of lipid hydroperoxides at high temperatures and/or in the presence of traces of transition metals. Alkoxyl radicals may further decompose to form a variety of non-volatile and volatile secondary oxidation products (Frankel, 1991). The latter compounds are responsible for the flavor deterioration that is a result of lipid oxidation (Aidos et al., 2002; Aro et al., 2003; Frankel, 1983; Kulås et al., 2002; Venkateshwarlu et al., 2004).

Photosensitizers become electronically excited  
by absorbing light: UV or visible

Photosensitizers have two excited states

Singlet state: <sup>1</sup>Sens and Triplet state: <sup>3</sup>Sens

There are two types of photosensitized reactions:

#### Type 1:



#### Type 2: Singlet oxygen production



Figure 2 Photosensitized lipid oxidation.

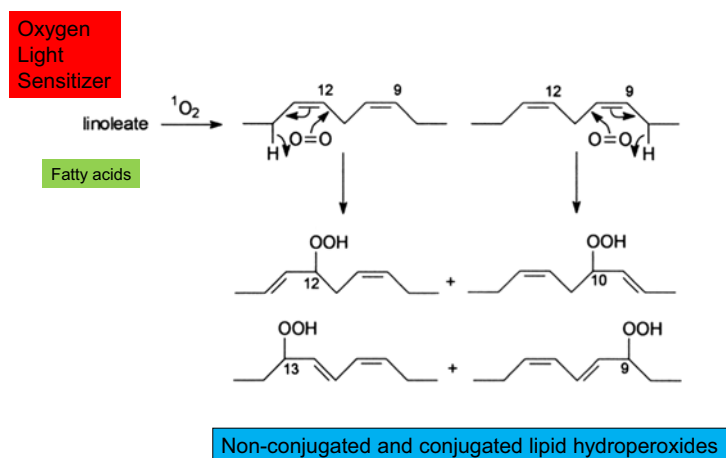
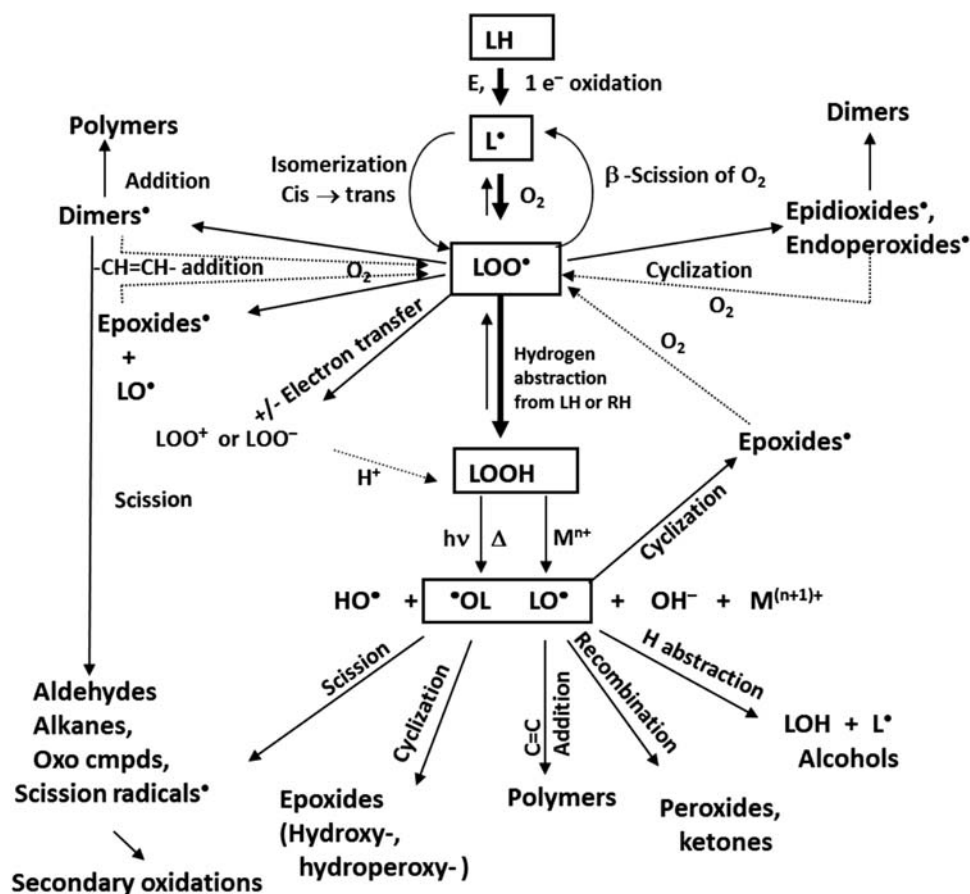


Figure 3 Formation of non-conjugated and conjugated lipid hydroperoxides.



**Figure 4** Reaction scheme for lipid oxidation integrating alternate pathways. From Schaich, K., 2005. Lipid oxidation in fats and oils: theoretical aspects. In: Shahidi, F. (Ed.), *Bailey's Industrial Fats and Oils*, sixth ed. John Wiley, New York, pp. 2681–2767. Used with permission.

From the chain reactions described above a specific sequence in formation of products is expected from an analytical point of view. Thus, first conjugated dienes, then lipid hydroperoxides, and then secondary volatile oxidation products, which would only evolve after hydroperoxide decomposition. However, because experimental results for both kinetics and products often have been inconsistent with the traditional reaction scheme a more complex, integrated reaction scheme that may more fully and more accurately describe the complex reactions of lipid oxidation have been developed in the recent years (Fig. 4) (Schaich, 2012, 2016). This reaction scheme shows the existence of simultaneous alternate pathways, which includes addition, rearrangement, and dismutation reactions of lipid peroxy radicals ( $\text{LOO}^\bullet$ ), which generate dimers, epoxides, and aldehydes from the beginning of oxidation in parallel with hydroperoxides, and they can divert radicals from formation of hydroperoxides. The existence of these alternate pathways has consequences for the type of measurements that should be made to follow lipid oxidation during storage. See further discussion in the section about methods for measuring lipid oxidation.

### Sensory Impact of Lipid Oxidation

Only the secondary volatile oxidation products formed during lipid oxidation are responsible for the undesirable changes in the aroma and flavor properties of foods. Among these compounds the vinyl ketones and the trans, cis-alkadienals have the lowest flavor thresholds in oils (Table 1). In contrast, hydrocarbons (alkanes and alkenes) have the highest flavor thresholds (Frankel, 2005).

The same volatile oxidation product can give rise to different off-odors and off-flavors depending on its concentration and on the food matrix in which it occurs. Lipid oxidation of neat oils will give rise to off-flavors ranging from nutty through green, grass cucumber to rancid and synthetic. For fish oils, the term train oil or fishy has frequently been used. The oil may even develop a painty odor if lipid oxidation is extreme.

Flavor defects caused by lipid oxidation in milk and cream are usually referred to as “oxidized flavor” and in butter they are referred to as “metallic” or “tallowy” (Frankel, 2005). Oxidized flavors are very easily detected in fluid milk even at very low oxidation levels ( $\text{PV} < 1 \text{ meq/kg}$ ) (Frankel, 2005). Exposure of milk or butterfat to sunlight produces off-flavors that have been described as burnt, light-activated or sunlight flavor (Frankel, 2005).

**Table 1** Threshold values of compounds formed from oxidized oils

Compounds	Thresholds (mg/kg)
Hydrocarbons	90–2150
Substituted furans	2–27
Vinyl alcohols	0.5–3
1-alkenes	0.02–9
2-alkenals	0.04–2.5
Alkanals	0.04–1.0
Trans,trans-2,4-alkadienals	0.04–0.3
Isolated alkadienals	0.002–0.3
Isolated cis-alkenals	0.0003–0.1
Trans,cis-alkadienals	0.002–0.006
Vinyl ketones	0.00002–0.007

Akoi, C.C., Min, D.B., 1998. Food Lipids: Chemistry, Nutrition, and Biotechnology (Food Science and Technology), Marcel Dekker.

In meat products, the oxidized flavors in precooked, stored and reheated meat is often described by the term “warmed over flavor” (WOF), which is mainly caused by oxidation of lipids. Particularly the formation of hexanal, 1-octen-3-one, (E)- and (Z)-2-octenal, (Z)-2-nonenal (E,E)-2,4-nonadienal and trans-4,5-epoxy-(E)-2-decenal seems to play a role for the formation of WOF (Konopka and Grosch, 1991). WOF has been suggested to mainly be the result of oxidation of membrane phospholipids (Gray and Pearson, 1987).

The most important flavor change during frozen storage of fatty fish such as salmon is the formation of train oil, bitterness and metallic tastes (Refsgaard et al., 1998). Milo and Grosch (1995) suggested that the increased concentration of 1-octen-3-one (Z)-1,5-octadien-3-one, hexanal (Z)-3-hexenal (Z)-4-heptenal (Z,Z)-2,6-nonadienal and (E,Z)-2,6-nonadienal observed during storage of cod most likely were important contributors to the train oil odor in the stored cod sample.

Apart from affecting odor and flavor, lipid oxidation may also affect color. Autoxidation of heme iron may decrease the red color of fish muscle (Sannaveerappa et al., 2007), whereas lipid oxidation of the lipids will lead to an increase in a more yellow hue.

## Measurement of Lipid Oxidation

### Accelerated Methods Versus Traditional Storage Experiments

Evaluation of the oxidative flavor deterioration of neat lipids can be performed at ambient temperatures or lipid oxidation can be accelerated by elevating the temperature to 30 °C or above. It is, however, important to bear in mind that already at temperatures above 40 °C oxidation kinetics will change for some oil types as illustrated for fish oil (Sullivan et al., 2011). Therefore, oxidation studies carried out at high temperatures may not be representative for reactions taking place at lower temperature. Regarding emulsified foods such as dressing and mayonnaise temperatures above 30 °C may break the emulsions and therefore it will not be possible to increase temperature above this level. For other food products such as raw meat and fish products higher temperatures than 5 °C will lead to microbial spoilage and storage experiments are therefore carried out at lower temperatures than 5 °C. For neat oils and emulsions storage experiments at ambient temperatures may be accelerated by light or by the addition of metals such as iron or copper, whereas for fish muscle models oxidation is accelerated by addition of heme iron (Jacobsen et al., 2008). It is important to bear in mind that light induced oxidation will give rise to other types of secondary oxidation products and thereby other off-flavors than those produced by autoxidation.

### Analytical Methods for Lipid Oxidation Measurements

Measurement of lipid oxidation can be carried out by a wide range of methods such as peroxide value (PV), anisidine value (AV), thiobarbituric acids reacting substances (TBARS) as well as instrumental methods such as HPLC, GC-MS, NIR, FTIR and DSC. Sensory evaluation of oxidative flavor deterioration is another important method that should always be included at some stage to understand how lipid oxidation has impacted sensory properties of the food product in question. In lipid oxidation studies on meat products there seems to be a fairly good correlation between TBARS and sensory data, although in many cases no attempts have been made to statistically evaluate the correlations (Eckert et al., 1997; Murano et al., 1998; Winne and Dirinck, 1997). However, newer data indicate that even in meat TBARS may be an unreliable method (Summo et al., 2010). This may be related to the fact mentioned above that lipid oxidation have several alternate pathways. Therefore, if lipid oxidation is only followed by measuring formation of TBARS and peroxide values the major proportions of oxidation products may be missed and the extent of lipid oxidation may be significantly underestimated. The same is the case for studies, which only measure peroxide value and hexanal (aldehyde formed from n-6 fatty acids). In complex foods, the situation is even more complicated. This is due to the fact that lipid oxidation radicals and secondary oxidation products (e.g. aldehydes) react with other components in foods, particularly



proteins. This type of co-oxidation will thus consume lipid oxidation intermediates and products and at the same time other substrates in the food system will be affected by oxidation. If only the traditional lipid oxidation measurements are performed, lipid oxidation can be underestimated, when it is in fact spread to other components of the food system, which may have an effect on sensory properties of the food product.

Good correlations data from headspace GC analysis and sensory data have been found for a range of different oxidized products such as fish (Milo and Grosch, 1995), fish oil enriched milk (Venkateshwarlu et al., 2004), mayonnaise and dressing (Hartvigsen et al., 2000; Let et al., 2007a) and boiled potatoes (Blanda et al., 2010). Headspace GC analysis can therefore be recommended as one of the best methods to chemically assess oxidative flavor deterioration. Different headspace methods are available for collection of the volatile oxidation products including static headspace, dynamic headspace, purge and trap and solid phase microextraction. Thomsen et al. (2016) have recently compared the performance of some of these methods in neat oil and two emulsified systems.

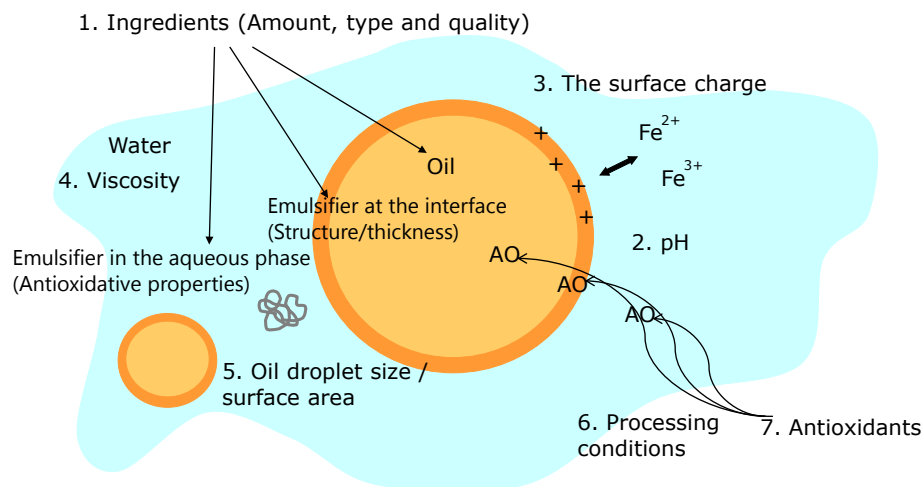
## Factors Affecting Lipid Oxidation in Neat Oils Versus Complex Food Systems

### Neat Oils

In neat oils, lipid oxidation is primarily affected by light, temperature, the oxygen level, the presence of minor components such as water, trace metals, free fatty acids (FFA), phospholipids (PL), monoacylglycerides (MAG), diacylglycerides (DAG) and natural antioxidants and pigments. Light will only increase the rate of lipid oxidation if a photosensitizer is present whereby singlet oxygen is formed as described above. If this is the case, lipid oxidation rates can be more than 1000 times faster compared to free radical oxidation with triplet oxygen (Frankel, 2005). Regarding temperature, as a rule of thumb lipid oxidation rates will double every time temperature is increased by 10 °C. The effect of minor components on lipid oxidation has been reviewed by Chen et al. (2011). Some important conclusions from the review are summarised here. Water can affect the rate, extent and oxidation mechanism in neat oils because it may act as a solvent for hydrophilic or amphiphilic antioxidants or prooxidants. Free fatty acids have been suggested to promote lipid oxidation due to their ability to accelerate decomposition of hydroperoxides and bind metals to make them more prooxidative. The effect of PL on lipid oxidation is controversial as some studies have found antioxidative effects of PL, particularly in the presence of tocopherol where PL have been found to have a synergistic effect. In contrast, other studies have shown either no effect or a prooxidative effect of PL. Whether PL has no, antioxidative or prooxidative effect seems to depend on the exact composition of the mixture of PL, the composition of the neat oil including the presence of other minor components as well as the conditions under which the study was performed. Importantly, FFA, PL, MAG and DAG are surface active compounds, which can form physical structures (association colloids) in neat oils in the presence of water (~300 ppm). A variety of association colloids can be formed such as reverse micelles, micro-emulsions, lamella structures and cylindrical aggregates (Chen et al., 2011). It is well known that the physicochemical properties of water–oil interfaces can impact lipid oxidation. Therefore, water–oil interfaces of association colloids can also impact lipid oxidation. How and to which extent the association colloids impact lipid oxidation depends on the exact composition and structure of the water–oil interface. See Chen et al. (2011) for further discussion of this topic.

### Complex Food Systems

In complex food systems, lipid oxidation is obviously more complex because foods consist of many ingredients, which can affect lipid oxidation in different ways. Since many foods are food emulsions, some of the factors that can affect lipid oxidation in emulsions will be summarised in the following. Fig. 5 illustrates an oil-in-water (o/w) emulsion. Milk, yoghurt, mayonnaise



**Figure 5** Factors affecting lipid oxidation in emulsions.

and dressing are examples of oil-in-water food emulsions. When lipids are dispersed as small droplets in an o/w emulsion there is a huge increase in the lipid-water surface area. For example, when the average droplet diameter decreases from 100  $\mu\text{m}$  to 100 nm, the specific surface area increases from 65.2  $\text{m}^2/\text{kg}$  to 65,220  $\text{m}^2/\text{kg}$  (Decker et al., 2017). As mentioned above lipid oxidation is an interfacial phenomenon because it is often catalysed by water soluble trace metals, which reacts with lipids or lipid hydroperoxides located near the oil-water interface. Therefore, lipid oxidation is typically faster in emulsions than in neat oils.

In food emulsions, water soluble ingredients will be located in the aqueous phase, oil soluble compounds in the oil phase and amphiphilic compounds at the interface, where they can interact with the emulsifier. Lipid oxidation in emulsions is affected by factors such as pH, physical structure of the emulsion, composition, microstructure and charge of the interface and partitioning of the emulsifier between the interface and the aqueous phase as discussed in a recent reviews by Berton-Carabin et al. (2014) and Jacobsen (2015) and as illustrated in Fig. 5.

As mentioned above, trace metals present in the aqueous phase may promote decomposition of lipid hydroperoxides located near the interface of the lipid droplets whereby alkoxyl radicals are formed. These radicals will then diffuse inside the oil droplet and react with another lipid whereby lipid oxidation propagates. The oil-water interface should therefore be designed to prevent this interaction between trace metals and lipid hydroperoxides. Factors that can affect the ability of the interface to resist the interaction between lipid hydroperoxides and trace metals include the thickness and physical properties of the interface as well as its charge. If the interface is positively charged it will repel the positively charged trace metal ions (e.g.  $\text{Fe}^{2+}$ ) (Mei et al., 1998). In contrast, if the interface is negatively charged, it will attract trace metal ions and increase lipid oxidation. The charge of the interface is determined by pH and the type of emulsifier used. Furthermore, pH also affects the solubility of trace metals. For example,  $\text{Fe}^{2+}$  is more soluble at low pH than at neutral pH.

Because lipid oxidation is an interfacial phenomenon, the rate of lipid oxidation could be expected to be depending on size of the total interfacial area, i.e. the droplet size as shown in Fig. 5. However, contradicting reports are available on the effect of the droplet size on oxidation in different food systems (Jacobsen et al., 2000; Lethuaut et al., 2002; Let et al., 2007b). The available data thus suggest that other factors than the mere interfacial area are of significance for oxidation.

Fig. 5 also shows that the emulsifier may be present in the aqueous phase. This is the case when there is an excess of emulsifier and the unadsorbed emulsifier will then partition into the aqueous phase. Particularly unadsorbed proteins may strongly interact with water soluble free radicals and metal ions whereby lipid oxidation can be affected (Donnelly et al., 1998; Ponginebbi et al., 1999; Faraji et al., 2004). In many cases, the unadsorbed protein will act as an antioxidant due to its ability to chelate metals or scavenge free radicals as reviewed by Berton-Carabin et al. (2014). Likewise, unadsorbed surfactants present in the aqueous phase in concentrations above CMC can form colloid structures such as micelles, mixed micelles or very small droplets, which has been shown to reduce oxidation rates in some cases (Ponginebbi et al., 1999; Berton et al., 2011).

Viscosity of the surrounding aqueous phase depends on the ingredients used to produce the food emulsion. A high viscosity of the aqueous phase will slow down the diffusion of reactants, which includes trace metals and even some polar lipid hydroperoxides, which may be present in the aqueous phase.

## Prevention of Lipid Oxidation by Antioxidants

### Antioxidant Mechanisms

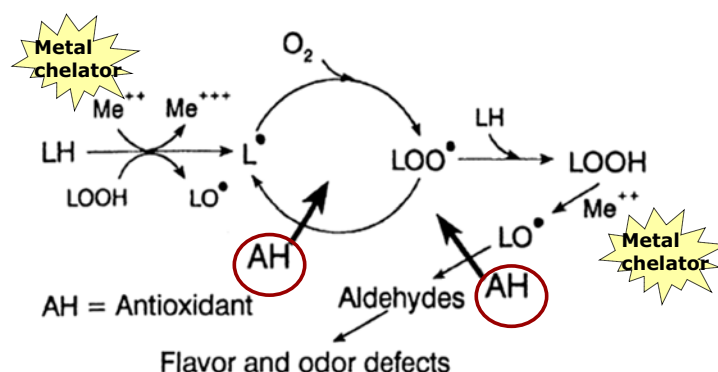
Antioxidants can retard or inhibit lipid oxidation. Antioxidants are classified as primary or secondary antioxidants based on their mechanism of action. Some antioxidants have more than one mechanism of action and are referred to as multiple-function antioxidants. Primary antioxidants, also referred to as chain-breaking antioxidants, are able to react directly with free radicals and convert them to more stable, non-radical products. In the initiation and propagation steps, antioxidants react with lipid, peroxy and alkoxyl radicals, whereby further decomposition into aldehydes and other volatile oxidation products is prevented (Fig. 6). Phenolic compounds with one or more hydroxyl groups are effective chain breaking antioxidants because they can donate H-atoms to the free radicals. After they have donated their H-atom they form stable and relatively unreactive phenoxyl radicals. Examples on synthetic phenolic compounds are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate and tertiary butyl hydroquinone (TBHQ). Examples on natural phenolic compounds are tocopherol, ascorbic acid, caffeic acid and rosmarinic acid.

Secondary antioxidants inhibit lipid oxidation by different mechanisms such as chelation of transition metals, oxygen scavenging and quenching of singlet oxygen. Some secondary antioxidants are able to regenerate primary antioxidants in a synergistic manner. In food systems, metal chelators, which prevent the metal ions from decomposing lipid hydroperoxides to reactive radicals, are often very important secondary antioxidants. Examples of metal chelators are synthetic ethylenediaminetetraacetic (EDTA), polyphosphates, phytate, caseinate and lactoferrin. Polyphenols are often multi-function antioxidants with both free radical scavenging and metal chelating properties.

### Factors Affecting Antioxidant Efficacy

Apart from the basic properties of antioxidative compounds mentioned above the efficacy of antioxidants in complex foods is affected by many other factors and is therefore hard to predict. In 1980, Porter proposed the "the polar paradox", which stated that polar antioxidants work best in bulk oils while non-polar antioxidants work best in lipid dispersions such as oil-in-water

- **Primary or chain breaking antioxidants (radical scavengers) (AH)**



- **Metal chelators can chelate transition metals such as  $Fe^{2+}$**

**Figure 6** Antioxidant mechanisms.

emulsions and liposomes (Porter, 1980). For several years, attempts have been made to use the polar paradox theory to predict antioxidant effects in emulsified systems. Unfortunately, in several cases the experimental data did not agree with the predictions made from this theory. Recent research has compared the antioxidative effect of antioxidants esterified to fatty acids with a chain length from 2 to 20 carbon atoms in simple oil-in-water emulsions (Laguerre et al., 2009, 2010). On the basis of these results the so-called cut off theory was proposed. In brief, this theory states that the efficacy of polar antioxidants such as ascorbic acid in emulsions can be increased by lipophilizing the antioxidants up to a certain length of the alkyl group esterified to the polar antioxidant. Beyond the optimal chain length of the alkyl group, the efficacy of the lipophilized antioxidant will collapse. The cut-off theory has been confirmed in simple oil-in-water emulsions with antioxidants such as rosmarinic acid (Laguerre et al., 2010), chlorogenic acid (Laguerre et al., 2009) and dihydrocaffeic acid (Sørensen et al., 2012). In general, it was found that antioxidant esters had maximal activity in the 8–12 carbon chain length with esters with short and longer chain lengths being less effective. However, lipophilisation of protocatechuic acid did not improve their efficacy in simple soy o/w emulsions (Silva et al., 2017).

The absence of AO hydrophobicity effect on the antioxidant efficiency of protocatechuates was explained by their concentrations at the interfacial region of soybean oil emulsions, which is believed to be the site where AOs react with lipid radicals. The distributions of the esters in the emulsions were assessed by using a novel approach based on a pseudophase kinetic model for interpreting observe rate constants ( $k_{obs}$ ) for the reaction between a hydrophobic arenediazonium ion probe ( $16-ArN_2^+$ ) and the antioxidants as also described in previous works by this group. The results obtained for protocatechuates suggested that lipophilization with alcohols of different chain length had a modest effect on their interfacial concentrations, which as a consequence, led to insignificant variations in their antioxidant efficiency.

When ferulic acid and caffeic acid esters were evaluated in fish oil enriched milk other optimal chain lengths than those reported in simple emulsions were found. For both phenolic compounds it was observed that the short chain esters (with chain lengths C1 or C4) were the most efficient antioxidants (Sørensen et al., 2015; Alemán et al., 2015). This suggests that the optimal chain length of an antioxidant in complex food systems cannot be predicted from studies in simple o/w emulsions. This is most likely due to the fact that antioxidants can impact lipid oxidation kinetics in many ways and therefore their efficacy is not only governed by their physical location. Moreover, antioxidants may adsorb and interact with the oil-water interface. A recent review by Decker et al. (2017) discusses the hurdles in predicting antioxidant efficacy in oil-in-water emulsions in more detail. The reader is also referred to another review by McClements and Decker (2018) which discusses the strategy of using antioxidant emulsifiers to prevent lipid oxidation. An antioxidant emulsifier is in this context defined as “a substance that can adsorb to oil-water interfaces, enhance the formation and physical stability of emulsions and inhibit lipid oxidation”.

For more details on the effect of antioxidants in a wide range of food emulsions the reader is referred to Jacobsen (2016).

## Conclusions

Lipid oxidation has significant impact on the sensory properties and the shelf life of foods. Lipid oxidation reactions can proceed through several alternate and parallel pathways. Therefore, one should carefully consider which methods to use for measuring the extent of lipid oxidation. The rate of lipid oxidation can be affected by several different factors, which can have different impact depending on the type of food. Lipid oxidation can be prevented by the addition of antioxidants, but with our current knowledge it is hard to predict the efficacy of antioxidants in different foods.

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## Pectic Enzymes

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### Glossary

**Arabinofuranosidases** (EC 3.2.1.55) act on  $\alpha$ -L-arabinofuranosides,  $\alpha$ -L-arabinans containing (1,3)- and/or (1,5)-linkages, arabinoxylans, and arabinogalactans.

**$\beta$ -elimination** in organic chemistry class, one learns that elimination reactions involve the cleavage of a  $\sigma$  bond and formation of a  $\pi$  bond. A nucleophilic pair of electrons (either from another bond or a lone pair) heads into a new  $\pi$  bond as a leaving group departs. This process is called  **$\beta$ -elimination** because the bond  $\beta$  to the nucleophilic pair of electrons breaks.

**Feruloyl esterase** forms a part of the enzyme complex that acts collectively and synergistically to completely hydrolyze xylan to its monomers.

**Homogalacturonans** are linear chains of  $\alpha$ -(1–4)-linked D-galacturonic acid.

**Methylophilic bacteria** are a group of microorganisms that are able to use compounds containing one-carbon as well as multi-carbons as energy and carbon sources.

**Psychrophilic** thriving at a relatively low temperature

**Rhamnogalacturonans** (RGs) are a group of closely related cell wall pectic polysaccharides that contain a backbone of the repeating disaccharide: 4)- $\alpha$ -D-GalpA-(1,2)- $\alpha$ -L-Rhap-(1,.

### Introduction

Pectin is a high-molecular weight, biocompatible, nontoxic, and anionic natural polysaccharide extracted from cell walls of higher plants. It mostly consists of three structurally well-characterized polysaccharide motifs: homogalacturonan (HG), rhamnogalacturonan I (RG I), and rhamnogalacturonan II (RG II) (Chen et al., 2015). Pectic enzymes, pectinases or pectinolytic enzymes, are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants. They have a share of 25% in the global sales of food enzymes (Jayani et al., 2005). The primary source of industrial enzymes is microorganisms. The classification of pectic enzymes is based on their attack on the galacturonan backbone of the pectic substance molecule, and pectic enzymes is broadly classified into three types on the basis of their mode of action: pectin esterase, hydrolases and lyases (Garg et al., 2016). Pectinases are the growing enzymes of biotechnological sector, showing gradual increase in their market. They hold a leading position among the commercially produced industrial enzymes. Pectinases have many applications in various industries, such as pectin treatment in the food industry, wastewater treatment in the paper and pulp industry, and natural fiber treatment in the textile industry. Recent years, pectinases were also used in some new industries, such as bioethanol and oligosaccharides production. This is the review which covers the recent reports on the classification, production, and application of pectinases. Furthermore, it provides a bird's eye view of the possible application of these enzymes in new commercial sector.

### Classification of Pectic Enzyme

In general, pectinases are divided into three groups according to the cleavage site: hydrolases consisting of polygalacturonase, PG (EC 3.2.1.15); lyase/*trans*-eliminases comprising pectin lyase, PNL (EC 4.2.2.10), and pectate lyase, PL (EC 4.2.2.2); pectin esterase, PE (EC 3.1.1.11) (Garg et al., 2016; N. Sharma et al., 2013). Because pectin is an extremely complex polysaccharide, composed of as many as 17 different monosaccharides and more than 20 different linkages. Therefore, there are new classification of pectin degrading enzymes depending on their action sites in the pectic polymer (Bonnin et al., 2014). Briefly, these pectinases including homogalacturonan (HG) and rhamnogalacturonan (RG)-degrading enzymes (Table 1).

### HG-Degrading Enzymes

HG-degrading enzymes consist of esterases, polygalacturonases, and lyases. Esterases include pectin methylesterases (PME; EC 3.1.1.11; CE 8; [www.cazy.org](http://www.cazy.org)) and pectin acylesterases (PAE; CE 12). PME catalyze the release of methanol from methyl-esterified GalpA, PAE remove acylesters from pectin. Polygalacturonases include endo-polygalacturonases (endo-PG; EC 3.2.1.15; GH 28) and exopolygalacturonases (exo-PG; EC 3.2.1.67; GH 28), both endo-PG and exo-PG catalyze the hydrolysis of the  $\alpha$ -(1–4) glycosidic bond between two adjacent D-GalpA units.

Xylogalacturonan hydrolase (no EC number; GH 28) acts on xylogalacturonan by cleaving  $\alpha$ -1,4-D-galacturonan linkages between two xylosylated GalpA units only to predominantly release the disaccharide Xyl-GalpA. Pectin and pectate lyases split the glycosidic linkage between two GalpA units by catalyzing a  $\beta$ -elimination reaction, thus introducing a double bond on the newly



**Table 1** Enzymes involved in pectin degradation

Enzyme	Abbreviation	EC number	CAZy family <sup>a</sup>	Substrate
<b>HG-degrading enzymes</b>				
Endo-polygalacturonase	endo-PG	3.2.1.15	GH 28	Homogalacturonan–LM pectin
Exo-polygalacturonase	exo-PG	3.2.1.67	GH 28	Homogalacturonan–LM pectin
Exo-polygalacturonosidase	–	3.2.1.82	GH 28	Homogalacturonan–LM pectin
Xylogalacturonan hydrolase	–	3.2.1.	GH 28	Xylogalacturonan
Pectin lyase	PL	4.2.2.10	PL 1	High methyl ester pectin
Pectate lyase	PAL	4.2.2.2	PL 1,2,3,9,10	Homogalacturonan
Pectin methylesterase	PME	3.1.1.11	CE 8	Pectin
Pectin acylesterase	PAE	3.1.1.6	CE 12	Pectin
<b>RG-degrading enzymes</b>				
Rhamnogalacturonan hydrolase	RGH	3.2.1.171	GH 28	Rhamnogalacturonan–pectin
RG-galacturonohydrolase	–	3.2.1.173	GH 28	Rhamnogalacturonan–pectin
RG-rhamnoidhydrolase	–	3.2.1.174	GH 28	Rhamnogalacturonan–pectin
$\alpha$ -L-rhamnosidase	$\alpha$ RHA	3.2.1.40	GH 28	Rhamnogalacturonan–pectin
Endo-arabinanase	eA	3.2.1.99	GH 43	Arabinan
Exo-arabinanase	–	3.2.1.-	GH 93	Arabinan
$\alpha$ -L-Arabinofuranosidase	AF	3.2.1.55	GH 3, 10, 43, 51, 54, 62	Oligoarabinosides
$\beta$ -L-Arabinofuranosidase	–	3.2.1.185	GH 127	Oligoarabinosides
Endo- $\beta$ -1,4 galactanase	eG	3.2.1.89	GH 53	Galactan
Exo- $\beta$ -1,4 galactanase	–	3.2.1.-	GH 35	Galactan
$\beta$ -galactosidase	$\beta$ -Gal	3.2.1.23	GH 1,2,35, 42	Oligogalactosides
Rhamnogalacturonan lyase	RL	4.2.2.23	PL 4, 11	Rhamnogalacturonan
RG-acylesterase	RGAE	3.1.1.86	CE 12	Rhamnogalacturonan
Feruloyl esterase	–	3.1.1.73	CE 1	Feruloylated oligosides

<sup>a</sup>CAZy family data from [www.cazy.org](http://www.cazy.org).Data modified from [Garg et al. \(2016\)](#).

formed nonreducing GalpA end. Pectin lyases (EC 4.2.2.10; PL 1) and pectate lyases (EC 4.2.2.2; PL 1,2,3,9,10) act on methylated and non-methylated substrates, respectively.

### RG-Degrading Enzymes

RG-degrading enzymes include endo- and exo-enzymes are involved in the cleavage of the RG-I backbone. Exo-acting enzymes include RG hydrolase (EC 3.2.1.171), RG-galacturonohydrolase (EC 3.2.1.173), and RG-rhamnoidhydrolase (EC 3.2.1.174). All the hydrolases involved in RG-I backbone degradation belong to the family GH 28, as PGs do. Very few RG-lyases (EC 4.2.2.23) are reported, which ensure the endo-type eliminative cleavage of L- $\alpha$ -Rhap-(1–4)- $\alpha$ -D-GalpA bonds of RGI domains. RG acylesterases (EC 3.1.1.86, CE 12) required for the deacetylation of the RG-I backbone were firstly described in *Aspergillus* species.

Degradation of arabinan involves different enzymes that differ by their recognition sites in the polymer: endoarabinanases, exoarabinanases, and arabinofuranosidases. Endo-arabinanases (EC 3.2.1.99, GH 43) randomly cleave the  $\alpha$ -1,5-linkages in the internal region of the arabinan backbone. Exo-arabinanases (no EC number, GH 93) hydrolyze arabinan from the nonreducing end to release arabinobiose. Arabinofuranosidases (EC 3.2.1.55) act on  $\alpha$ -L-arabinofuranosides,  $\alpha$ -L-arabinans containing (1,3)- and/or (1,5)-linkages, arabinoxylans, and arabinogalactans. They are found in several GH families: 3, 10, 43, 51, 54, and 62.

Similarly to arabinan, the complete degradation of galactan requires different enzymes. Endo- $\beta$ -1,4 galactanases (EC 3.2.1.89, GH 53) act randomly on the galactan core of AGI. Exo- $\beta$ -1,4 galactanases (no EC number, GH 35) release galactose or galactobiose from the nonreducing end of  $\beta$ -1,4-galactan.  $\beta$ -Galactosidases (EC 3.2.1.23) release the terminal nonreducing galactose from various substrates of low molar mass.

22 pectic enzymes were listed in **Table 1**, these enzymes belong to endo/exo-hydrolases, lyases, and esterases, respectively. Among the 15 hydrolases involved in pectin degradation, 8 enzymes belong to GH 28 family. By comparison, lyases and esterases are less than hydrolases, there were only 3 lyases and 4 esterases in **Table 1**.

### Microbial Pectinases

Pectinases are one of the most widely distributed enzymes in bacteria, fungi and plants. The primary source of industrial enzymes is microorganisms, out of which, 50% originate from fungi and yeast, 35% from bacteria, while the remaining 15% are either of plant or animal origin. The pectinases are being produced by various kinds of microorganisms, and almost all the commercial preparations of pectinases are produced from fungal sources. *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes ([Jayani et al., 2005](#)).

### Acidic Pectinases

Acidic pectic enzymes used in the fruit juice industries and wine making often come from fungal sources, especially from filamentous fungi. An acidic endo-polygalacturonase (EPG4) from a mutant strain of *Penicillium oxalicum* displayed optimal pH and temperature at 5.0 and 60–70 °C towards polygalacturonic acid (PGA), respectively, and was notably stable at pH 2.2–7.0 (Cheng et al., 2016). Wang et al. (2017) showed that the optimal pH and temperature of the recombinant PGA-ZJ5A from *A. niger* were 4.5 and 40 °C, respectively, and this make PGAZ-J5A potentially effective in juice clarification without pH adjustment.

### Alkaline Pectinases

Fungi and yeasts produce mainly acidic PGases, whilst alkaline pectinases are mainly produced by bacteria. The highest reported values for PGase production (from 3600 to 23076 IU/g) were obtained by alkalophilic strains of *Bacillus* spp. and *Streptomyces* spp. under SSF conditions (Favela-Torres et al., 2006). Recently, alkaline thermostable pectin lyase from *A. niger* strain\_WHAK1 was produced, and the optimum pH and temperature values of pectin lyase were 8.0 and 40 °C at 60 min, respectively (Poturcu et al., 2017).

### Thermostable Pectinases

Thermal stability and activity of pectinases are of great significance in biotechnological processes. Recently, a thermo-alkaline pectate lyases (BacPelA) gene from an alkaliphilic *Bacillus clausii* strain was cloned and overexpressed in *Escherichia coli*, and the mature BacPelA exhibited maximum activity at pH 10.5 and 70 °C and showed high cleavage capability on methylated pectins (Zhou et al., 2017).

### Psychrophilic Cold-Active Pectinases

Recently, there has been a new trend in the food industry to adopt low-temperature processing. The reason behind this change in trend is driven by certain economic and environmental advantages, such as, energy saving, retention of labile and volatile flavor compounds, prevention of contamination and elimination of any residual enzyme activity, which is inactivation of enzyme when temperature is raised (Adapa et al., 2014). Merin and de Ambrosini (2015) reported that cold-active and acid-tolerant pectinases from non-*Saccharomyces* yeasts were able to remain active at glucose, ethanol and SO<sub>2</sub> concentrations usually found in winemaking. Five strategies have been suggested to promote proper expression and folding of cold-active enzymes expressed in heterologous host, increasing their solubility, activity, and yield. These strategies include the use of: (i) molecular chaperones; (ii) cold-active promoters; (iii) fusion partners; (iv) psychrophilic hosts, and (v) a combination of these strategies (Santiago et al., 2016).

### Production of Pectinases

There are two fermentation techniques we can use for pectinases production, as many other enzymes these techniques are Solid State Fermentation (SSF) and submerged fermentation (SmF). Usually, the pectinases can be produced via fungal SSF particularly by using *Aspergillus* strains. Industrially, pectinases can be produced from pectin-containing wastes, such as citrus and orange wastes, apple pomace, grape pomace or other fruit residues without any harsh pre-treatment owing to the nature of these substrates and the low moisture content (Kiran et al., 2014). Orange peel wastes are the most suitable candidate for production of pectinase and/or polygalacturonase because they contain more pectin content besides the fermentable sugars (Table 2). Demir and Tari (2014) found that wheat bran, among various agro-industrial wastes, was the most suitable substrate for the production of polygalacturonase using *Aspergillus sojae*. Li et al. (2015a,b,c) compared the typical agricultural residues for production of multi-enzyme complexes (pectinase, CMCase, and xylanase) by *Aspergillus japonicus* PJ01 in SmF and SSF. Compared to SmF, SSF presents unparalleled advantages, such as higher productivity per reactor volume, lower capital and operating costs, lower space requirements, simpler equipment and easier downstream processing. However, there were still some obstacles needed to overcome, such as unsatisfactory reproducibility of the results, difficulty in scale-up, regulating and monitoring of biomass concentration, and complicated methods of product purification (Hölker and Lenz, 2005).

### Applications of Pectinases

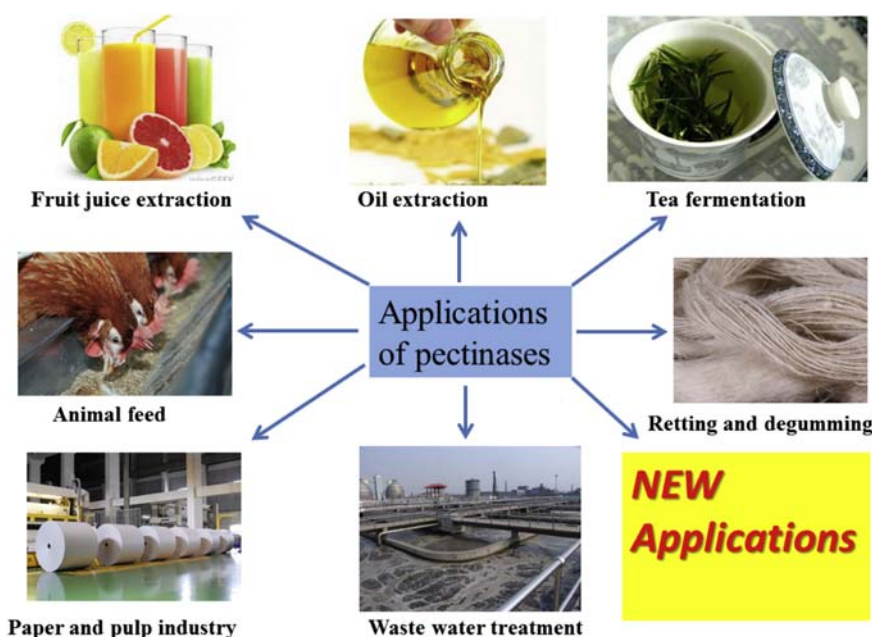
#### Food Industry

Pectinase has been used in many fields of food industry, such as fruit and vegetable processing, wine industry, extraction of vegetable oil, tea and coffee processing. Enzymatic treatment for juice extraction is most commonly used nowadays (Fig. 1). Enzymatic treatment prior to mechanical extraction significantly improves juice recovery compared to any other extraction process. Enzymatic hydrolysis of the cell walls increases the extraction yield, reducing sugars, soluble dry matter content and galacturonic acid content and titratable acidity of the products. Furthermore, cellulolytic and pectolytic enzymes mixtures are having wide application to enhance pulp liquefaction and provide a higher yield of juice with high soluble solids content (Sharma et al., 2017b).

**Table 2** Pectic enzymes production using agricultural wastes as the main substrate

Microorganisms	Substrate <sup>a</sup>	Enzyme	Fermentation	References
<i>Aspergillus</i> spp.	WB & OPW	Pectinolytic enzymes	SSF	Heerd et al. (2012)
<i>A. niger</i>	WB & OPW	Pectinase and cellulase	SSF & SmF	Sanjay Kumar et al. (2011)
<i>A. giganteus</i>	OPW & pectin	Polygalacturonase	SmF	Pedrolli et al. (2008)
<i>A. awamori</i>	OPW & Grape pomace	Exo-polygalacturonase and xylanase	SSF	Díaz et al. (2011)
<i>A. japonicus</i>	OPW	Pectinase, CMCase, etc.	SmF	Li et al. (2015a,b,c)
<i>A. foetidus</i>	OPW	Pectinase	SmF	Sunil Kumar et al. (2015)
<i>A. fumigatus</i>	OPW	Pectinase	SSF	Phutela et al. (2005)
<i>A. sojae</i>	OPW	Polygalacturonase	SmF	Gogus et al. (2014)
<i>A. sojae</i>	WB	polygalacturonase	SSF	Demir and Tari (2014)
<i>Penicillium oxalicum</i>	OPW	Pectinase, CMCase, etc.	SmF	Li et al. (2015a,b,c)
<i>P. oxalicum</i>	OPW	Pectin lyase	SSF	Yadav and Shastri (2007)
<i>P. viridicatum</i>	OPW, WB, etc.	Pectin lyase & polygalacturonase	SSF	Silva et al. (2002)
<i>Aspergillus</i> & <i>Penicillium</i>	OPW	Polygalacturonase, endoglucanase	SSF	Adeleke et al. (2012)
<i>Trichoderma</i> sp.	OPW, etc.	Polygalacturonase, etc.	SSF	Mohamed et al. (2013)
<i>Eupenicillium javanicum</i>	Citrus processing wastes	Cellulase, pectinase, xylanase, etc.	SSF	Tao et al. (2011)
<i>Pseudozyma</i> sp.	OPW	Pectinase	SSF	Sharma et al. (2012)
<i>Bacillus licheniformis</i>	Pectin, etc.	Polygalacturonase	SmF	Rehman et al. (2012)
<i>Thermoascus aurantiacus</i>	OPW, WB, etc.	Polygalacturonase	SmF	Martins et al. (2012)
<i>Rhizopus oryzae</i>	OPW	Pectin lyase	SSF	Hamdy (2005)
<i>Fusarium solani</i>	OPW	Exo-polygalacturonase	SSF	Hamid et al. (2008)

<sup>a</sup>OPW, orange peel waste; WB, wheat bran.

**Figure 1** Applications of pectinases in various industries.

The use of pectolytic enzymes, in this case preferably alkaline, allows the extraction of vegetable oils in an aqueous process by degradation of cell wall components. Aqueous enzymatic process (AEP) for oil extraction is undoubtedly an emerging technology in the fats and oil industry since it offers many advantages such as cost saving, environmental friendliness, and healthful nutrition. *A. giganteus* pectinases improved oil yield and rheological features without altering chemical composition of olive oil (Ortiz et al., 2017). Cavitation-accelerated aqueous enzymatic extraction (CAEE) of seed oil from *Cucurbita pepo* was performed, an enzyme cocktail comprised of cellulose, pectinase and proteinase can work synergistically in releasing the oil (Li et al., 2016c).

Pectinase treatment accelerates tea fermentation by breaking down the pectin which is present in the cell walls of tea leaves and also destroys the foam forming property of instant tea powders by destroying the pectins (Garg et al., 2016). Pectinase from *A. niger*, *Byssochlamys fulva* and *Mucor circinellus* used for fermentation of tea leaves from *Camellia sinensis* plant, increased production of phenolic compounds increases tea quality (Thakur and Gupta, 2012).

### Animal Feed

The use of pectinases in production of ruminant feed decreases the feed viscosity and increases the absorption of nutrients by ruminants, liberates nutrients by enzymatic action which also reduces the amount of faeces (Kumar and Suneetha, 2014).

### Processing of Textile Material

Retting and degumming are important in processing of textile material. Fibres containing gum should be degummed for its usage in textile industry. Pectin cements the fibres together and this pectin should be degraded. As chemical degumming causes pollution, an alternative use of pectinases and xylanases mixture serves as an eco-friendly and economical solution for pollution with non biodegradable pollutants (Rebello et al., 2017). Recently, a thermo-alkaline pectate lyases gene (*pelA*) from an alkaliphilic *B. clausii* strain S10 was cloned and overexpressed in recombinant *E. coli*, and this pectate lyases have potential application in bioscouring of the textile industry (Zhou et al., 2017). Abdulrachman et al. (2017) expressed a recombinant acidic endo-polygalacturonase from *Aspergillus aculeatus* in methylotrophic yeast *Pichia pastoris* using high cell density fermentation, the recombinant enzyme was evaluated in eco-friendly textile processing in comparison to the conventional alkaline scouring process.

### Paper and Pulp Industry

Pectinases in combination with xylanases are primarily used in the paper industry as a bio-bleaching agent. Unlike the conventional chemical bleaching agents, the use of enzymes is found to be eco-friendly, less harsh and good in improving the quality of the paper (Rebello et al., 2017). Recent studies indicate that ultra filtered concoction of pectinase and xylanase give better result than crude enzyme (Sharma et al., 2017a). Agrawal et al. (2016) reported xylano-pectinolytic synergism from *Bacillus pumilus* AJK was used in bleaching of plywood veneer SAQ pulp.

### Waste Water Treatment

Current deinking process depends upon the use of large amount of environment damaging chemicals. Deinking using enzymes is less polluting, energy saving, gives better performance to achieve the desired deinked pulp properties and results in lower disposal problems (Rebello et al., 2017). Recently, a combination of xylanase and pectinase has been used for deinking of school waste paper (Singh et al., 2012).

### New Applications

The first generation bioethanol (1G) consists in fermenting sucrose from the juice. Sucrose is extracted from large vacuoles present in cells of the stem. Sucrose is then used in fermentation tanks to produce ethanol using *Saccharomyces cerevisiae* (Amorim et al., 2011). The raw material employed in 2G technologies is the plant cell wall, a complex conglomerate of three polysaccharide domains (pectins, hemicelluloses, and cellulose) which are cross-linked with lignin. Hydrolysis of the fibers for production of monosaccharides is the most important and critical step because monosaccharides serve as a base material for production of value-added products such as ethanol, vitamins and organic acids. Li et al. (2016b) investigated the saccharification of orange peel wastes with crude enzymes from *A. japonicus* PJ01. John et al. (2017) review on the potential of citrus waste for bioethanol production, and pectinase enzyme will help in complete degradation of peel by breaking the polysaccharide pectin from cell wall to galacturonic acid in hydrolysis period.

Pectin-degrading enzymes and -modifying enzymes may be used in a wide variety of applications to modulate pectin properties or produce pectin derivatives and oligosaccharides with functional as well as nutritional interests. Many pectin-rich agro-residues such as orange peel waste (OPW), apple pomace, sugar beet, and olive pomace could serve as feedstocks for pectic oligosaccharides (POS) production. Since OPW contain a lot of insoluble polysaccharides, synergistic action of pectinase, cellulase, and xylanase for its depolymerization is required. Production of POS from pectin-rich biomass using commercial pectinase, cellulase, and beta-glucosidase has been reported by some teams (Combo et al., 2013; Concha Olmos and Zúñiga Hansen, 2012; Martínez Sabajanes et al., 2012). Martínez Sabajanes et al. (2012) assessed the effects of the enzyme loadings (polygalacturonases and cellulases) and reaction time on the conversion of polysaccharides into monosaccharides and oligomers using an empirical model. Li et al. (2016a) studied the production of POS from OPW by using multi-enzyme complexes from *A. japonicus* PJ01, and evaluated the prebiotic and antimicrobial potentials of these POS.

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# Phospholipases

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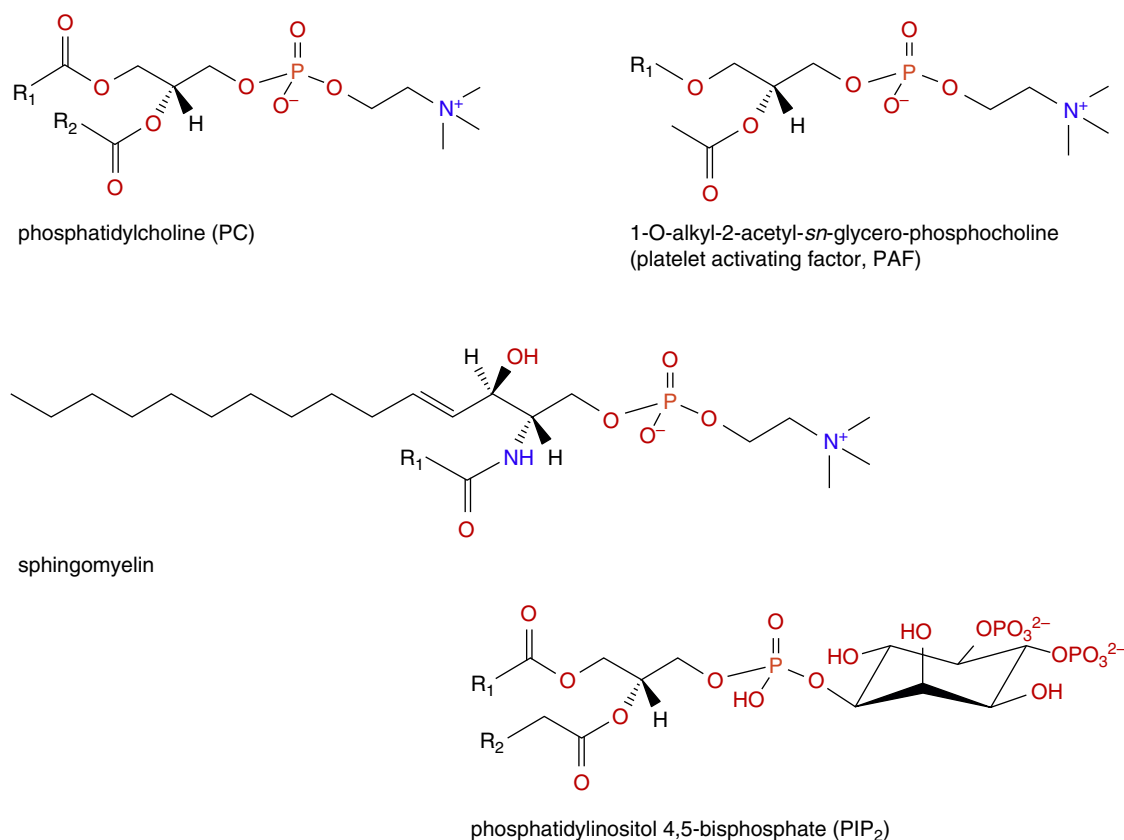
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## Introduction

Phospholipases (EC 3.1) are enzymes present in all animal, plant, lower eukaryotic and bacterial cells studied so far; they hydrolyze ester bonds of plasma or internal membrane structural lipids (glycerophospholipids and sphingophospholipids, [Fig. 1](#)) to produce simpler metabolites. These are either lipid molecules, which diffuse in the membrane where they exert their further action, or they are water-soluble molecules. The water-soluble metabolites leave the membrane and play their role, in many cases as parts of signal transduction pathways, in a different cellular compartment.

The main classes of phospholipases, hydrolyzing glycerophospholipids, are shown in [Table 1](#), together with their mode of function.

Phospholipases of the A type (PLA) remove one of the two fatty acids from their substrate molecule to produce a lyso (monoacyl) phospholipid, therefore they are acyl hydrolases (EC 3.1.1, a fourth number in the EC numbering system refers to the enzyme substrate). The remaining fatty acid is removed by lysophospholipases, which prevent high concentration of lytic lysophospholipids in the membrane. PLA<sub>1</sub>s are not very specific as some of them degrade neutral lipids as well (acting as lipases) or remove also the acyl chain from the *sn*-2 position, thus showing additional lysophospholipase activity (phospholipases B, PLBs). Where the enzyme appears to have low selectivity for the *sn*-1 and *sn*-2 positions, the term PLA is used. The term PLB should be restricted to enzymes that produce minimal accumulation of lysophospholipids. Phospholipases C and D are phosphodiesterases (EC 3.1.4). PLC removes the *sn*-3 associated phosphobase group to produce 1,2-diacylglycerol (DAG). PLD produces PA, an important intermediate of lipid metabolism, together with the base group associated at the *sn*-3 position. PA has a cone-shaped geometry and negative charge and, therefore, it induces negative membrane curvature promoting vesicle formation ([Kooijman and Burger, 2009](#)). In the presence of short-chain primary alcohols, PLDs exchange the base group of membrane lipids with the alcohol group and this transphosphatidyl reaction produces lipids not normally existing in membranes ([Jenkins and Frohman, 2005](#)). Accordingly, this reaction is used for PLD activity identification. Besides their specificity concerning ester bonds,



**Figure 1** Structures of membrane lipids. Glycerophospholipids may contain, besides choline, H (phosphatidic acid, PA), ethanolamine (PE), serine (PS), *myo*-inositol (PI) or phosphatidylglycerol (cardiolipin) attached to phosphate group esterified at the *sn*-3 position of the glycerol molecule.

**Table 1** Hydrolysis of glycerophospholipids by phospholipases

phospholipase A <sub>1</sub> (PLA <sub>1</sub> )	
phospholipase A <sub>2</sub> (PLA <sub>2</sub> )	
phospholipase C (PLC)	
phospholipase D (PLD)	

there are phospholipases acting only on specific members of the different phospholipid groups. For example, PAF acetylhydrolase (PAF-AH) is a PLA<sub>2</sub> which removes the ester-linked acetic acid from the *sn*-2 position of a PC molecule containing an ether-linked fatty acid at the *sn*-1 position (Fig. 1). Similarly, the phosphoinositide-specific PLC (PI-PLC) hydrolyzes preferentially PIP<sub>2</sub> (Fig. 1).

Sphingophospholipids are ceramide derivatives: sphingomyelins, which are present mainly in the myelin sheath, contain phosphocholine (Fig. 1) or phosphoethanolamine as a base group, thus resembling glycerophospholipids in their three-dimensional structure and general properties. Specific sphingomyelinases hydrolyze phosphocholine group of sphingomyelins by a mechanism resembling that of a glycerophospholipid PLC. The metabolism of sphingolipids is prone to genetic defects of enzymes involved in their degradation; a rare defect in lysosomal acid sphingomyelinase induces accumulation of sphingomyelin in brain, spleen and liver causing mental retardation and early death in infants (Niemann-Pick disease, a sphingolipidosis).

### Interaction of Phospholipases With Lipid–Water Interfaces

As most phospholipases are soluble proteins, they act in lipid–water interfaces. These enzymes are more active *in vitro* on aggregated substrates, becoming fully active above critical micellar concentration (cmc) of the substrate. The soluble nature of phospholipases suggests that their interaction with cellular membranes is possibly a regulatory mechanism which controls membrane lipid degradation. The interaction involves the creation of an enzyme interfacial binding which precedes productive binding in the lipid surface and actual catalysis. Both polar and non-polar interactions are involved in the interfacial binding, while surface charge and perturbations in the interface (like those from factors affecting membrane fluidity, phospholipid packing density or polymorphism of the aggregate) affect phospholipase activity. The membrane surface charge is also influenced by ions present in the aqueous environment. However, although binding of PLA<sub>2</sub>s to membrane phospholipids is enhanced by 10-fold in the presence of Ca<sup>2+</sup>, in its absence binding of the highly cationic human secreted sPLA<sub>2</sub> is promoted by the presence of anionic PA in the bilayer (Kinkaid et al., 1998). PI, also an anionic phospholipid, may have a similar role. Additionally, phospholipase activity is affected by possible conformational changes in the enzyme structure upon its binding in the lipid aggregate (van den Berg et al., 1995) and by the diffusion rate of the enzyme product. This is important especially for the A type phospholipases, as both their products are hydrophobic.

### Classification of Phospholipases

Phospholipases are further classified into different types which are presented in Tables 2 and 3 (mammalian PLA<sub>1</sub>/PLA<sub>2</sub> and PLC/PLD, respectively) and Table 4 (plant phospholipases). This classification is based on their distribution, sequence similarity, structure characteristics and requirements for optimum activity *in vitro*, which is connected to the differential activation of these enzymes.

Although food industry faces phospholipases mainly as hydrolytic tools, their physiological functions show that they are indispensable for cellular metabolism and homeostasis (structural lipids undergo constant metabolic turnover, the rate of their synthesis being counterbalanced by an equal rate of degradation by phospholipases), membrane remodeling and, also, for environmental signal regulation. A good example of membrane remodeling is PAF production in hypersensitivity reactions: PAF is synthesized from alkylacylphosphocholine after *sn*-2 acyl chain removal by a PLA<sub>2</sub> (and not by de novo synthesis). PAF can then be inactivated by PAF-AH.

### Structure and Biological Functions of Mammalian Phospholipases

#### PLAs

Mammalian PLA<sub>1</sub>s are classified into intra- and extra-cellular enzymes based on their cellular localization (Table 2). Besides they all possess a GxSxG lipase motif, the extracellular PLA<sub>1</sub>s contain also the catalytic SHD triad and lipid binding surface loops (β9, lid domain) which affect their specificity, whereas conserved intracellular disulfide bonds provide stability (Bamji-Mirza and Yao, 2017). PLA<sub>2</sub> is the most varied class of phospholipases: it is consisted of 16 groups of enzymes, subdivided into six main types: secreted PLA<sub>2</sub> (sPLA<sub>2</sub>), cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>), Ca<sup>2+</sup>-independent PLA<sub>2</sub> (iPLA<sub>2</sub>), PAF-AH PLA<sub>2</sub> and the lipoprotein-associated PLA<sub>2</sub> (LpPLA<sub>2</sub>, a subtype of PAF-AH PLA<sub>2</sub>), lysosomal PLA<sub>2</sub> (LPLA<sub>2</sub>) and adipose-specific PLA<sub>2</sub> (AdPLA<sub>2</sub>) (Mouchlis and Dennis, 2016). The main structural characteristics and functions of PLA<sub>2</sub>s are presented in Table 2. The sPLA<sub>2</sub>s are highly disulfide-

**Table 2** Mammalian phospholipases A<sub>1</sub> and A<sub>2</sub>

Classes	Types	Groups	Substrates	Domains	Regulators/cofactors	Functions
PLA <sub>1</sub>	Intracellular		Non-specific	GxSxG lipase motif		- Spermiogenesis - Vesicular transport
	Extracellular	- PS-PLA <sub>1</sub> - mPA-PLA <sub>1</sub> - Lipases	- PS - PA - Phospholipids, triacylglycerols	- GxSxG lipase motif - Catalytic SHD triad (x3) - C residues for disulfide bonds - $\beta$ 9 loop - Lid domain - Catalytic HD dyad (x2) - 6–8 disulfide bonds - Ca <sup>2+</sup> binding in the active site		Regulation of: apoptosis, blood coagulation, PKC signaling (PS-PLA <sub>1</sub> ), platelet aggregation, motility, cell proliferation (PA-PLA <sub>1</sub> )
PLA <sub>2</sub>	Secreted (sPLA <sub>2</sub> )	I–III, V, IX–XIV	Higher activity with anionic phospholipids (PE, PG, PS) or PC vesicles	- Catalytic HD dyad (x2) - 6–8 disulfide bonds - Ca <sup>2+</sup> binding in the active site	Ca <sup>2+</sup>	- Non-catalytic role (aggregates with vesicles acting as ligands for specific cellular targets) - Antibacterial, antiviral functions - Role in inflammatory and autoimmune diseases
	Cytosolic (cPLA <sub>2</sub> )	IVA–F	- Highly AA-specific - Additional PLA <sub>1</sub> , lysophospholipase, transacylase activity	- Catalytic HD dyad (x3) - $\alpha/\beta$ hydrolase domain - C2 domain - PIP <sub>2</sub> binding site - Lid domain	Ca <sup>2+</sup> , also activated by PIP <sub>2</sub> , C1P	Generation of eicosanoids, role in inflammation
	Ca <sup>2+</sup> -independent (iPLA <sub>2</sub> )	VIA–F	- Low specificity for <i>sn</i> -2 AA - Additional PLA <sub>1</sub> , lysophospholipase, transacylase, lipase activity	- 7–8 ankyrin repeats - S in the active site - Catalytic SD dyad	- Ca <sup>2+</sup> -independent - ATP-regulated	- Constitutive low release of fatty acids - Role in insulin release
	PAF-AH PLA <sub>2</sub> secreted or intracellular	VIIB, VIIIA–B	Specificity for PAF, PAF analogues (short <i>sn</i> -2 acyl chain)	- Catalytic SHD triad (x3) - GxSxG lipase motif - $\alpha/\beta$ hydrolase domain		- Response to oxidative stress - Involved in spermatogenesis
	Lipoprotein-specific (LpPLA <sub>2</sub> ) (PAF-AH PLA <sub>2</sub> subgroup)	VIIA	- Specificity for oxidized phospholipids (monomers or small aggregates) - Additional PLA <sub>1</sub> DAG/triacylglycerol hydrolase activity		Ca <sup>2+</sup> -independent	- Associated with LDL, HDL - Role in atherosclerosis, cardiovascular diseases
	Lysosomal (LPLA <sub>2</sub> )	XV		- Catalytic SHD triad (x3) - GxSxG lipase motif - N-terminal signal sequence - N-glycosylation sites - Secreted	Ca <sup>2+</sup> -independent	Major enzyme in pulmonary surfactant metabolism
	Adipose-specific (AdPLA <sub>2</sub> )	XVI	- AA-specific - Additional PLA <sub>1</sub> , acyltransferase activity	- Catalytic HC dyad - Intracellular	Ca <sup>2+</sup> -independent	- Found in adipocytes - Induced by insulin - Antilipolytic agent - Role in peroxisome biogenesis, tumorigenesis

mPA-PLA<sub>1</sub>, membrane phosphatidic acid-specific PLA<sub>1</sub>; C1P, ceramide 1-phosphate.

**Table 3** Mammalian phospholipases C and D

Classes	Types	Substrates	Domains	Regulators/ cofactors	Functions
PLC	PC-specific PLC (PC-PLC)	PC	Not defined		<ul style="list-style-type: none"> <li>- Signaling through protein kinase pathways</li> <li>- Programmed cell death</li> <li>- Stem cell differentiation</li> <li>- Cell proliferation</li> </ul>
	PI-specific PLC $\delta$ (PI-PLC $\delta$ )	<ul style="list-style-type: none"> <li>- Specific for PIP<sub>2</sub></li> <li>- Secondary substrates: PI, PIP</li> <li>- Slightly active on PIP<sub>3</sub></li> </ul>	<ul style="list-style-type: none"> <li>- X, Y catalytic domain</li> <li>- PH domain</li> <li>- 2 EF hand motifs</li> <li>- C2 domain</li> </ul>	Ca <sup>2+</sup>	Role in: <ul style="list-style-type: none"> <li>- Cell cycle control</li> <li>- Alzheimer's disease</li> <li>- Esophageal, gastric, colorectal and breast cancer</li> </ul>
	PI-PLC $\beta$	see PI-PLC $\delta$	Same as PI-PLD $\delta$ , plus C-terminal extension (CTR)	Ca <sup>2+</sup>	Role in: <ul style="list-style-type: none"> <li>- Cell cycle control</li> <li>- Brain function/disorders</li> </ul>
	PI-PLC $\gamma$	see PI-PLC $\delta$	Same as PI-PLD $\delta$ , plus <ul style="list-style-type: none"> <li>- SH2/SH3 domains</li> <li>- 2-split PH domain</li> </ul>	Ca <sup>2+</sup>	Role in: <ul style="list-style-type: none"> <li>- Embryonic lethality</li> <li>- Breast cancer</li> <li>- Immunodeficiency</li> <li>- Autoimmunity</li> </ul>
	PI-PLC $\epsilon$	see PI-PLC $\delta$	Same as PI-PLD $\delta$ , plus: <ul style="list-style-type: none"> <li>- C-rich region (CR)</li> <li>- 2 RA domains</li> <li>- Ras-GEF domain</li> </ul>	Ca <sup>2+</sup>	Role in: <ul style="list-style-type: none"> <li>- Cardiac contractivity</li> <li>- Inflammation</li> <li>- Esophageal, gastric and colorectal cancer</li> </ul>
	PI-PLC $\zeta$	See PI-PLC $\delta$	Same as PI-PLD $\delta$ , without C2 domain	Ca <sup>2+</sup>	Role in fertilization
	PI-PLC $\eta$	See PI-PLC $\delta$	Same as PI-PLD $\delta$ , plus S/P signal peptide	Ca <sup>2+</sup>	Role in retina development
PLD	PLD1, PLD2	Mainly PC	<ul style="list-style-type: none"> <li>- 2 catalytic HKD motifs (in II, IV domains)</li> <li>- PX, PH domain</li> <li>- I-IV conserved domains</li> <li>- Loop region (PLD1)</li> </ul>	Ca <sup>2+</sup>	Membrane transport role in: <ul style="list-style-type: none"> <li>- Inflammation</li> <li>- Cell migration</li> <li>- Neuronal and cardiac stimulation</li> <li>- Chemo-resistance</li> </ul>

bonded and Ca<sup>2+</sup>-dependent phospholipases. The cPLA<sub>2</sub> is specific for *sn*-2-associated arachidonic acid (AA), while it contains a lid domain in the catalytic region that regulates substrate uptake (Bamji-Mirza and Yao, 2017). The release of AA leads to the generation of eicosanoids and subsequent inflammatory response. On the contrary, iPLA<sub>2</sub> has low specificity for *sn*-2 AA and it is responsible for the constitutive, low-level release of fatty acids that is necessary for membrane homeostasis. As shown by its name, iPLA<sub>2</sub> does not require Ca<sup>2+</sup> for activity. Instead, it is regulated by ATP and, only indirectly, by Ca<sup>2+</sup> (Mouchlis and Dennis, 2016). Structurally, the ankyrin repeats discriminate the iPLA<sub>2</sub> from the rest PLA<sub>2</sub>s and facilitate protein–protein interactions. Finally, the LPLA<sub>2</sub> and AdPLA<sub>2</sub> are the less studied types, known by their role in lung surfactant metabolism (Bamji-Mirza and Yao, 2017) and AA release in white adipose tissue (Wolf, 2009), respectively.

An additional non-specific acylhydrolase has been recently studied, namely the patatin-like PLA<sub>2</sub> (PNPLA<sub>2</sub>). The mammalian patatin-like enzymes have an active site similar to that of cPLA<sub>2</sub> and they are highly expressed in adipocytes, while their functions are under investigation (Bamji-Mirza and Yao, 2017).

## PLCs

According to their substrate specificity, mammalian PLCs are classified into PC- and PI-PLCs. PC-PLCs are not extensively studied. However, it is well-documented that they rapidly translocate, upon stimulation, from the cytosol to the plasma membrane to regulate cellular functions like those presented in Table 3 (Cocco et al., 2015). PI-PLCs act mainly on PIP<sub>2</sub> to yield, also upon stimulation, the membrane-associated DAG and the soluble inositol triphosphate (IP<sub>3</sub>). Both these molecules are important second messengers, activating protein kinase C (PKC) and regulating Ca<sup>2+</sup> release from internal stores, respectively. PI-PLC group is consisted of enzymes divided into six subgroups ( $\beta$ - $\eta$ ). They are intracellular enzymes, present mainly in plasma but also in nuclear membrane and they are regulated by direct binding to G protein subunits, small GTPases, receptor and non-receptor tyrosine kinases and also by membrane lipids (Balla, 2013; Bunney and Katan, 2011).

**Table 4** Plant phospholipases

Classes	Types	Substrates	Domains	Regulators/cofactors	Functions
PLA	PC-hydrolyzing PLA <sub>1</sub> (PC-PLA <sub>1</sub> )	Strict <i>sn</i> -1 specificity main substrates: PC, DGDG secondary: MGDG, triacylglycerols	Not defined		<ul style="list-style-type: none"> <li>- Response to wounding, pathogens (chloroplastic PC-PLA<sub>1</sub>)</li> <li>- Membrane lipid degradation in senescence (cytosolic PC-PLA<sub>1</sub>)</li> </ul>
	PA-specific PLA <sub>1</sub> (PA-PLA <sub>1</sub> )	Mainly PA	Homologous to mammalian PA- PLA <sub>1</sub>		Role in vesicular trafficking during gravity sensing
	Secreted low-molecular-weight PLA <sub>2</sub> (sPLA <sub>2</sub> )	<ul style="list-style-type: none"> <li>- PE &gt;&gt; PC</li> <li>- No clear specificity for fatty acids, unsaturated preferred</li> </ul>	<ul style="list-style-type: none"> <li>- Catalytic HD dyad</li> <li>- PA2c domain having Ca<sup>2+</sup> binding loop</li> <li>- N-terminal signal peptide</li> <li>- 6 disulfide bonds</li> </ul>	Ca <sup>2+</sup> (μM-mM)	Role in: <ul style="list-style-type: none"> <li>- Germination</li> <li>- Stomatal opening</li> <li>- Shoot elongation, gravitropism</li> <li>- Pollen development</li> <li>- Pollen tube growth</li> </ul>
	Patatin-like PLA (pPLA I-III)	<ul style="list-style-type: none"> <li>- Substrates: phospholipids, galactolipids</li> <li>- PLA<sub>1</sub>/PLA<sub>2</sub> activity</li> <li>- No lipase activity</li> </ul>	Catalytic SD dyad		Role in: <ul style="list-style-type: none"> <li>- JA production (pPLA I)</li> <li>- Oxylipin biosynthesis (pPLA II)</li> <li>- Auxin-related root responses (pPLA II)</li> <li>- Long-term responses to stress (pPLA II)</li> <li>- Modulation of cellulose production (pPLA III)</li> </ul>
PLC	Non-specific PLC (NPC)	<ul style="list-style-type: none"> <li>- Main substrates: PC, PE, lysoPA</li> <li>- Secondary: PS, PG</li> <li>- Poor activity for PA, PIP<sub>2</sub></li> </ul>	Phosphoesterase domain	Ca <sup>2+</sup> -independent	Role in: <ul style="list-style-type: none"> <li>- Phospholipid degradation</li> <li>- Response to ABA, drought, salinity, auxin, cytokinin</li> <li>- Rapid response to aluminium, elicitors, brassinosteroids</li> </ul>
	PI-PLC	PIP <sub>2</sub>	<ul style="list-style-type: none"> <li>- X, Y catalytic domain</li> <li>- EF hand motif</li> <li>- C2 domain</li> </ul>	<ul style="list-style-type: none"> <li>- Ca<sup>2+</sup> (μM-mM)</li> <li>- Regulation by G proteins, phosphorylation</li> </ul>	Role in: <ul style="list-style-type: none"> <li>- Response to cold, salinity, dehydration, nutrients, ABA</li> <li>- Root hair growth</li> <li>- Pollen tube growth</li> <li>- Gravitropic responses</li> <li>- Cytoskeletal organization</li> </ul>

(Continued)

**Table 4** Plant phospholipases—cont'd

<i>Classes</i>	<i>Types</i>	<i>Substrates</i>	<i>Domains</i>	<i>Regulators/cofactors</i>	<i>Functions</i>
PLD	PLD $\alpha$	PC, PE, PG	- 2 catalytic HKD motifs - C2 domain	Ca <sup>2+</sup> (~100 mM)	Role in: - H <sub>2</sub> O balance - Response to pathogens, wounding, drought, salinity, freezing stress - ROS production - Seed aging
	PLD $\beta$	- Main phospholipids, NAPE - Active in the presence of phosphoinositides and PE	- 2 catalytic HKD motifs - C2 domain - K/R-rich PIP <sub>2</sub> binding motif	- Ca <sup>2+</sup> (50 $\mu$ M) - PIP <sub>2</sub>	Role in early responses to pathogens
	PLD $\gamma$ PLD $\delta$	see PLD $\beta$ - Mainly PC, PE - Active in the presence of oleic acid	- 2 catalytic HKD motifs - C2 domain	- Ca <sup>2+</sup> (100 $\mu$ M, $\mu$ M-mM) - Oleic acid	Role in: - Response to freezing, drought, pathogens - ROS regulation - Cytoskeletal organization
	PLD $\epsilon$	Main phospholipids	- 2 catalytic HKD motifs - C2 domain	- Ca <sup>2+</sup> - No distinction from PLD $\alpha$ - $\delta$	Role in: - Nitrogen signaling - Root development
	PLD $\zeta$	Mainly PC	- 2 catalytic HKD motifs - PX domain - PH domain	Ca <sup>2+</sup> -independent	Role in: - Regulation of phosphate levels - Vesicular trafficking - Response to auxin
	PLD $\phi$ (SP-PLD)	Not defined	- 2 catalytic HKD motifs - N-terminal signal peptide	Not defined	Not defined

DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; ABA, abscisic acid; NAPE, N-acylphosphatidylethanolamine



## PLDs

Mammalian PLDs belong to either PLD1 or PLD2 types. PLD1 exerts low basal activity, being stimulated by PIP<sub>2</sub>, IP<sub>3</sub>, PKC $\alpha$  and GTP-binding proteins. PLD2 is stimulated also by PIP<sub>2</sub> and negatively regulated by cytoskeletal proteins (Bamji-Mirza and Yao, 2017). They both contain the conserved domains shown in Table 3, while the existence of a negatively regulating loop region in PLD1 discriminates the two types. Mammalian PLD involvement in cellular processes has been attributed mainly to the PA formation. In addition, PLD activity implication in disease-related signaling pathways (for example, those related to cancer invasion and metastasis) makes PLDs prominent therapeutic targets. A specific mammalian lysoPLD (autotaxin) acts on lyso-phospholipids and it is the main source of lysoPA in blood. This enzyme does not include an HKD motif (for PLD functions and autotoxin, see Further Reading).

## Structure and Biological Functions of Plant Phospholipases

### PLAs

Plant PLAs are classified, based on sequence similarity and biological functions, into four classes, namely PC-hydrolyzing PLA<sub>1</sub> (PC-PLA<sub>1</sub>), PA-specific PLA<sub>1</sub> (PA-PLA<sub>1</sub>), secreted low-molecular-weight PLA<sub>2</sub> (sPLA<sub>2</sub>) and patatin-like PLA (pPLA). Concerning PLA<sub>1</sub>s, they show high specificity for their substrate (PC or DGDG for PC-PLA<sub>1</sub> and PA for PA-PLA<sub>1</sub>), while they are involved, besides cell homeostasis, in wound responses and the regulation of vesicular trafficking in plant cells (Table 4). sPLA<sub>2</sub> shows preference for unsaturated fatty acid-containing membrane lipids, while its structure resembles mammalian sPLA<sub>2</sub>. Finally, pPLA exerts both PLA<sub>1</sub> and PLA<sub>2</sub> (but not lipase) activity and it is involved in oxylipin or jasmonic acid (JA) biosynthesis and in responses to several environmental stresses (Wang et al., 2012).

### PLCs

As in mammals, plants possess two different types of PLC, the non-specific PLC (NPC) and the PI-PLC. The first is low-expressed and hydrolyzes mainly PC, PE and lysoPA, but not other lysophospholipids (Hong et al., 2016). NPC is responsible for phospholipid degradation in case of phosphate deficiency and it is also involved in responses to hormones and abiotic stresses (Wang et al., 2012; Pokotylo et al., 2013). PI-PLC, which resembles mammalian PI-PLC $\zeta$ , acts selectively on PIP<sub>2</sub> in a Ca<sup>2+</sup>-dependent manner. Plant PI-PLC is implicated in cytoskeletal organization, root hair and pollen tube growth, as well as in stress-induced responses (Table 4) (Wang et al., 2012; Hong et al., 2016).

### PLDs

PLDs were first discovered in plants. Twelve PLD genes exist in *A. thaliana* when there is only one in *S. cerevisiae* and two in animal cells. Plant PLDs are divided into seven main types, PLD $\alpha$ - $\zeta$ , and the newly-discovered PLD $\phi$  (Wang et al., 2012; Hong et al., 2016; Tang et al., 2016). They all hydrolyze common membrane phospholipids, mostly PC and PE in the presence of Ca<sup>2+</sup>, with the exception of PLD $\zeta$  which shares more homology to yeast and mammalian isoforms. These enzymes respond to several environmental stresses and regulate cytoskeletal organization and reactive oxygen species (ROS) production (Wang et al., 2012; Hong et al., 2016; Bourtsala et al., 2017). PLD $\alpha$  is the constitutive plant PLD. PLD $\beta$  and  $\gamma$ , apart from Ca<sup>2+</sup>, require also PIP<sub>2</sub> as cofactor. Likewise, oleic acid is indispensable for PLD $\delta$  activity.

## Bacterial Phospholipases

In bacteria, besides their role in cellular homeostasis and membrane remodeling, phospholipases have additional functions serving also extracellular nutrition and host penetration. This is the reason why phospholipases, mainly of Gram-negative bacteria, are involved in several diseases. In *E. coli*, the presence of two PLA<sub>1</sub>-type phospholipases has been demonstrated. One is a detergent-resistant protein in the outer membrane of the cell, which acts more as a PLB-type enzyme, showing also lysophospholipase activity. The second is a detergent-sensitive enzyme present in plasma membrane and soluble fractions. sPLA<sub>2</sub>s have also been found in both Gram-positive and Gram-negative bacteria, but they differ in structure from the most eukaryotic sPLA<sub>2</sub>s. In addition, plant-type sPLA<sub>2</sub>s and PLA<sub>2</sub>s containing patatin-like domains (p.e. ExoU from *Pseudomonas aeruginosa*, which is activated by ubiquitin) have been identified in bacteria (Mansfeld, 2017). PLCs are mainly secreted by pathogenic bacteria and, therefore, play important roles in cytotoxicity. Bacterial enzymes have various specificities as they are either PLCs acting on PC/sphingomyelin or PI-PLCs acting on PI (but not on phosphatidylinositol phosphates) and, also, on glycosylphosphatidylinositol (GPI)-anchored mammalian proteins. Microbial PLDs are also involved in cell penetration like those secreted by *Streptomyces* strains. Some of them are the best characterized PLDs and the only microbial PLDs commercially used for the production of natural and synthetic phospholipids. This is favored by their high transphosphatidylase activity (Mansfeld, 2017).

### Phospholipases in Fungi and Protozoa

Phospholipases are also present in lower eukaryotes. PLA<sub>1</sub>s have been found in several fungi strains, like *Aspergillus* and the plant-associated *Fusarium*, and also in protozoa. Genes encoding sPLA<sub>2</sub>s and cPLA<sub>2</sub>-like proteins have been identified in the genomes of various fungi, including the *PLaA* of *A. nidulans* and several *Neurospora* genes. In contrast to bacteria, PI-specific PLCs are present in many lower eukaryotes like yeasts, slime molds (*Dictyostelium discoideum*) and protozoa (Heinz et al., 1998; Leondaritis and Galanopoulou, 2011). Finally, a PC-specific PLD from *S. cerevisiae* (SPO14/PLD1), involved in cell sporulation and maintaining membrane trafficking events, has been extensively studied (Rudge and Engebrecht, 2012).

### Phospholipases in Food Processing

Based on the presence of phospholipids in most foods, the industrial use of phospholipases serves the improvement of food quality and the production of novel foods with improved properties. In addition, because of the recent interest for cleaner technologies, they are used as biocatalysts, often after genetic modification of existing enzymes to create desirable properties, i.e. higher stability. A similar high stability is also shown by extremophile enzymes and this explains their recent use in industry (Song et al., 2005). There are already commercially available enzymes, mainly of microbial origin which have a lower cost, serving edible oil processing or baking and dairy product manufacture. The first phospholipase to be commercialized was the thermostable Lecitase® Ultra (Novozymes A/S, Denmark), a PLA<sub>1</sub> from *Thermomyces lanuginosus*/*Fusarium oxysporum* expressed in *Aspergillus oryzae*, used for the degumming of vegetable oils (Casado et al., 2012). There are also several examples of commercial PLA<sub>2</sub>s, like Lecitase® 10 L (Novozymes), a secreted enzyme of animal origin, also for the degumming of vegetable oils, and LysoMax® (Genencor, USA) of bacterial origin which releases lysophospholipids with emulsifying properties but not fatty acid which are transferred to the hydroxyl group of sterols present in the oil. Industrial application of PLC is rather limited, while PLDs from different plant and microbial sources are commercially available: their application is mainly based on their transphosphatidylolation potential (Ulbrich-Hofmann et al., 2005).

#### Degumming

Vegetable oils separated from either oilseeds or oil-bearing fruits contain, regardless the separation procedure, variable amounts of undesirable material, which may affect the quality of the final product. The major purification process is refining, either chemical which neutralizes fatty acids with alkaline solutions, or physical (usually a distillation process). Degumming, a process converting phospholipids to hydrated gums which can be separated accordingly by filtration or centrifugation, is an essential preliminary step. However, the relative inefficacy of traditional water or acid degumming methods introduced phospholipases in the field for the removal of non-hydratable compounds. Using phospholipases, an effective separation of the aqueous phase containing the hydrolyzed phospholipids is an easy next step (Casado et al., 2012). The main commercial enzymes for degumming purposes, mainly PLA<sub>1</sub> and PLA<sub>2</sub>, have been reviewed recently (Dijkstra, 2010). The aqueous mixture of the degumming product, containing lysophospholipids, can be used in animal feed industry, while the fatty acid fraction can be removed during the subsequent deodorization step. One additional advantage of phospholipases involvement in degumming is the fact that they can be reused. Furthermore, the use of PLCs, leading to the formation of DAG which stays in the oil on refining (instead of fatty acids which must be removed), is recently considered for oil degumming.

#### Bakery Industry

Bread making includes mixing of flour, water and yeast, fermentation of dough and baking of the fermented dough. All these steps are characterized by complex physical, chemical or biochemical changes of the dough main components, namely starch and gluten (the protein network responsible for trapping carbon dioxide during fermentation) which affect the quality and the volume of the final product. To achieve good quality, emulsifiers and enzymes (amylases, oxidases, proteases) are widely used in the bakery industry. It is interesting that, among the enzymes used in food processing, those used in bakery industry constitute one-third of the market (Casado et al., 2012). Although lipid fraction is a minor component of wheat (and flour), the presence of phospholipids is important for bread making: they form monolayers in the gas/liquid interface, stabilizing gas cells and increasing gas retention in the dough. Therefore, their function results in the improvement of bread volume. However, due to the low levels of lipids in flour, exogenous phospholipids must be added during bread making to act as emulsifiers instead of traditional emulsifying agents. The use of phospholipases in bread making is quite recent and it is related to the modification of endogenous or exogenously added phospholipids: the lysophospholipid released improve dough rheological properties. Usually, phospholipases with broad specificity are used in the bakery industry. A good example is the commercially available lipase from *F. oxysporum* (Lipopan F®, Novozymes) which shows both lipase and phospholipase activity (de Maria et al., 2007).

#### Dairy Industry

Milk fat consists mainly of triacylglycerols (>95%) present in the form of fat globules dispersed in the aqueous phase of milk. Triacylglycerols are localized in globule core, while globules are surrounded by a milk fat globule membrane (MFGM), rich in

phospholipids, sphingolipids and cholesterol, which is derived from the membrane of the secreting mammary epithelial cells and act by stabilizing the dispersion. Phospholipid fraction of MFGM consists mainly of sphingomyelin, PC and PE. Depending on the dairy product, MFGM has a different technological role (Casado et al., 2012). For example, in aerated products like ice-cream, milk phospholipids reduce the effect of ice crystals while, during butter manufacture, they influence phase separation and fat crystallization (Fedotova and Lencki, 2008).

Lipolytic enzymes, mainly lipases, have been traditionally used in the cheese industry for enhancing ripening process, including flavor improvement. Recently, phospholipases are used during cheese processing, but with a different purpose, that of increase in cheese yield. Normally, 85%–95% of milk fat is entrapped in the cheese curd, while the rest is lost in the whey. Phospholipases release lysophospholipids which, compared to phospholipids, have better emulsifying potential and promote increased fat retention in the final product. Novozymes proposed the general use of PLA<sub>1</sub>, PLA<sub>2</sub> and PLB for milk fat pretreatment during cheese production (Bourlieu et al., 2009). Apart from the A type phospholipases, the use of PLC and PLD has also been suggested for cheese production, as these enzymes eliminate PC and PE during the procedure.

### Modification of Egg Yolk Properties

Whole eggs and egg yolks are widely used in food industry, as they act as very effective emulsifying agents during the manufacture of bakery products and mayonnaise. Egg yolk is an emulsion containing 32% phospholipids, mainly PC. These phospholipids are present in the layer that surrounds lipoproteins of yolk plasma fraction. As these lipoproteins are the main factor responsible for the emulsifying properties of egg yolk, any modification of the phospholipids, including treatment with phospholipases, leads to the modification of lipoproteins which, in turn, changes emulsifying properties of egg yolk (Casado et al., 2012). Enzymes used in the bakery industry are also used in mayonnaise and sauce industries. One additional advantage in this case is the improvement of heat stability of products, which facilitates pasteurization. In addition to PLAs, PLD has also been used for the modification of egg yolk properties. By using this enzyme, the production of unsaturated fatty acids by PLA<sub>2</sub>, possibly inducing bitter taste to the final product, is avoided (Jaekel and Ternes, 2009).

### Phospholipases in Human Nutrition

Dietary triacylglycerols are absorbed through the intestinal wall after fine dispersion induced by biological emulsifiers (bile acids) and are subsequently degraded by water-soluble lipases in the intestine. Degradation products diffuse into intestinal epithelial cells, where they are reconverted to triacylglycerols and packaged to chylomicrons for transfer to tissues. Similarly, dietary phospholipids are degraded to lyso (1-acyl) phospholipids by an intestinal PLA<sub>2</sub>. Therefore, development of novel lipids by linking fatty acids of biological interest (p.e. omega-3 fatty acids) at the *sn*-1 position of dietary phospholipids is an interesting new approach in human nutrition (Casado et al., 2012). In a similar attempt, yolk suspension was hydrolyzed by a PLA<sub>2</sub> (Lesitase 10 L, Novozymes) before omega-3 fatty acids being linked to egg lysophospholipids.

Phospholipase-produced lysophospholipids have also been used for better absorption of nutrients since they form smaller micelles in the intestinal track compare to phospholipids. Furthermore, hydrolysis of egg yolk PC by cabbage or *Streptomyces chromofuscus* PLD yields a lipid mixture rich in PA (Shnigir and Kisel, 2004), which has been used for medical purposes. Another example of phospholipases involved in human nutrition is the production of PS whose availability from natural sources is limited. Clinical trials have indicated that PS supplementation in the diet may improve mental functions. Therefore, methods for the PLD-catalyzed conversion of PC to PS have been developed (Hokosawa et al., 2000).

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## Relevant websites

- <http://lipidlibrary.aocs.org/> – AOAC lipid library.
- <http://www.lipidmaps.org/> – Lipid maps.

# Polyphenoloxidase in Fruit and Vegetables: Inactivation by Thermal and Non-thermal Processes

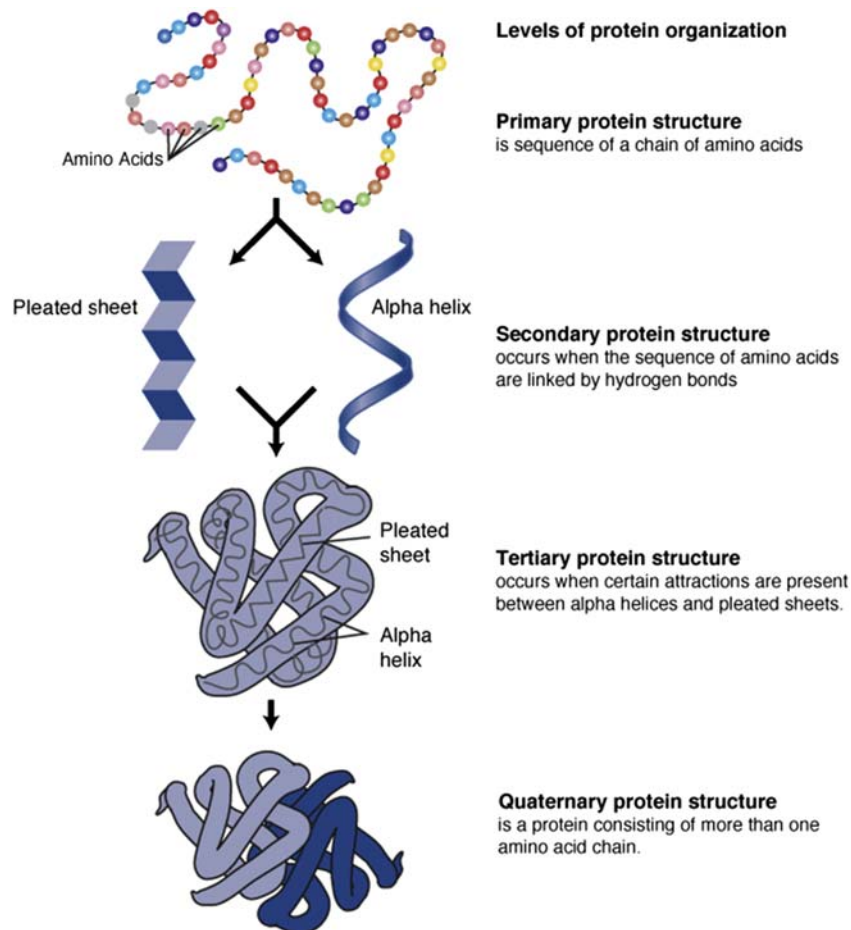
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## Enzyme Fundamentals

Enzymes are proteins with a biological function. They are highly selective biological catalysts involved in many metabolic processes in all organisms (Bugg, 2012). Enzymes accelerate, or catalyze, chemical reactions by converting substrates into products. Enzymes consist of one or more polypeptide backbones of a defined amino acid sequence, arranged in a specific folding. The shape of natural folding is known as native state. Enzyme activity is related with its three-dimensional structure. The deteriorative enzyme activity is not desirable in food since it causes the loss of quality during storage.

As shown in Fig. 1, four distinct levels of structure characterize the enzyme globular protein. The primary structure consists of a unique sequence of amino acids in polypeptide chain. The folding of the polypeptide chain gives rise to the secondary structure, which are the  $\alpha$ -helix and  $\beta$ -sheet structures. The structures are held by the hydrogen bonds of the amino acids. Tertiary structure is defined by the unique three-dimensional arrangement of the polypeptides. It describes the relationship and interaction of secondary structures and usually governed by the hydrogen bonding, hydrophobic interactions, electrostatic interactions and van der Waals forces. The structure formed by the union of more than one protein molecule is known as quaternary structure. Enzymes usually shift between several similar structures in order to function. These functional arrangements which are the tertiary and quaternary structures are called conformations. Thus, the changes in the native conformation are addressed as conformational



**Figure 1** The four level of protein structure. Retrieved from National Institutes of Health, National Human Genome Research Institute. Talking Glossary of Genetic Terms: Protein. <https://www.genome.gov/glossary/>.



changes. Enzyme conformational changes can result in partial or total inactivation of enzyme, no change in the activity or even enzyme activation. The enzyme conformation is affected by the pH, temperature (T), pressure (P), salt concentration and chemical or physical processes, which can cause denaturation of the protein. Sometimes denaturation can be reversible because the primary structure of the polypeptide is conserved in the process and after the denaturing factor is removed, the protein regenerates and resumes its function. Other times the denaturation is irreversible leading to partial or total enzyme inactivation. With respect to thermal treatment, high pressure processing (HPP), ultrasound (US), thermosonication (TS) and pulsed electric fields (PEF) of apple juice and strawberry puree, [Sulaiman et al. \(2017a, 2017b\)](#) demonstrated PPO did not recover the lost activity since the residual activity of PPO obtained after the process remained constant during 1 month at ambient temperature and refrigerated storage at 3 °C.

Enzymes are categorized in six groups according to the reactions they catalyze: oxidoreductases (1), transferases (2), hydrolases (3), lyases (4), isomerases (5), and ligases (6). In addition, every enzyme is classified with Enzyme Commission number (EC). The EC number consists of a sequence of four numbers, where the first number specifies the enzyme group.

### Food Endogenous Enzymes

While some commercial enzymes are added to foods to promote important reactions, most of endogenous native enzyme in foods can degrade nutrients and cause quality deterioration. The undesirable enzymatic reaction occurs when the cells contained in the foods of plant (e.g. fruit, vegetable) or animal origin are disrupted during processing and get in contact with its substrates. Fruit and vegetable often contain endogenous enzymes, which sometimes are more heat resistant than microorganisms ([Silva and Gibbs, 2004, 2009](#)). Although the microbes are first considered in the design of new thermal and non-thermal processes to pasteurize food products, spoilage enzymes should also be inactivated to a minimal level, to avoid deteriorative chemical reactions during storage and allow a longer shelf-life of the food ([Silva and Sulaiman, 2017](#)). The activity of deteriorative enzymes naturally present in foods such as polyphenoloxidase or polyphenol oxidase (PPO), peroxidase (POD), pectinmethylesterase (PME), polygalacturonase (PG),  $\beta$ -glucosidase (BGL) and lipoxygenase (LOX), can affect food color, flavor, texture and nutritional quality ([Ludikhuyze et al., 2003; Vernwal et al., 2006; Zhang et al., 2005](#)).

## Polyphenoloxidase (PPO) and Enzymatic Browning in Fruit and Vegetable Products

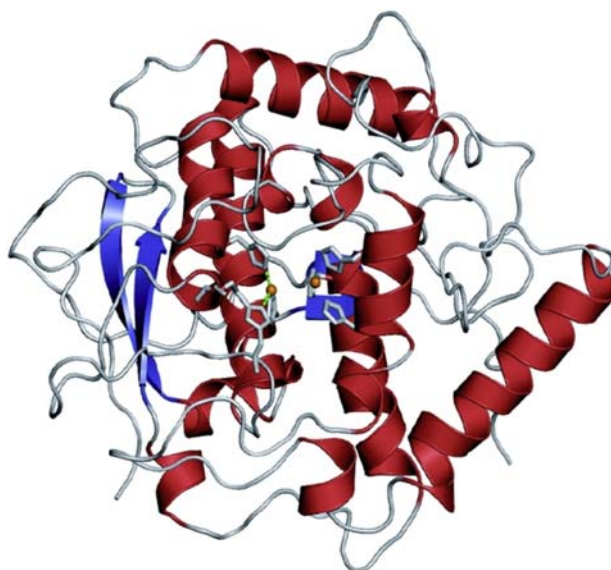
### Food Browning

Browning reactions occur in food during processing and storage. There are two types of browning reactions, the non-enzymatic browning (e.g. Maillard and caramelization) and the enzymatic browning. It is well known that fruits, vegetables and certain crustacean seafood (e.g. shrimp) experience enzymatic browning when plant tissues are exposed to air during handling, processing and storage. This undesirable reaction is due to the activity of polyphenoloxidase (PPO) endogenous enzyme, which is naturally present in the plant tissues. PPO catalyses the degradation of phenolic fruit constituents to o-quinones in the presence of oxygen. The resulting o-quinone will subsequently polymerize with other o-quinone, protein or amino acids producing undesirable brown compounds ([Golagoldhirsh et al., 1984; Vámos-Vigyázó, 1981](#)). The enzymatic browning of fruit and vegetable tissues has been an on-going concern in the industry with economic losses due to the negative impact on the colour, appearance, flavor, nutritive properties of food and subsequent consumer's acceptability ([Mayer and Harel, 1991; Dauthy, 1995; Whitaker and Lee, 1995; Lee, 1999](#)). Enzymatic browning can occur due to mechanical injuries in whole fruits/vegetables during postharvest handling and storage, and is also common in processed food products such as juices, purees/smoothies, cut fruits/vegetables (e.g. cubes, slices, etc).

### PPO Enzyme

Polyphenoloxidase or polyphenol oxidase or PPO (EC 1.14.18.1) is an oxidoreductase copper-containing metalloprotein that is responsible for the browning of fruits and vegetables (e.g. apple, pear, peach, potato, lettuce, mushroom), and certain seafoods such as crustaceans ([Martinez and Whitaker, 1995](#)). Catechol oxidase, tyrosinase, phenolase, catecholase, o-diphenol oxidase and are some of the names of PPO. PPO catalyzes the hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to o-quinones. Then the quinones may condense and react non-enzymatically with other phenolic compounds, amino acids, proteins and other cellular constituents to produce brown melanoidin pigments ([Rouet-Mayer et al., 1990; Whitaker and Lee, 1995](#)). PPO enzyme can be active in a range of pH from 3.5 to 9 and temperatures from 15 to 60 °C ([Dalmadi et al., 2006; Navarro et al., 2014; Siddiq et al., 1993; Wu et al., 2013; Yang et al., 2000](#)). [Fig. 2](#) shows an example of PPO structure from grape berry which has been successfully crystalized and modelled ([Virador et al., 2010](#)). The crystal structure is a monomeric protein of 38.4 kDa mass with an ellipsoidal shape and dimension of  $57 \times 48 \times 48$  Å ([Virador et al., 2010](#)). The secondary structure is primarily  $\alpha$ -helical with four helix bundle which are composed of  $\alpha$ -helices  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 12$  and  $\alpha 14$  ([Fig. 2](#)). The active site is the dicopper in the centre of the four helix bundle ([Fig. 2](#)). Removal or distortion that causes the conformational changes of the overall ellipsoidal shape will inactivate the enzyme. The active site of grape PPO was found to be similar to the sweet potato PPO described by [Klabunde et al. \(1998\)](#).





**Figure 2** Ribbon model of PPO grape (*Vitis vinifera* L., cv. Grenache) showing the overall ellipsoidal shape, two  $\beta$ -shape and the dicopper center within a four-helix bundle. Adapted from Virador et al. (2010).

## PPO Control and Inactivation in Fruit and Vegetable Products

### PPO Inhibition in Foods by Chemical Agents

Anti-browning agents such as sulphites, sodium chlorite, cysteine, kojic acid, ascorbic acid and cinnamic acid are used for food preservation to control enzymatic browning (Lambrecht, 1995; Vámos-Vigyázó, 1995; Guerrero-Beltrán et al., 2005; Queiroz et al., 2008). These anti-browning compounds have different mechanisms in inhibiting PPO activity. Ascorbic acid reduces o-quinones to monophenols or diphenols (McEvily et al., 1992); cysteine produces colourless adducts when reacting with o-quinones (Walker and Reddish, 1964); kojic acid inhibits PPO and bleached melanin due to chemical reaction of the browning pigment to colorless compound (Iyidogan and Bayindirli, 2004); benzoic and cinnamic acids make a complex with copper at the active site of PPO and sodium chlorite as a strong oxidizing agent which generates chlorine dioxide under acidic condition reduce the pH of the food thus inhibit PPO (Lu et al., 2006). Natural compounds such as honey also have inhibitory effects on PPO (Chen et al., 2000). Honey from different floral sources reduced PPO activity (range of 2% to 45%) in fruit and vegetable homogenates (Chen et al., 2000).

### PPO Inactivation by Thermal and Non-thermal Processes

Consumers have been choosing preservative free foods and there is a global trend to reduce the use of chemical additives that have adverse effect towards human health. Thermal processing is the most reliable method for controlling fruit/vegetable browning, often referred to by the industry as blanching or pasteurization. However, heat employed in thermal processing negatively affects the fruit flavor (Silva et al., 2000) and can degrade some nutrients such as vitamin C, polyphenols and other antioxidants (Castro et al., 2008; Volden et al., 2008). There is an increasing consumer demand for convenient, healthy and ready-to-eat fruit/vegetable products such as canned baby foods, fruit juices, fruit puddings, fruit jams, and so forth. These products should maintain the original characteristics flavor, texture, color, nutrient and aroma of the fruits/vegetables (Landl et al., 2010) and offer a reasonably extended shelf life. Due to the demand for fresh and minimally processed, preservative free fruit/vegetable products, the study and potential of emerging non-thermal food preservation technologies alone and combined with mild heat to inactivate PPO enzyme in foods is an active area of research. The following technologies and their impact on PPO inactivation were reviewed in the next sections: ultrasound (US), high pressure processing (HPP), and pulsed electric fields (PEF). As the enzyme inactivation and kinetics might be affected by the medium or food in which the enzyme is processed (Rapeanu et al., 2006), the studies reviewed have processed the fruit/vegetable product containing the enzyme. Also, in terms of industrial application, it is desirable to process the real fruit/vegetable product instead of an extract containing the enzyme.

## PPO Inactivation in Fruit and Vegetable Products by Thermal Processing/Blanching

### Thermal Processing Fundamentals

Heat is the common method used by food industry to inactivate endogenous fruits and vegetables' enzymes. Thermal processing is an ancient and established technique for stabilizing and preserving foods, as heat inactivates the microbes (Silva and Gibbs, 2009;

Silva et al., 2014) and denatures permanently deteriorative enzymes. Heat from steam is usually applied indirectly by heat exchangers to juices and purees. As enzymes can be active at freezing temperatures (Arighi et al., 1936) and spoil whole/pieces of frozen fruits and vegetable during storage, blanching is usually carried prior to the freezing process. Blanching involves the direct contact of steam or hot water with the whole or cut pieces of fruit and vegetables for a certain period of time. The biggest drawback of using heat to preserve foods is the destruction of thermosensitive nutrients, which include vitamins, flavors, color, texture, carbohydrates and other water-soluble components (Queiroz et al., 2008). In addition, blanching is an energy and water intensive technology, thus economically unfavorable. The thermal stability of PPO is dependent on the fruit/vegetable species, and within one species on the cultivar. Depending on the fruit/vegetable, exposure to 70 to 100 °C partially or completely inactivates PPO (Dimick et al., 1951; Ludikhuyze et al., 2003; Silva and Gibbs, 2004). The thermal inactivation kinetics of PPO was described with simple first order model (Dimick et al., 1951; Sulaiman et al., 2015a) and first order biphasic model.

### Effect of Thermal Processing/Blanching on PPO Enzyme

A review of recent works focusing on the effect of thermal processing and blanching of fruit and vegetable products and its effect on endogenous PPO enzyme was carried out and is presented on Table 1. The results vary from 45% residual activity (RA) on slices of red cabbage blanched at 100 °C for 10 min (Bernstein and Noreña, 2016) to <5% RA in Boskoop cloudy apple juice exposed for 1 min to 70 °C (Buckow et al., 2009), and 0.3% RA in Camarosa strawberry puree exposed for 5 min to 70 °C (Sulaiman and Silva, 2013). The variability within the same fruit is also very high, depending on the fruit cultivar or variety. Taylor's Gold pear puree processed for 10 min at 85 °C resulted in 0.3% RA (Sulaiman et al., 2015a), whereas Packam pear puree RA was 53% after 100 °C for 0 min (Zhou et al., 2018). Regarding strawberry puree, 90 °C for 15 min had no effect on Festival PPO enzyme and minor effect on PPO in Aroma strawberry puree (Terefe et al., 2010) as opposed to 0.3% RA in Camarosa puree after 5 min at 70 °C. Other very resistant PPO enzymes include pumpkin, mushroom, mango and pineapple.

**Table 1** Residual activity of polyphenoloxidase in fruit and vegetable products after thermal processing and blanching<sup>a</sup>

Fruit/vegetable	Cultivar	Medium	Heat treatment	Residual activity (RA, %)	Reference
Pear	Packham	Puree	100 °C – 0 min	53	Zhou et al. (2018)
Pear	Nashi	Puree	85 °C – 15 min	15	Yoon and Silva (2011)
Pear	Taylor's Gold	Puree	85 °C – 10 min	0.3	Sulaiman et al. (2015a)
			80 °C – 10 min	15	
			75 °C – 10 min	90	
Pear	<i>Pyrus bretschneider</i>	Juice	95 °C – 2 min	0	Saeeduddin et al. (2015)
			65 °C – 10 min	59	
Apple	Royal Gala	Puree	85 °C – 3 min	2	Sulaiman et al. (2015a)
			75 °C – 10 min	24	
Apple	Starking Delicious	Cloudy juice	70 °C – 2 min	22	Akgün and Ünlütürk (2017)
Apple	Boskoop	Cloudy juice	70 °C – 1 min	< 5	Buckow et al. (2009)
Pineapple	Queen	Puree	95 °C – 12 min	3	Chakraborty et al. (2015)
Pineapple	nr	Puree	90 °C – 5 min	1.2	Chutintrasri and Noomhorm (2006)
			85 °C – 5 min	7	
			80 °C – 5 min	31	
Strawberry	Festival	Puree	90 °C – 15 min	100	Terefe et al. (2010)
	Aroma		90 °C – 15 min	82	
Strawberry	Camarosa	Puree	70 °C – 5 min	0.3	Sulaiman and Silva (2013)
			60 °C – 5 min	44	
Strawberry	Ruby Gem	Puree	88 °C – 2 min	0 not detected	Terefe et al. (2013)
	Camarosa				
Bayberry	nr	Juice	75 °C – 10 min	< 2	Cao et al. (2018)
Orange, strawberry, banana	–	Juice/smoothie	80 °C – 7 min	0.4	Escobedo-Avellaneda et al. (2016)
Pumpkin	nr	Puree	90 °C – 10 min	55%	García-Parra et al. (2018)
Coriander leaves	nr	Paste	90 °C – 2 min	0 not detected	Nath et al. (2016)
<b>Blanching</b>					
Red cabbage	Capitata rubra	0.5 cm slices	Water 90°C-10 min	46	Bernstein and Noreña (2016)
			Steam 100°C-10 min	45	
Mushroom	nr	Whole	Water 100 °C – 10 min	2	Matser et al. (2000)
			Water 100 °C – 5 min	3.2	
Mango	nr	1 cm slices	Steam 94 °C – 7 min	0	Ndiaye et al. (2009)

<sup>a</sup>nr – not reported.

## PPO Inactivation in Foods by High Pressure Processing (HPP) and High Pressure Thermal Processing (HPTP)

### HPP Fundamentals

Although Hite described first the application of HPP to preserve milk long time ago (Hite, 1899), not until 1980s more interest on the HPP technology was observed, leading to several studies and developments on various aspects of this novel pasteurization treatment, and the introduction of the first HPP pasteurized foods in Japan in the early 1990s (Van Loey et al., 2003). In 2013 units with 525 L capacity and 3000 kg/h throughput were launched by Hiperbaric, and in 2015, 315 HPP industrial units were already installed worldwide, mostly in the USA and Europe, and (Balda, 2018).

High pressure processing (HPP) or high hydrostatic pressure (HHP) is a cold pasteurization technology by which vacuum packed food products are introduced in a vessel containing a pressure transmitting liquid (usually water), and submitted to a high level of isostatic pressure (300 to 600 MPa). The pressure builds up to a desired maximum pressure which is held for a certain time, followed by a quick decompression to end the HPP cycle. The product is removed and stored generally under refrigerated conditions. HPP is a commercial pasteurization considered as an alternative to conventional heat treatment, because of its ability to inactivate pathogenic and spoilage microorganisms at room or mild temperatures (Evelyn and Silva, 2018; Silva and Evelyn, 2018). Thus, the nutritional and sensory characteristics of the original fresh fruit/vegetables are better retained compared to an equivalent thermal process (Houška and Silva, 2018). Cold pressed HPP fruit juices have been the drivers of this technology, but smoothies, guacamole, oysters, ham and fresh meat products are also available in the supermarkets worldwide (Balasubramaniam, 2003). Several studies have been demonstrating that room temperature HPP has limited effectiveness towards the inactivation of certain microbial spores and enzymes associated with food quality degradation (Silva and Evelyn, 2018; Terefe and Buckow, 2018). Therefore, the combination of HPP with mild heat (60–90 °C) also referred to as high pressure thermal processing (HPTP) has been investigated for more effective spore and enzyme inactivation.

### Effect of HPP on Enzymes

With respect to enzymes, HPP at ambient temperature typically breaks the non-covalent bonds (e.g. hydrogen bonds, in the enzymes), but does not break covalent bonds (e.g. peptide bond in enzyme) (Hendrickx et al., 1998; Mozhaev et al., 1996). Therefore, HPP mainly affects tertiary and quaternary structure of the enzymes (Terefe and Buckow, 2018). The hydrogen bond maintaining the helical secondary structures is also ruptured at HPP > 400 MPa (Hendrickx et al., 1998). Since HPP disrupts the enzyme secondary, tertiary and quaternary structures, the enzyme may be activated or inactivated (Mozhaev et al., 1996), depending on the levels of pressure treatments. The factors influencing the effect of HPP process on the enzyme includes the origin of the enzyme (fruit/vegetable cultivar), the level and duration (time, t) of pressure (P) treatment, and for heat assisted pressure processing (HPTP), the temperature (T) of the treatment. Generally, it is expected a higher enzyme inactivation or lower enzyme residual activity (RA) when higher T, P and t are used for processing.

### Effect of HPP and HPTP on PPO Enzyme

A few modeling studies of PPO inactivation by HPTP revealed non-linearity, best described by a first order biphasic model (Weemaes et al., 1998; Rapeanu et al., 2005; Sulaiman et al., 2015b). This model was applied successfully to HPTP inactivation of PPO in Taylor's Gold pear and Camarosa strawberry purees (Sulaiman et al., 2015b). Tables 2 and 3 show a review of HPP/HPTP processes applied to fruit and vegetable products, respectively, and its effect on PPO residual activity (RA). Except for whole strawberry PPO which was fully inactivated by 15 min at 600 and 800 MPa at room temperature (Garcia-Palazon et al., 2004), room T HPP only partially inactivate PPO enzyme in Scirose apple puree (40% RA after 600 MPa - 15 min), whole raspberry (70% RA, 800 MPa - 15 min), strawberry puree from Ruby Gem, Festival, Camarosa and whole Pajaro strawberry varieties (19%–83% RA, 600 MPa), peach puree (5% after 22 min at 517 MPa), and Hass avocado puree (16% after 30 min at 689 MPa) (López-Malo et al., 1998; Garcia-Palazon et al., 2004; Guerrero-Beltrán et al., 2004; Terefe et al., 2013; Sulaiman et al., 2015b). Therefore several authors attempted HPTP, which still seems to have only partial inactivation of the enzyme. For example RA of Nashi pear puree was 88% after 15 min exposed to 600 MPa-62 °C, apple juices/purees from Boskoop, Scirose, Royal Gala, and Amasya exposed to 450–700 MPa and 50–71 °C resulted in RA in the range of 11%–60% (Bayindirli et al., 2006; Buckow et al., 2009; Sulaiman et al., 2015b). Camarosa strawberry puree was fully inactivated after exposure for 15 min to 600 MPa-71 °C (Sulaiman et al., 2015b). The increase of T in HPTP reduced the RA of the PPO enzyme of Royal Gala apple puree and Queen pineapple puree (Chakraborty et al., 2015). The combination of a very high P with a very high T was still incapable to fully inactivate PPO enzyme in pumpkin puree (54% RA after 900 MPa - 121 °C - 3 min) and red plum puree (50% RA, 900 MPa - 95 °C - 1 min) (García-Parra et al., 2014, 2018). The differences between the results published might be just due to different fruit cultivars, growing conditions and ripening stage which can have different isoenzymes (Terefe et al., 2014). In addition fruit composition (pH, soluble solids, etc.) could also be the reason for different PPO inactivations in different cultivars. For example, Rapeanu et al. (2006) concluded PPO resistance from Victoria grape was higher in the grape must than the buffer solution at the same pH.

Undesirable fruit enzyme activation was registered under certain processing conditions: 600 MPa HPP at RT for 15 min caused PPO activation in Taylor's Gold and Nashi pear purees, and Royal Gala apple puree (Sulaiman et al., 2015b); 1 cm slices of Bartlett pear submitted to 400 MPa for 10 min activated PPO enzyme by a factor of 5 (Asaka and Hayashi, 1991). Activation at room temperature 600 MPa was also observed in the vegetables sweet potato and mushroom (Matser et al., 2000; Tribst et al., 2016).

**Table 2** Residual activity of polyphenoloxidase in fruit products after non-thermal high pressure processing (HPP) and high pressure thermal processing (HPTP)<sup>c</sup>

<i>Fruit</i>	<i>Cultivar</i>	<i>Medium</i>	<i>Type of treatment<sup>a</sup></i>	<i>Treatment conditions<sup>b</sup></i>	<i>Time (min)</i>	<i>Residual activity (RA, %)</i>	<i>Reference</i>
Pear	Packham	Puree	HPTP	600 MPa – 90 °C	5	59	Zhou et al. (2018)
Pear	Taylor's Gold	Puree	HPTP	600 MPa – 71 °C	15	127	Sulaiman et al. (2015b)
			HPP at RT	600 MPa – 62 °C	15	122	
				600 MPa	15	120	
Pear	Nashi	Puree	HPTP	600 MPa – 62 °C	15	88	Sulaiman et al. (2015b)
			HPP at RT	600 MPa	15	121	
Pear	Bartlett	1 cm slices	HPP at RT	400 MPa	10	Activation by factor of 5	Asaka and Hayashi (1991)
Apple	Boskoop	Cloudy juice	HPTP	700 MPa – 70 °C	15	11	Buckow et al. (2009)
Apple	Scirose	Puree	HPTP	600 MPa – 62 °C	15	59	Sulaiman et al. (2015b)
			HPP at RT	600 MPa	15	40	
Apple	Royal Gala	Puree	HPTP	600 MPa – 71 °C	15	40	Sulaiman et al. (2015b)
			HPP at RT	600 MPa – 62 °C	15	59	
				600 MPa	15	174	
Apple	Amasya	Juice	HPTP	450 MPa – 50 °C	30	47	Bayindirli et al. (2006)
Red plum	Crimson	Puree with peel	HPTP	900 MPa – 95 °C	1	50	García-Parra et al. (2014)
	Globe		HPTP	600 MPa – 85 °C	1	65	
Pineapple	Queen	Puree	HPTP	600 MPa – 70 °C	20	8	Chakraborty et al. (2015)
			HPTP	600 MPa – 60 °C	20	21	
Raspberry	Red	Whole	HPP at RT	800 MPa	15	70	García-Palazon et al. (2004)
Strawberry	Aroma	Puree	HPTP	690 MPa – 90 °C	5	77	Terefe et al. (2010)
Strawberry	Ruby Gem	Puree	HPP at RT	600 MPa	5	83	Terefe et al. (2013)
	Festival				5	65	
	Camarosa				5	62	
Strawberry	Camarosa	Puree	HPTP	600 MPa – 71 °C	15	0	Sulaiman et al. (2015b)
			HPTP	600 MPa – 62 °C	15	2	
			HPP at RT	600 MPa	15	19	
Strawberry	Pajaro	Fruit	HPTP	400 MPa – 60 °C	15	No effect	Cano et al. (1997)
			HPP at RT	285 MPa	15	40	
Strawberry	nr	Whole	HPP at RT	800 MPa	15	0	García-Palazon et al. (2004)
				600 MPa	15	0	
Orange, strawberry, Banana	–	Juice/smoothie	HPTP	600 MPa – 64 °C	5	5	Escobedo-Avellaneda et al. (2016)
Peach	Nr	Puree	HPP at RT	517 MPa	5	19	Guerrero-Beltrán et al. (2004)
					22	5	
Avocado	Hass	Puree	HPP at RT	689 MPa	30	16	López-Malo et al. (1998)
		acidified					
Avocado	Hass	Paste	HPP at RT	60 <sup>a</sup> MPa	3	51	Jacobo-Velázquez and Hernández-Brenes (2010)

<sup>a</sup>HPP at RT means high pressure processing at room temperature non-thermal conditions (T < 30 °C).<sup>b</sup>For HPTP, temperature is the average during constant pressure phase of the HPP cycle.<sup>c</sup>The fruit and vegetable products were vacuum packed before HPP and HPTP processing.

Furthermore, activation was observed after a HPTP treatment of Taylor's Gold pear puree (600 MPa – 71 °C-15 min) and pumpkin puree (900 MPa – 58 °C-5 min) (Sulaiman et al., 2015b; García-Parra et al., 2018). The increase in enzyme activity with HPP or HPTP is unacceptable as this can result in undesirable browning, and changes in flavour and nutritional quality of the juices during product processing and storage (Terefe and Buckow, 2018).

### Comparing Thermal With HPTP Inactivation of PPO

For certain fruit/vegetable cultivars, there is no extra benefit in using the thermal assisted HPP technology (HPTP) to potentiate and increase the inactivation of PPO. For example, thermally treated pumpkin puree at 90 °C for 10 min resulted in 55% residual activity. The same authors attempted 900 MPa combined with 121 °C for 3 min and obtained a similar RA of 54% (García-Parra et al., 2018). So, for pumpkin puree, an exclusively thermal process seems to be a better option. The PPO RA of plum puree containing peel after 85 °C for 5 min was approximately 50%. Using HPTP at 600 MPa-85 °C for 1 min resulted in 65% RA (García-Parra et al., 2014). This might indicate that HPTP is not suitable for PPO inactivation in Crimson Globe plum puree, being preferable to use a higher temperature thermal process alone. The Royal Gala apple puree PPO RA of 23% after a thermal treatment of

**Table 3** Residual activity of polyphenoloxidase in vegetable products after non-thermal high pressure processing (HPP) and high pressure thermal processing (HPTP)<sup>c</sup>

Vegetable	Cultivar	Medium	Type of treatment <sup>a</sup>	Treatment conditions <sup>b</sup>	Time (min)	Residual Activity (RA, %)	Reference
Pumpkin	nr	Puree	HPTP	900 MPa - 121 °C	3	54	García-Parra et al. (2018)
				900 MPa - 58 °C	5	111	
Sweet potato	nr	Puree	HPP at RT	600 MPa	30	380	Tribst et al (2016)
		0.5 cm cubes			30	200	
Mushroom	nr	Whole	HPP at RT	950 MPa	5	10	Matser et al. (2000)
				800 MPa	5	25	
				600 MPa	5	153	
Cocoyam	nr	Puree	HPP at RT	600 MPa	30	60	Tribst et al (2016)
		0.5 cm cubes			30	75	
Carrot	Peruvian	Puree	HPP at RT	600 MPa	30	10	Tribst et al (2016)
		0.5 cm cubes			30	25	
Coriander leaves	nr	Paste	HPP at RT	600 MPa	5	38	Nath et al. (2016)

<sup>a</sup>HPP at RT means high pressure processing at room temperature non-thermal conditions ( $T < 30\text{ }^{\circ}\text{C}$ ).

<sup>b</sup>For HPTP, temperature is the average during constant pressure phase of the HPP cycle. For pumpkin puree the temperature is after the compression phase/adiabatic heating (García-Parra et al., 2018).

<sup>c</sup>The vegetable products were vacuum packed before HPP and HPTP processing.

15 min at  $75\text{ }^{\circ}\text{C}$  is lower than 40%, the residual activity value obtained after 15 min HPTP at 600 MPa-  $71\text{ }^{\circ}\text{C}$  (Sulaiman et al., 2015a, 2015b). For Taylor's Gold pear puree while 600 MPa -  $71\text{ }^{\circ}\text{C}$  for 15 min activated the enzyme (RA = 127%), a great inactivation is observed at  $85\text{ }^{\circ}\text{C}$ -10 min (RA<1%) (Sulaiman et al., 2015a, 2015b). The heat processing of Boskoop cloudy apple juice at  $70\text{ }^{\circ}\text{C}$  for 1 min resulted in <5% RA of PPO, whereas a 15 min HPTP at 700 MPa- $70\text{ }^{\circ}\text{C}$  resulted in a higher RA of 11%, revealing HPTP was not acceptable for PPO inactivation in this apple cultivar juice (Buckow et al., 2009). This could be possibly due to the protective effect of pressure against temperature. Hydrogen bonds in proteins are generally stabilized by pressure and destabilized by heat (Damodaran, 1996; Heremans, 1982).

## PPO Inactivation in Foods by Ultrasound Processing (US) and Thermo-sonication (TS)

### Ultrasound Fundamentals

Ultrasound is composed of sonic waves with frequencies higher than human ear audible sound. Power ultrasound or high intensity ultrasound uses lower frequencies (kHz) than medical applications, and are characterized by sound intensity or acoustic intensity ranging between 10 and  $1000\text{ W/cm}^2$  (Feng and Yang, 2011). Sonication refers to the power ultrasound processing of liquid foods or beverages for microbial and enzyme inactivation to increase shelf life. Thermo-sonication (abbreviated TS) is the combined use of power ultrasound with moderate temperatures to process the food and improve the efficiency of thermal process alone. The application of an external mild pressure during sonication (e.g. 2 atm =  $2 \times 101.3\text{ kPa}$ ), referred as manothermo-sonication (abbreviated MTS), is one step further in terms of microbial and enzyme inactivation. The sonication generates bubbles in the liquid food as the wave energy propagates and this phenomenon is called acoustic cavitation. The formation and collapse of tiny bubbles can occur in few microseconds and the cavitation is increased with acoustic energy (power). Vigorous mixing of gas bubbles and liquid food occurs which accelerate heat and mass transfer. In addition to the mechanical effects of cavitation, chemical effects can also occur during sonication, with the formation of reactive free radicals.

The ultrasound unit consists of a generator which converts electricity into high frequency alternating current and a transducer for converting the current into mechanical vibrations. The probe is the most efficient method to sonicate liquid food because of its direct contact with the food. The probe is also referred as sonotrode, ultrasonic tip, horn or finger. Food research ultrasound units are available with a selection of sonotrodes with different geometries for different applications, which present different maximum amplitude (energy). Some of the units available for research are equipped with a flow cell which allows continuous mode ultrasound experimentation (Milani and Silva, 2017), one step further close to industrial application. The flow cell is surrounded by a jacketed vessel where cold water (or other cooling liquid) is circulated to better control the temperature of the liquid food product during the ultrasound treatment, avoiding the increase of temperature. The flow cell with controlled temperature can also be used in batch process for kinetic experiments (Evelyn and Silva, 2016; Milani et al., 2016; Milani and Silva, 2017). The probe conveys the sonic vibrations to the treated food and the energy transmitted to the food medium can be expressed as sound intensity ( $\text{W/m}^2$ ), which is proportional to the amplitude of the wave ( $\mu\text{m}$ ). Usually the amplitude/energy can be adjusted in the settings of the ultrasound unit. The specific acoustic energy/power or volumetric acoustic energy/power density (AED/AEP, in  $\text{W/ml}$  of processed liquid sample) is also very useful to quantify the energy requirements of the process for a given volume of treated food. The higher the sound intensity/energy/power, the higher will be the mechanical and sonochemical effects. The energy supply for the ultrasound



treatment can be set in continuous or in pulses. Wave frequency is given in kHz and ultrasound units usually present a fixed frequency and varying amplitude, the last directly proportional to the energy or power consumed and energy delivered to the food. The frequency of most units used for food research is around 20–24 kHz. Higher food medium temperature (T) and/or pressure (P) increase the efficiency of the TS/MTS process.

### Ultrasound Effect on Enzymes

Ultrasound is known to break up proteins, starches and other large biopolymers (Weiss et al., 2011) such as enzymes, and can affect the protein and enzyme functionality. The damage of protein structure by ultrasound is desirable since it results in enzyme inactivation. Changes in enzyme biological activity are due to changes in the folding of the proteins, the secondary and tertiary structures of the enzyme. The rapid formation and collapse of bubbles change the enzyme's environment, such as temperature, pressure, shear stress and pH, which can cause the enzyme inactivation (Feng and Yang, 2011; Sala et al., 1995; Terefe et al., 2015a). Under these extreme changes, hydrogen bonds and van der Waals bonding in the enzyme polypeptide chains can be broken down (Islam et al., 2014), resulting in loss of enzyme activity (Zhong et al., 2004). Additionally, the formation of high energy free radicals due to sonication could lead to the sonolysis of water molecules (O'Donnell et al., 2010). The free radicals can react with the amino acids of the enzyme structure, subsequently affecting the enzyme activity and its catalytic function (Barteri et al., 2004; López et al., 1994; Potapovich et al., 2003).

The sonication processing conditions can cause partial or total inactivation of enzyme activity, depending on the type and the fruit/vegetable source of the enzyme. Often room temperature sonication has minor effects on enzyme activity (O'Donnell et al., 2010), so it is necessary to use TS or MTS for effective enzyme inactivation (Silva and Sulaiman, 2017). The effect of ultrasound treatment on enzyme depends on ultrasonic intensity (acoustic energy), duration of process (t), temperature (T), pressure (P), food pH and ionic strength. The medium in which the enzyme is suspended for ultrasound processing affects hugely the inactivation of enzymes (Özbek and Ülgen, 2000; Terefe et al., 2015a). For example, the extracted thermostable orange pectinmethylesterase (PME) was suspended in orange juice and citrate buffer with the same pH (3.5) and processed by MTS (117  $\mu$ m, 72 °C, 200 kPa) and thermal treatment alone. The MTS reduced the  $D_{72^{\circ}\text{C}}$ -value of PME suspended in orange juice from 500 min (only thermal process) to 1.24 min, and when suspended in citrate buffer, the  $D_{72^{\circ}\text{C}}$ -values were very different and reduced from 20 to 0.8 min (Vercet et al., 1999). The methodology used to extract the enzyme and the different media submitted to TS affect the enzyme inactivation rate. Therefore, the review showed in the following section will focus on studies of enzymes processed in their natural food environment. This approach will reproduce better real-case-scenario conditions (Table 3) (Table 3).

### Effect of Ultrasound (US) and Thermosonication (TS) on PPO Enzyme

The TS inactivation of PPO in Taylor's Gold pear puree, Royal Gala apple puree, Camarosa strawberry puree and Golden Delicious apple juice was linear and followed the first order kinetics model (Başlar and Ertugay, 2013; Sulaiman et al., 2015a). Table 4 shows the residual activity of PPO in several juices/purees after TS and US processing. The TS process was much more efficient than sonication at room temperature (US), yielding the following RA after 10 min treatment: 1%–4% at 72 °C in Taylor's Gold pear, Royal Gala apple and Camarosa strawberry purees (Sulaiman et al., 2015a); 2% at 65 °C for *Pyrus bretschneideri* pear juice (Saeeduddin et al., 2015); 5%–6% at 60 °C for Fuji apple juice (Abid et al., 2014) and carrot juice (Jabbar et al., 2015). On the other hand, the values of RA after a 10 min sonication process without heat were 89% for *P. bretschneideri* pear juice, 58% for Taylor's Gold pear puree, 59% for Royal Gala apple puree, 85% and 97% for juices of 2 apple cultivars, 84% for pineapple diluted juice (Costa et al., 2013) and 25% for strawberry puree. From the studies analyzed it is also obvious the higher the TS temperature, the higher is the PPO inactivation with lower RA values. Surprisingly, Cheng et al. (2007) registered 80% increase in activity of guava juice PPO after a 30 min ultrasound treatment at 15–20 °C.

### Comparing TS With Thermal Inactivation of PPO

The comparison of TS with thermal treatment alone at similar temperatures demonstrated the potential of power ultrasound to assist thermal pasteurization processes aiming enzyme inactivation. The best example is Taylor's Gold pear puree PPO, while 90% RA was obtained after 10 min – 75 °C thermal process, only 4% was registered after a 10 min TS at 72 °C (Sulaiman et al., 2015a). For *Pyrus bretschneideri* pear juice thermally treated for 10 min at 65 °C resulted in 59% RA, and TS (10 min at 65 °C) resulted in 2% RA (Saeeduddin et al., 2015). Royal Gala apple puree submitted for 10 min at 75 °C resulted in 24% RA vs. 4% RA after TS for 10 min at 72 °C (Sulaiman et al., 2015a).

## PPO Inactivation in Foods by Pulsed Electric Fields (PEF)

### PEF Fundamentals

Pulsed electric fields (PEF) is a non-thermal preservation method that uses high voltage at extremely short processing time (milliseconds) and relatively low temperature to inactivate enzymes and produce microbiologically safe foods. It retains the fresh-like



**Table 4** Residual activity of polyphenoloxidase in fruit and vegetable juices/purees after ultrasound processing (US) and thermosonication (TS)

<i>Fruit or vegetable</i>	<i>Cultivar</i>	<i>Medium processed</i>	<i>Soluble solids</i> <i>pH (° Brix)</i>	<i>Treatment</i> <i>details</i>	<i>Specific acoustic</i> <i>energy</i> <i>(W/g or W/mL)</i>	<i>Type of treatment</i>	<i>T<sup>a</sup></i> <i>(° C)</i>	<i>Time</i> <i>(min)</i>	<i>Residual activity</i> <i>(RA, %)</i>	<i>Reference</i>
Pear	Taylor's Gold	Puree	4.6 16.7	24 kHz, probe, 210 µm	1.30	TS US	72 32	10 10	4 58	Sulaiman et al. (2015a)
Pear	<i>Pyrus bretschneideri</i>	Juice	4.8 12.6	20 kHz, probe	0.30	TS US	65 25	10 10	2 89	Saeeduddin et al. (2015)
Apple	Royal Gala	Puree	4.0 10.9	24 kHz, probe, 210 µm	1.30	TS US	72 32	10 10	4 59	Sulaiman et al. (2015a)
Apple	Golden Delicious	Juice	nr nr	24 kHz, probe, 100 µm, 1 s on/1 s off	nr	TS TS US	60 50 40	10 10 10	17 71 85	Başlar and Ertugay (2013)
Apple	Fuji	Juice	3.9 12.0	20 kHz, probe, 5 s on/5 s off	0.30	TS TS US	60 40 20	10 10 10	6 53 97	Abid et al. (2014)
Strawberry	Camarosa	Puree	3.3 9.3	24 kHz, probe, 210 µm	1.30	TS TS US	72 52 32	10 10 10	1 14 25	Sulaiman et al. (2015a)
Pineapple	Perola	Diluted juice	4.0 8.0	19 kHz, probe	0.67	US	≤21	10	84	Costa et al. (2013)
Carrot	Heitian-5	Juice	6.0 8.0	20 kHz, probe, 5 s on/5 s off	0.26	TS TS US	60 40 20	10 10 10	5 50 100	Jabbar et al (2015)

nr = not reported.

<sup>a</sup>T is the process average temperature which was controlled during treatment with a surrounding water bath or a jacketed vessel.

flavor without significant loss of nutrients. PEF involves the application of a short burst ( $\mu\text{s}$  or  $\text{ms}$ ) of high voltage (15–80 kV/cm) to foods flowing between two electrodes (Butz and Tauscher, 2002; Qin et al., 1996; Milani et al., 2015). The main important components of PEF are the impulse generation systems and the treatment chamber (Alkhafaji and Farid, 2007). Most PEF is done in a continuous mode where the liquid food is pumped into the chamber at room temperature, refrigerated temperature or even at elevated temperature. The pre-cooling or pre-heating might be required to maintain the temperature of the supplied food product to the desired temperature of processing and packaging. The continuous mode operation is an advantage for the high throughput required for industrial production of beverages. There are several studies about the inactivation of enzymes by PEF within the last two decades which demonstrate no inactivation to full inactivation of enzyme, depending on the food origin of the enzyme and suspending medium (Terefe et al., 2015b). Commercial PEF treatment of orange juice was carried out (Min et al., 2003).

### PEF Effect on Enzymes

The mechanism of enzyme denaturation is still unclear. The application of electric fields may cause association or dissociation of functional groups of protein, movement of charged chains and changes the alignment of the helices (Tsong and Astumian, 1987). The structure of proteins is stabilized by a sensitive balance of various covalent and non-covalent bonds/interactions (electrostatic forces, Van Der Waals, hydrogen bonds, and interaction hydrophobic effect). Also, the application of external electric field may effect the local electrostatic fields in proteins and disrupt the electrostatic interaction of peptide chains leading to conformational changes (Barsotti and Cheftel, 1999; Zhao and Yang, 2009). The electro-chemical and thermal effects associated with PEF solely or in combination as mentioned above may cause changes in the structure and conformation of enzymes (Tsong, 1990; Yeom et al., 1999; Van Loey et al., 2001; Zhong et al., 2007; Zhao and Yang, 2009).

### Effect of PEF on PPO Enzyme

Table 5 summarizes the results of a few studies carried out with apple and white grape juices. It seems the resistance of PPO to PEF is higher in Parellada white grape juice, followed by Jonagold Red apple juice, then Royal Gala apple juice and finally Golden Delicious apple juice. The increase of electric field intensity causes higher treatment temperature ( $T_{\text{max}}$ ), thus higher PPO inactivation. Ertugay et al. (2013) fully inactivated PPO activity in Golden Delicious apple juice using a thermal assisted PEF treatment at 40 kV/cm-55 °C for 200  $\mu\text{s}$ . So PEF combined with moderate heat was slightly more effective for enzyme inactivation than non-thermal PEF treatment. For example an electric field intensity of 30 kV/cm and 100  $\mu\text{s}$  real PEF treatment time applied to Jonagold Red apple juice resulted in 43% RA when using heat (65 °C) as opposed to 59% RA for PEF alone ( $T_{\text{max}} = 38$  °C) (Riener et al., 2008). The increase in treatment time also reduces the PPO RA. Ertugay et al. (2013) applied a non-thermal PEF process (30 kV/cm,  $T \leq 45$  °C) for 100 and 400  $\mu\text{s}$  to Golden Delicious apple juice and obtained 32% and 1.6% RA, respectively. In another non-thermal PEF study carried out with white grape juice at 35 kV/cm ( $T_{\text{max}} = 40$  °C) the RA was reduced from 94% when applying 1000  $\mu\text{s}$  treatment to 16% after 5000  $\mu\text{s}$  PEF treatment (Marsellés-Fontanet and Martín-Belloso, 2007).

### Other Non-thermal Food Preservation Technologies for PPO Inactivation

High pressure carbon dioxide, cold plasma and ultraviolet processing are other emerging non-thermal food technologies under investigation with potential to inactivate PPO enzyme.

### Ultraviolet Light Processing

Within the optical radiation spectrum, broad-spectrum ultraviolet (UV) is the strongest and most damaging to living microorganisms. UV light (200 to 400 nm wavelength) is a commercial non-thermal technology used for decontamination of water (e.g. supply drinking water, pool water), air and surfaces, which can be applied for food and beverage processing. UV is divided into wavelength ranges identified as UV-A (315 to 400 nm), UV-B (280 to 315 nm), UV-C (200 to 280 nm) and UV-vacuum (100 to 200 nm) (NTP, 2018). UV-C has shown effectiveness in eliminating microbial contamination at the surface of meat, poultry and fish, and also extending the shelf life of beverages such as apple cider and fruit juices (Choi and Nielsen, 2005; Guerrero-Beltrán and Barbosa-Canovas, 2006; Huang and Toledo, 1982; Kaess and Weidemann, 1973; Nakayama and Shinya, 1981; Stother, 1999; Tran and Farid, 2004; Wallner-Pendleton et al., 1994). As opposed to water disinfection, the UV light has a very low penetration effect on turbid and coloured liquid beverages. Despite the effectiveness for microbial disinfection, enzyme inactivation by UV-C is still under study. One of the proposed mechanisms of PPO inactivation is that the photo-oxidation by UV can affect the native structure of protein, thus resulting in loss of enzyme functionality (Davies and Truscott, 2001; Rhim et al., 1999). A RA of 19% in mango nectar has been reported after a 30 min UV treatment with light intensity of 25 mW/cm<sup>2</sup> (Guerrero-Beltrán and Barbosa-Cánovas, 2006). The irradiation of Starking Delicious cloudy apple juice by combined rays of UV-C and UV-A with UV-LED for 40 min resulted in 33% PPO RA (Akgün and Ünütürk, 2017).

**Table 5** Residual activity of polyphenoloxidase in fruit juices after pulsed electric fields (PEF) processing

Fruit or vegetable	Cultivar	Medium processed	Treatment details	Electric field intensity (kV/cm)	Type of treatment	$T_{max}^a$ (°C)	Treatment time (μs)	Residual activity (RA, %)	Reference
White grape	Parellada	Juice	200 Hz, 4.0 μs pulse	35	Non-thermal	40	5000	16	Marsellés-Fontanet and Martín-Belloso (2007)
				35			1000	94	
				25			1000	96	
Apple	Jonagold	Juice	15 Hz, 1.0 μs pulse	40	PEF + heat	72	100	29	Riener et al. (2008)
	Red			40	Non-thermal	45	100	40	
				30	PEF + heat	65	100	43	
				30	Non-thermal	38	100	59	Sulaiman et al. (2017a)
Apple	Royal Gala	Juice	721 Hz, 2.8 μs pulse	24.8	PEF + heat	54	169	18	
Apple	Golden Delicious	Juice	200 Hz, 2.0 μs pulse	40	PEF + heat	55	200	0	Ertugay et al. (2013)
				40	PEF + heat	54	100	6.2	
				30	Non-thermal	45	400	1.6	
				30	Non-thermal	44	200	7	
				30	Non-thermal	43	100	32	

<sup>a</sup>The PEF treatment increases the juice temperature. Therefore,  $T_{max}$  is the maximum temperature measured after processing in the outlet of the PEF unit.

### Cold Plasma

Cold plasma, plasma or non-thermal plasma is the partially or wholly ionized gas that is essentially composed of photons, ions and free electrons as well as atom in their fundamental or excited state owing a net neutral charge (Misra et al., 2011). Cold plasma is generated through the non-equilibrium of temperature between electrons and ion. The fundamental of cold plasma technology and the application on food disinfection has been reviewed by Misra et al. (2011) and Niemira (2012). Being a recent technology, few studied the effect of cold plasma on enzyme inactivation. Peroxidase has been inactivated by cold plasma in a model food system and crude extract from tomato (Pankaj et al., 2013; Surowsky et al., 2013). Surowsky et al. (2013) postulated that cold plasma induced modification of the secondary structure of the enzyme as the possible inactivation mechanism.

### High Pressure Carbon Dioxide

HPCD which is also known as dense phase carbon dioxide (DPCD) processing involves the use of carbon dioxide (CO<sub>2</sub>) at it subcritical or supercritical phase, to process a food material by having CO<sub>2</sub> to contact with the food. The supercritical CO<sub>2</sub> is achieved by processing sample at the CO<sub>2</sub> critical temperature (31 °C) and pressure (4.7 MPa). The sensory quality and nutritional component of the food is not affected by this processing (Damar and Balaban, 2006). Cloudy Fuji apple juice treated with supercritical carbon dioxide at 30 MPa and 55 °C for 60 min resulted to 38.5% residual activity (RA) (Gui et al., 2006). PPO RA 12% was registered for carrot juice treated with HPCD at 8 MPa for 3 min at room temperature, while peach PPO revealed to be more resistant as 33% RA was obtained after a HPCD of 8 MPa room temperature for 60 min (Zhang et al., 2010). The inactivation of enzyme under HPCD was suggested to be caused by the decrease in pH of the media and alteration of ionic equilibrium (Ortuño et al., 2013).

### PPO Stability Versus Regeneration During Storage

There are only few studies assessing the possible regeneration of PPO during storage after processing. This is related with reversible or irreversible inactivation of the enzyme with the processing. The 15 min treatments of Camarosa strawberry puree at 65 °C alone, 600 MPa-48 °C and 1.3 W/g - 33 °C reduced PPO activity to <18%. No changes were observed throughout 30 days storage (at room temperature and 3 °C) on PPO residual activity (Sulaiman et al., 2017b). Likewise, no change in PPO was observed during 23 days storage at 14 °C of peeled green banana (cv. Enana) after blanching for 11 min in boiling water (~4% RA) (Cano et al., 1990). Stable PPO activity has also been reported for guava (cv. Chung Shan) puree treated at 88 to 90 °C for 24 s, presenting PPO RA of 16% and 14% after processing and 60 days storage respectively (Yen and Lin, 1996). Similarly, TS (1.3 W/mL, 58 °C, 10 min) and thermal treatment alone (75 °C, 20 min) of Royal Gala apple juice reduced the PPO RA to 15% and 3%, respectively, which remained unchanged during 30 days storage at room temperature and 3 °C (Sulaiman et al., 2017a). The RA of 18% obtained after PEF treatment of the same apple juice (26.4 kV/cm, 53.4 °C, 169 μs) decreased slightly to 14% during 30 days storage at 3 °C and to 12% after 30 days storage at room temperature. An increase of PPO RA was registered after 60 days storage at 4 °C from 63% to 81% RA for HPP treated (600 MPa, 15 min) guava puree (Yen and Lin, 1996).

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## Processing Effects on Meat Flavor

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### Glossary

**Aging** Process of breaking down the protein by endogenous enzymes.

**Dry aging** Process of aging unpacked meat or packaged meat with highly permeable material through exposing the meat to a controlled environment.

**Dynamic headspace extraction** Extraction method involves entrapping volatiles onto a matrix

**Heterocyclic compounds** Cyclic compounds that are generated from the Maillard reaction. The structure contains nitrogen, oxygen or sulfur atoms.

**Lipid oxidation** Oxidative degradation of lipid in meat. The process where free radicals damage the lipid compounds.

**Simultaneous distillation extraction** Solvent extraction method that is performed inside a special apparatus using two different solvents.

**Solid phase micro extraction** Extraction method involving adsorption of compounds onto a special chemical matrix.

**Wet aging** Process of aging meat by sealing the meat under vacuum and storing it for certain time (from 7 to 28 days) at chilled conditions.

### Introduction

Meat is an important source of high quality and bioavailable protein in our diet. The main composition of meat comprises proteins, water and fat. Meat is normally eaten after being seasoned and cooked but sometimes can be eaten raw (e.g. beef tartare). Meat is a rich reservoir of compounds that can contribute to Maillard reactions (MR) during heat treatment. Flavor of meat is one of the most important eating quality attributes that complements the dining and eating experience. There are many factors that can affect the flavor of the meat such as breed, sex, feed, muscle types, aging, cooking treatment, novel technologies and so on. Therefore, it is important to benchmark the impact of these factors on flavor formation.

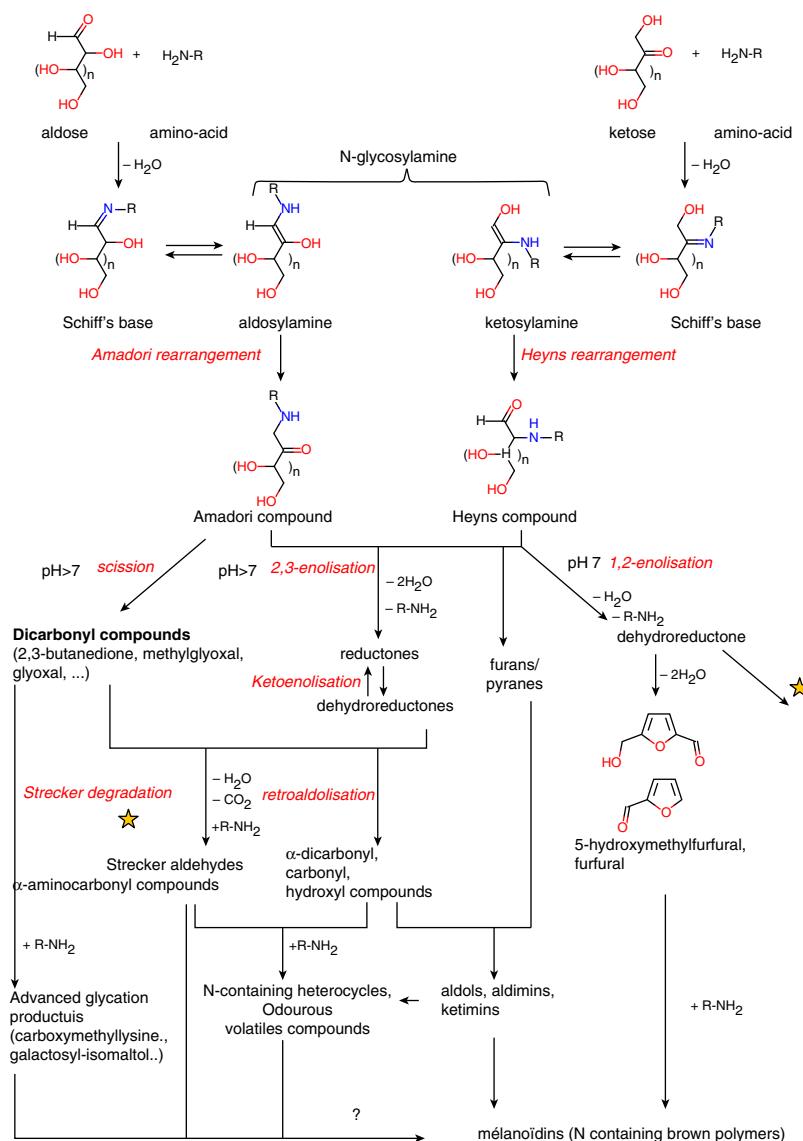
### Meat Flavor

The majority of meat flavor precursors can be divided into two groups; water-soluble components and lipids. During the heat treatment, many reactions such as Maillard reactions, lipid oxidation, and thiamine degradation contribute to the formation of these compounds. Meat flavor and palatability are influenced by fat content, especially in Intra-muscular fat. Lipid oxidation is a major cause of meat quality deterioration during storage and processing due to generation of rancid and off-flavors but low levels of lipid oxidation can enhance the flavor of the cooked meat. Heterocyclic compounds have been reported to be the main contributors to the volatiles of cooked meat.

The Maillard reaction is a reaction between the carbonyl group of a reducing sugar and the amine group of amino acids. It produces the desirable flavor in the cooked meat, baked bread, coffee and chocolate. In the Maillard reaction, a cascade of reactions occur, and this creates numerous compounds. Primary compounds such as furfural, furanone derivatives, hydroxyketones, and dicarbonyl compounds are created initially. Further reactions with other amines, amino acids, aldehydes, hydrogen sulphide, and ammonia through the Amadori rearrangement, Strecker degradation, and Schiff bases pathways lead to more complex products. The reaction also leads to the formation of melanoidins, brown and high molecular weight polymers, from the condensation of cyclic compounds. The overall reaction scheme is shown in Fig. 1. Different classes of compounds contribute their own characteristics to the meat flavor. The summary and compound examples are shown in Table 1.

The final stage of the MR (Fig. 2) is the most important step for flavor formation and it is often called the Strecker degradation. The intermediate carbonyl compounds react with each other, amino compounds, and amino acid degradation products such as hydrogen sulphide and ammonia. This final stage produces heterocyclic compounds such as pyrazines, pyrroles, furans, oxazoles, thiazoles and thiophenes.

The Strecker degradation is very important in flavor generation, as it provides routes by which nitrogen and sulphur can be introduced into heterocyclic compounds in the final stage of the Maillard reaction. The Strecker degradation is initiated by reaction between carbonyl compounds and amino acid forming two important intermediate compounds;  $\alpha$ -aminoketones and Strecker aldehyde. The Strecker aldehyde itself contributes to the flavor of the meat. The  $\alpha$ -aminoketones are key precursors for heterocyclic

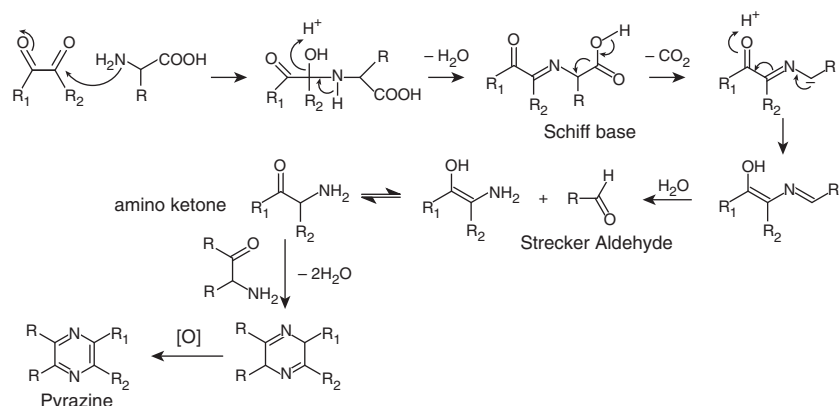


**Figure 1** Schematic illustration of volatile compounds generated by the Maillard reaction between sugars and amino acids; heat degradation of lipids and thiamin, \* refers to Strecker's degradation. Source: Bertrand, E., El Boustany, P., Faulds, C.B., Berdagué, J.-L., 2018. The Maillard reaction in food: an introduction. Reference Module in Food Science. Elsevier.

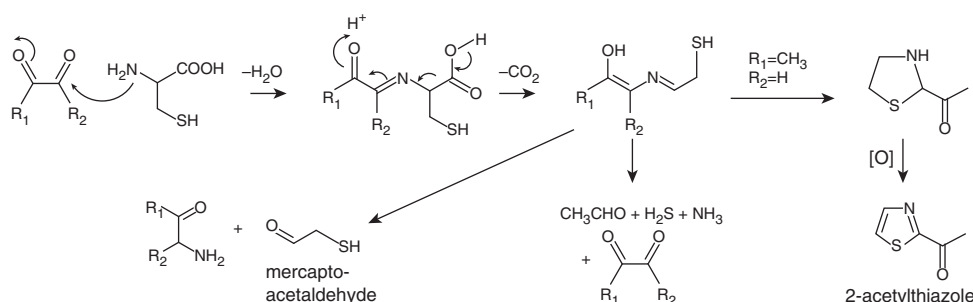
**Table 1** Flavor compounds generated from the Maillard reaction

Flavor class	Characterized flavor/aroma notes	Compound example
Alkane	Fatty, burnt, pungent	Hexane
Aldehyde	Green, fatty, sweet, pungent, smoky	Hexanal, Butanol, Undecanal
Ketone	Musty, fruity, fatty, mouldy	2-Octanone, 1-octen-3-one
Alcohol	Roasted, woody, fatty, oily	1-Octen-3-ol
Pyrazine	Nutty, roasted, meaty	Dimethylpyrazine
Pyridine	Cereal-like	2-Isobutyl-3,5-diisopropylpyridine
Furan	Sweet, burnt, pungent, caramel-like	Pentafuran
Oxazoles	Green, nutty, sweet	2,4,5-Trimethyl-1,3-oxazole
Thiophene	Meaty	2-Formyl-5-methylthiophene

Adapted from Van Ba et al. (2012) and <http://www.flavornet.org/flavornet.html>.



**Figure 2** Strecker degradation. Source: Mottram, D.S., 2007. The Maillard reaction: source of flavour in thermally processed foods. Flavours and Fragrances. Springer.



**Figure 3** Strecker degradation of cysteine compounds. Source: Mottram, D.S., 2007. The Maillard reaction: source of flavour in thermally processed foods. Flavours and Fragrances. Springer.

compounds, such as pyrazines, oxazoles and thiazoles. In the case of alkylpyrazines, the most direct and important route for their formation is thought to be via self-condensation of  $\alpha$ -aminoketones or condensation with other aminoketones.

The Strecker degradation can lead to the production of hydrogen sulphide, ammonia and acetaldehyde if the amino acid is cysteine, whereas methionine will yield methanethiol. These compounds, together with carbonyl compounds produced in the Maillard reaction, provide intermediates for reactions giving rise to important aroma compounds, including sulphur-containing compounds such as thiophenes, thiazoles, trithiolanes, thianes, thienothiophenes and furan thiols and disulphides. The reaction scheme is shown in Fig. 3.

## Lipid Oxidation

Fatty acids and lipids contribute greatly to the generation of the volatile flavor compounds that are characteristic of cooked meat. The fatty acids in meat are influenced by several factors such as diet, life style and breeds. The levels of fat content and fatty acid profiles in meats plays an important role in the formation of volatiles. Intramuscular fat and adipose tissues contain different proportions of saturated and unsaturated fatty acids. Both types can be degraded and oxidized, which create a prolific number of volatile flavor compounds. Examples of volatile flavor compounds derived from lipid degradation that have been found in cooked meat include aliphatic hydrocarbons, aldehydes, ketones, alcohols, carboxylic acids and esters. The significance of lipid-derived compounds is not as high as the relatively low concentrations of the heterocyclic compounds because the odor detection threshold values for the lipid-derived compounds are much higher. The oxidation of adipose tissues and intramuscular fat occur in raw meat and continues under the catalytic conditions such as in the presence of metals, oxygen, light, and heat. Lipid has a significant role in meat flavor, when adipose tissue is added to lean meat, it does not affect the lipid-derived flavor compounds (Mottram and Edwards, 1983). Mottram and Edwards (1983) studied the effect of intramuscular fats and phospholipids removal from beef and found significant impact on flavor compounds formed and sensory characteristics of the cooked meat. Thus, the intramuscular (i.e. marbling fat) and membrane lipids are thought to be the main source of volatile flavor components and generate species-specific flavors. High levels of polyunsaturated fatty acid contents (PUFA) can cause undesirable aromas and flavors as they are prone to oxidation and inhibit of some MR products (Mottram and Edwards, 1983).

## Feed

Animal feed affects the carcass composition, fatty acid profile, tenderness and color but feed mainly affects the fatty acid profile of the meat (Khan et al., 2015; Van Ba et al., 2012). Grass-fed beef could be less prone to lipid oxidation than grain-fed beef as there is a higher level of vitamins A, C and E, carotenoids, and flavonoids. However, steaks from grass/forage-fed beef were reported to be less tender compared to steak from grain-fed animals, but no difference in juiciness was observed. Feeding animals with vitamins improves the overall quality of the meat. For example, vitamin E in feed is deposited in lipid tissues and membranes and acts a strong antioxidant during storage, which enhances the shelf life and color of the meat. Mutton aroma found in cooked sheep meat was reported to arise from the presence of short branched-chain fatty acids (BCFAs) (Young et al., 2003). Animals with grain-based diet contains a high value of BCFAs compared to pasture-based diet. Diet high in polyunsaturated fatty acids such as fish oils, raw soybeans or canola oil can result in off-flavors in beef. An increase in unsaturated fatty acids increases oxidation during storage. Palm-oil and whole linseed supplements increases muscle levels of alpha-linolenic [C18:3] and EPA (eicosapentaenoic acid [C20:5]); fish oil increases EPA and DHA (docosahexaenoic acid [C22:6]). Meat flavor can be influenced by the diet, which means that diet can be used to tailor desirable flavor to some degree.

## Heat Treatment

Heat and cooking techniques (grilling, roasting or boiling) do have a massive effect on the flavor of the meat. Grilling, roasting and boiling create different volatile profiles due to the mode of heat transfer and the direct or lack of exposure to the heat source. The Maillard reaction is influenced by many factors such as heat, pH and water content. Dry heat cooking methods encourage the Maillard reaction and wet cooking methods hinder the reaction. This is seen when meat is cooked in a frying pan as it develops stronger color and flavor compared to boiled meat. The effect of different cooking methods (frying, boiling, roasting and microwaving) on the formation of volatiles profile and lipid oxidation was investigated by Domínguez et al. (2014). Ester compounds were reported to be the most abundant for fresh foal meat. Aldehydes were the main compounds present in the headspace of the cooked foal meat, representing between 53 and 65% of total volatiles. Alkanes were also reported to have a higher concentration in cooked foal meat. Meat cooked under boiled and lightly roasted conditions was found to be dominated by lipid oxidation products (aldehydes, alcohols, ketones and lactones) (Mottram, 1985).

It is known that there is a higher rate of pyrazine formation when the cooking temperature is about 120 °C. Therefore, cooking methods such as roasting and grilling would produce pyrazines at a higher yield compared to boiling. A lower yield of heterocyclic compounds was found when pork is cooked by light grilling, roasting or boiling (Mottram, 1985). The concentration of pyrazines produced was higher under dry heating conditions compared to boiling. Boiling does not allow a high temperature to be reached and low water activity to be obtained, so that MR is not promoted. The caramelization process is also eliminated during boiling which reduces its contribution to flavor production. Reheating food has an impact on the volatiles because during cooking the membranes get disrupted and dehydrated making the phospholipids more prone to oxidation contributing to warm off flavor.

## pH

pH is one of the important factors that influence the volatile flavor compounds formed in the MR, and determines the final flavor characteristics of cooked meat. Numerous studies have revealed that pH plays a significant role in the formation of pyrazines (Yeo and Shibamoto, 1991; Koehler and Odell, 1970; Madruga and Mottram, 1995). These authors investigated the effect of pH by experimenting with glucose-protein and meat related systems. The formation of pyrazines is promoted when the pH level is high in the food system. The greatest number of pyrazine compounds was detected at pH 9. The authors stated that basic conditions enhanced the reactivity of the amino groups toward the carbonyl groups of glucose and increased rearrangement and fragmentation of sugars. Pyrazines were the most abundant class for all conditions (54%–79% of total volatiles) and were at their highest concentration under basic conditions.

## Aging Process

The aging process is a storage process under defined conditions (temperature, mass transfer and permeability of oxygen and moisture) to improve the tenderness and palatability of meat. Biochemical reactions such as lipolysis, proteolysis and oxidation occur during the aging process (Khan et al., 2016). The breakdown of myofibrillar proteins during aging releases peptides and amino acids. Also, the degradation of fats during aging creates free fatty acids and peroxides that contribute significantly to the flavor of the meat upon cooking. Breakdown products and metabolites form more precursor compounds that react during the cooking process. The concentrations of ribose, methionine and cysteine increase significantly during 21 days of the wet aging period (Koutsidis et al., 2008). These compounds were reported to be very important for flavor formation. After aging for 21 days, the increase in the total free amino acid pool was greater than that observed for the reduced sugars and related substances. Kim et al. (2016) reported that tryptophan, phenylalanine, valine, tyrosine, glutamate, isoleucine and leucine were more abundant in the dry-aged beef

samples than in the wet-aged samples, whereas inosine monophosphate was more abundant in the wet-aged group. Such changes are likely to result in an increase in MR-derived flavor compounds, such as pyrazines and Strecker aldehydes.

Two types of aging techniques are wet and dry aging. Both methods include storage in controlled temperature but for wet aging, the meat is vacuum sealed which prevents water loss and does not affect the overall yield. Dry aging, on the other hand, involves storing the meat in breathable sealed bags or when the meat is fully exposed to the air. The control of humidity is important for dry aging because if humidity is too high, microbial activity will occur but if too low, the meat yield will suffer greatly. Both methods were reported to improve the tenderness of meat. Dry aging resulted in a lower yield of the meat due to the water loss and the outer layer must be trimmed off after aging. Dry aging can be done to a whole carcass since it does not require sealing of the meat.

A significant difference in the overall flavor of cooked dry and wet aged beef loins was noted by consumers who rated dry aged beef higher than wet aged (Kim et al., 2016), and dry aged beef had a significantly higher score for the overall likeness. The development of musty and putrid flavor was reported in dry aged meat which was stronger closer to the exterior surface (Smith, 2012). An evaluation of beef flavor after aging using traditional dry aging, dry aging bag and vacuum aging using sensory panels revealed were no differences for most of the sensory attributes between samples (Dikeman et al., 2013). However, specific notes such as umami and butter fried meat taste were more noticeable in the dry-aged steaks compared with the vacuum-aged steaks. Both bag (dry aging bag and vacuum aging) methods had higher metallic flavor than traditional dry aging. Aging time affected most of the sensory traits in that study, and significant improvement occurred after 8 days of storage. In a consumer test, meat aged for 21 days in dry aging bags was preferred than the samples aged in a vacuum.

The volatile compounds of wet-aged beef (30 days) cooked at 180 °C was reported by Watanabe et al. (2015). The results showed that there was a significant increase in pyrazine, 2,6-dimethylpyrazine and 2,3-dimethylpyrazine with wet aging. The aging process increases the concentration of free amino acids which leads to an increase in total yield of pyrazine compounds. Research that compares the volatile profile between the two aging types is very limited but exposure of the meat to air during dry aging allows oxidation and this can contribute directly to the overall flavor of the meat.

## Irradiation

Irradiation is a technology designed to eliminate disease-causing germs from foods. Applying irradiation on the raw meat or processed meats can eliminate pathogenic bacteria such as *Escherichia coli*, *Salmonella* and *Listeria*, viruses and parasites. Irradiating fresh meat can result in the formation of off-flavors which have been described as rotten egg, bloody, fishy, barbecued corn, burnt, sulfury, metallic, acetic acid, liver-like, alcohol, and serummy). Irradiation can promote autooxidation, which can create undesirable flavor compounds. The effect of irradiation on meat flavor is dependant on the treatment dose, the presence of oxygen, temperature and pH of the treated meat, which mainly affect the rate of lipid oxidation. Methods to decrease the unwanted changes in meat are; oxygen exclusion, irradiation under vacuum or in the presence of an inert gas. Aldehydes such as propanal and hexanal were reported to be a good indicator of lipid oxidation in irradiated meat. Vacuum packaging will retain the irradiated sulfur-containing compounds but re-packaging meat in a permeable barrier allows for dissipation of these flavor compounds.

## Pulsed Electric Field

Pulsed electric field (PEF) is a non-thermal processing technology that uses high voltage short electric pulses to preserve food (Bekhit, 2017). The technology is very suitable for preserving liquid foods such as honey, juices and milk. Even though PEF treatment is considered to be a nonthermal process, it can still cause a slight temperature increase in the treated product due to the electric current flow. The advantages of pulsed electric field are small treatment time, low treatment temperature, increase in shelf life, increased yield (in juice), use of both batch and continuous processes and improved metabolite extraction. Pulsed electric field may improve the tenderization of meat by electroporation and enhance proteolysis however there is no agreement in the literature (Bhat et al., 2018). Study on meat volatiles with application of PEF is very limited. Ma et al. (2016) investigated the volatile profiles of PEF cooked lamb and non-PEF cooked lamb. There were some significant increases in volatile compounds between them. The compounds showing differences were mostly lipid oxidation related compounds; aldehydes, ketones, furans and pyrazines. The PEF sample had more livery and oxidized brown flavor and an increase in the overall volatile profile in lamb. PEF appears to have some potential tenderization effect which may find a commercial application in future provided further research comes up with optimized technological inputs for different muscles, cuts and species. There is no scientific evidence regarding the toxic or hazardous effects of the technology.

## High Pressure Processing

High pressure technology is another non-thermal technology as it allows microbial inactivation at low temperature (Cheftel and Culioli, 1997). The changes leading to lipid oxidation in pressure processed meat start around 300 MPa at room temperature. Suitable packaging or antioxidants must be used as these pressure induced oxidations will limit the potential of this technology for meat-based products (Cheah and Ledward, 1996).



High-pressure carbon dioxide (HPCD) is a pasteurization method that inactivates microorganisms and enzymes through molecular effects of CO<sub>2</sub> under pressures below 50 MPa without exposing foods to the effects of heat. Since thermal pasteurization can cause undesirable changes on nutritional quality of the foods (i.e. development of cooked flavors as well as causing denaturation of proteins) can reduce the product quality and consumer acceptance of foods. HPCD is a novel nonthermal processing technology that retains flavor, avoids loss of nutrients, as well as changes in physical, mechanical, and optical properties of the food materials involved in food processing. When HPCD is applied to meat, both the pressure and temperature could affect molecular interactions and protein conformation, leading to protein denaturation. The solubility of the sarcoplasmic and total protein in porcine longissimus dorsi muscle was decreased after HPCD (Choi et al., 2008), and the major denatured proteins were phosphorylase b, creatine kinase, and triosephosphate isomerase. HPCD had no significant effect on the myofibril fragmentation index value of chilled pork, and thus does not appear to affect texture.

## Flavor Extraction and Detection of Compounds

### Solid Phase Micro-extraction (SPME)

SPME is a simple, quick, solvent-free and inexpensive extraction technique that is now widely used in flavor analysis of foods such as wine, honey, fruits, drinks and meat (Balasubramanian and Panigrahi, 2011). This method is also commonly used for analyzing non-volatile compounds such as pesticides and pharmaceuticals. SPME requires an SPME fiber that looks like a needle. Fused silica fibers coated with a thin polymer film (such as polydimethylsiloxane (PDMS)) are now the more commonly used SPME devices used in meat volatile studies.

The two typical SPME applications are Head-space (HS-SPME) and Direct-immersion (DI-SPME). The HS-SPME is when the device is inserted through a septum-type cap into the headspace of a sample solution in a vessel. The fiber is exposed to the surroundings, which allows analytes to be absorbed and adsorbed onto the coated fiber. After a certain period, the fiber is then removed and inserted directly into either gas chromatography or liquid chromatography instruments. To release the analytes from the fiber, the fiber is heated (thermal desorption) for gas chromatography. A mobile phase is used for eluting the analytes in liquid chromatography. The fiber is immersed in the solution directly for DI-SPME. SPME is often used with a gas chromatography process as it does not require any solvents, which is convenient. The DI-SPME produces good reproducibility, but it does not allow changes to the sample matrix, such as changing the pH.

### Dynamic Headspace Extraction (DHE)

Dynamic headspace entrainment is regularly known as the “purge and trap” method. The process involves passing an inert gas carrier over the interested food complex trapping the volatile analytes onto a sorbent and then desorbed into the analytical instrument (Snow & Slack, 2002). It is a suitable method for analyzing a very low concentration of an analyte as a study reported that SPME is suitable for a straightforward analysis of major volatile compounds, but the dynamic headspace trapping is more suitable for trace analysis. Madruga et al. (2009) compared three extraction methods, DHE, SPME and simultaneous distillation extraction (SDE), and they concluded that the DHE (tenax) extracted a higher number of Maillard-derived compounds, such as pyrazines, pyrroles, pyridines, and alkyl sulphides.

### Simultaneous Distillation Extraction (SDE)

It is widely accepted that the SDE method was established in 1964 when Likens and Nickerson (1964) designed an apparatus to analyze hop oil. SDE is an old method but it still has a great value in isolating volatile fraction from the matrix before analysis. The apparatus contains two flasks attached to it; one for the solvent used for extraction and the other one is for the food to be extracted in water. The process involves boiling both the extraction solvent (such as dichloromethane) and a slurry of the food and allowing the steam to travel up the column and condense on the condenser surface where the extraction process happens at that point. Both the solvent and water are collected back into the separator part and due to density differences, they flow back into their respective flasks. The process can be done under an atmosphere (A-SDE) or under vacuum (V-SDE) (Chaintreau, 2001).

The advantages of this method are that the extract does not contain high-boiling point and non-volatile materials making it less potent to the GC liners and columns. The extract can be concentrated enabling the enhancement of a trace level detection. The main disadvantage of this method is that since the matrix is heated for a long time (about an hour), artefacts could form and some heat sensitive compounds might be destroyed whereas highly polar or hydrophilic compounds such as acids and alcohols are poorly extracted. A study by Farkaš et al. (1997) found that a number of compounds were formed in the SDE method but not in high vacuum distillation (EVD) method concluding that they were artefacts.

The most reported drawback in A-SDE is the presence of artefacts in the extracts because of the harsh condition and exposure to air. Many compounds can be sensitive to oxidation, including benzaldehydes, terpenes and unsaturated fatty acids.

Common solvents used for extraction of meat volatiles are pentane, dichloromethane and diethyl ether. Dichloromethane appears to be the best solvent for extraction as it provides the widest variation. Different compounds have different affinity to different solvents which means there is no best solvent for everything. A study by Jayatilaka et al. (1995) investigated the recovery rate for five different compounds; cinnamaldehyde, eugenol, cinnamyl acetate, 2-methoxycinnamaldehyde, cinnamyl alcohol and

coumarin using five different solvents, pentane, dichloromethane, chloroform, ethyl acetate and methyl ter-butyl ether. The recovery rate for dichloromethane was the highest on average but each solvent has its own preference to each compound. Adding salt to water can increase the recovery of polar compounds. The extraction time for SDE process is usually between 1–2 h. It had been reported that the maximum yield for honey flavor compounds reached maximum yield at after 30–45 mins mark and followed a slow decrease profile (Bouseta and Collin, 1995).

Cha et al. (2006) compared the volatile profile for blue crab using V-SDE and SPME extraction methods, the V-SDE was able to extract more aldehyde, ketone and alcohol compounds and SPME did better in the aromatic compounds. The SPME method extracted less low-molecular-weight compounds and the SDE extracted more high-molecular-weight compounds. The same trend is found in the goat meat's volatiles in which Tenax is reported to have the best extraction for low boiling point aldehydes, low boiling point alcohol, pyrazines and pyridines (Madruga et al., 2009).

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## Relevant Website

[http://www.beefissuesquarterly.com/CMDocs/BeefResearch/PE\\_Executive\\_Summaries/The\\_Chemistry\\_of\\_Beef\\_Flavor.pdf](http://www.beefissuesquarterly.com/CMDocs/BeefResearch/PE_Executive_Summaries/The_Chemistry_of_Beef_Flavor.pdf).

## Proteases and Meat Tenderization

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### Glossary

**Actin** A small globular protein important in the cytoskeleton of all eukaryotic cells and forms the major component of the thin filaments of myofibrils.

**Aging** The process where meat held at refrigerated temperatures becomes more tender as a result of proteolysis.

**Apoptosis** A controlled process of cell death mediated by caspases.

**Calpains** A family of calcium-dependent, cytoplasmic cysteine proteases found in most animal cells.

**Calpastatin** A protein which is a specific, reversible inhibitor of calpain

**Caspases** A family of proteases important in apoptosis

**Cathepsins** The proteases located in the lysosomes which are active under acid conditions.

**Collagen** Most abundant family of structural proteins in the connective tissue of animals.

**Glycogen** Storage form of glucose found in the muscle and liver of animals.

**Myofibrils** The elongated structural elements of muscle cells which are responsible for contraction.

**Myosin** An ATP-dependent enzyme which makes up the thick filaments in myofibrils and acts as a molecular motor to cause contraction following binding of actin.

**Proteases** The enzymes which break down other proteins by hydrolysis of peptide bonds

**Proteasome** A large structure in the cytoplasm with a set of proteases which are responsible for ATP-dependent proteolysis of ubiquitin-labeled proteins.

**Rigor or rigor mortis** Stiffening of muscles after death due to the irreversible binding of myosin to actin after ATP levels have declined in post-mortem muscle.

**Sarcomere** The repeating subunit of myofibrils in striated muscle.

**Ubiquitin** Small cytoplasmic proteins which binds to unwanted proteins and presents them to the proteasome.

### Muscle to Meat

People have cooked and consumed muscles from mammals, birds and fish for thousands of years. Meat continues to be an important source of protein and other micronutrients in the human diet. The characteristics of the meat we eat are the result of the composition of the muscle it was and the processes that occur to it after the death of the animal. Meat is derived from skeletal muscle and meat quality varies with the composition of those muscles. All the muscles have long myofibrils consisting of repeating sarcomere which are responsible for the contraction function of muscles (Ertbjerg and Puolanne, 2017). Surrounding these myofibrils is a variable amount of connective tissue principally made up of collagen.

The process of muscle turning to meat begins as the animal is slaughtered and the circulation ceases (Bate-Smith and Bendall, 1947). In the absence of oxygen and external fuel, the muscle metabolizes its glycogen reserves to maintain homeostasis and preserve its ATP level. This anaerobic glycolysis leads to an increase in lactic acid and a decline in pH. Typically when the pH drops to around 5.5, glycolysis stops and the ATP levels fall. At this stage the sarcomere lengths are fixed as myosin binds to actin and the muscle enters rigor (Matarneh et al., 2017).

### Tenderness

The texture or tenderness of meat is one of the most important quality attributes for consumers (Miller et al., 2001) and is largely dictated by the physical and biochemical status of proteins in the meat. Tenderness is a particular issue with the meat from mammals such as cattle, pigs and sheep. Customers are willing to pay a premium for meat that is more likely to be tender. Tenderness is measured on cooked meat either subjectively with consumer panels (Boleman et al., 1997) or objectively by measuring the shear force to cut through a defined cross-section of meat (Shackelford et al., 1995). These measurements have shown a high level of variability with high proportions of unacceptably tough beef but also lamb and pork (Bickerstaffe et al., 2001). The overall shear force consists of a background component, which is directly related to the proportion and type of collagen (Purslow, 2014), and another variable component resulting from the myofibrillar proteins. This second component increases to a maxima as the muscle goes into rigor and then decreases over time as the rigor is resolved

in a process known as aging or tenderization (Wheeler and Koohmaraie, 1994). The peak toughness is related to the sarcomere length which is determined by the conditions, temperature, pH and restraint, at the time the muscle goes into rigor (Marsh and Leet, 1966). The increase in tenderness with aging is the result of the breakdown of key structural proteins in the myofibrils by endogenous proteases and varies with species and muscles (Kemp et al., 2010). Although there is agreement on the importance of enzymes in aging there is still controversy over the contribution of individual proteases. A candidate enzyme must be found in the muscle, have access to the myofibrils, be active post-mortem and should be able to reproduce the *in vivo* pattern of proteolysis *in vitro* (Koohmaraie and Geesink, 2006).

## Calpains

The family of proteases that best fits the above criteria is the calpains (Goll et al., 2003). These proteases were originally found in skeletal muscle (Dayton et al., 1976) but are now known to be ubiquitous in animal cells. Their defining characteristic is that they require calcium for activation. Fourteen genes for calpain have been identified in the human genome (Ono et al., 2016) and three of these, calpain 1, 2 and 3, have been thought to have a role in tenderization. Calpain 1 and 2 have similar structures with an 80 kDa large subunit which contains the active site and a 28 kDa small subunit. They vary in the amount of calcium required for half maximal activity of approximately 50  $\mu$ M for calpain 1 and 500  $\mu$ M for calpain 2 which gave them their earlier names of  $\mu$ -calpain and *m*-calpain respectively (Goll et al., 2003). These values are considerably greater than the physiological levels of calcium which are of the order of 0.1  $\mu$ M. An initial autolysis step lowers the calcium requirement of both calpains with autolyzed calpain 1 having a half maximal activity near 3  $\mu$ M (Goll et al., 2003). There is also evidence that this autolysis makes the calpain 1 less stable and leads to further autolysis and denaturation (Geesink and Koohmaraie, 2000). Measurement of autolyzed calpain is used to indicate calpain activity (Raser et al., 1995). The calpains have a complex specificity (Shinkai-Ouchi et al., 2016) and often modify the activity of their substrates by making single cuts. Many of the substrates of calpains are cytoskeletal proteins and they are involved in changes in cell structure such as the development of muscle fibers (Sorimachi and Ono, 2012).

The calpain hypothesis for aging of meat begins with the decrease in ATP levels post mortem (Koohmaraie and Geesink, 2006). The cell cannot maintain low sarcoplasmic calcium as the active pumps in the sarcoplasmic reticulum and mitochondria require ATP. With the rise in calcium calpain 1 is activated (Hopkins and Thompson, 2001) and this operates on specific cytoskeletal proteins which are involved in maintaining the structure of the myofibrils. The most important of these are thought to be titin, the giant protein which connects the m-line to the z-disk, desmin, which connects bundles of myofibrils to each other, and vinculin in the costameres, the structures which attach myofibrils to the sarcolemma (Taylor et al., 1995). Nebulin and troponin T, key components of the thin filaments, are also calpain substrates. This proteolysis destabilizes the myofibrils and leads to tenderness. Importantly actin and myosin, the most abundant proteins in the myofibrils are neither good calpain substrates nor extensively proteolysed in post mortem meat.

The pattern of proteolysis when myofibrils were incubated with purified calpain 1 or 2, is similar to what is seen in aged meat (Huff-Lonergan et al., 1996). It was also found that infusion with calcium tenderised meat (Koohmaraie, 1990) while zinc and other more specific calpain inhibitors inhibited aging (Uytterhaegen et al., 1994). Calpain 1, rather than calpain 2, is considered the key enzyme, based on its lower requirement for calcium and its autolysis pattern (Koohmaraie et al., 1987). Calpain 1 is autolyzed in the first few days post-mortem where more proteolysis occurs while most calpain 2 can be intact weeks after death (Morton et al., 1999). This was confirmed by experiments in mice where knocking out the calpain 1 gene prevented post-mortem proteolysis of the muscle (Geesink et al., 2006). There is however evidence that calpain 2 may be important in the later stages of aging in some muscles (Colle and Doumit, 2017).

An important part of the calpain system is calpastatin, a protein with the sole known function of reversible, calcium-dependent inhibition of calpain. The inhibitory region of calpastatin binds to either side of the active site of calpain and blocks access to substrates (Moldoveanu et al., 2008). The importance of calpastatin is evident from the strong correlation between calpastatin levels and the rate of aging both between (Ouali and Talmant, 1990) and within species (Shackelford et al., 1991). Increasing the expression of calpastatin either in transgenic animals (Kent et al., 2004) or with  $\beta$ -agonists (Koohmaraie et al., 1991) leads to reduced post-mortem proteolysis and tougher meat. Increased calpastatin can be the mechanism when changes to increase animal growth sometimes lead to tougher meat as in the callipyge sheep (Koohmaraie et al., 1993). Genetic variation in calpastatin is linked to tenderness in cattle (Casas et al., 2006) and is the basis of commercial testing for tenderness potential.

A third isoform, calpain 3, is found in large amounts in muscle and is bound to titin, one of the key target proteins in aging (Sorimachi et al., 1995). Mutations in the calpain 3 gene are responsible for a muscle wasting disease in humans, limb girdle muscular dystrophy type 2A. Calpain 3 levels were correlated with aging in lamb (Ilian et al., 2004) but not in pork (Parr et al., 1999). However it is unlikely that calpain 3 plays a dominant role in aging as knocking out the calpain 3 gene did not affect post-mortem proteolysis in mice (Delgado et al., 2001) and it is not inhibited by calpastatin (Ono et al., 2004).

## Other Endogenous Proteases

Calpains are not the only proteases in muscle and there is evidence that other proteases have either a direct role in aging or interact with calpain. The cathepsins are the proteases which digest cellular components in the lysosome and are active at the acid pH of

meat (Sentandreu et al., 2002). However they would only have access to the myofibrils under conditions where the lysosomal membranes have been ruptured. They also proteolyse actin and myosin and there is only very limited breakdown of these proteins during aging (Mikami et al., 1987). The proteasome is another candidate protease. This is found in large amounts in the muscle and normally works by an ATP-dependent process involving recognition and proteolysis of ubiquitin-labeled proteins (Robert et al., 1999). In the absence of ATP, as in post-rigor muscle, the proteasome is no longer ubiquitin dependent and has been shown to maintain activity for at least one week post-mortem (Lamare et al., 2002). It can reproduce some of the characteristics of aging and proteolysis in chilled meat is slowed by proteasome inhibitors (Houbak et al., 2008) but the overall pattern of proteolysis differs from normal aging.

Recent theories of early aging have considered it as a process of apoptosis or controlled cell death (Becila et al., 2010). The caspases are the key enzymes activated in apoptosis and there is evidence that caspase 3 can reproduce many of the characteristics of post-mortem proteolysis in myofibrils (Kemp and Parr, 2008). Caspases have also been suggested to be the target of the serine peptidase inhibitors or SERPINs which have correlated with toughness in beef (Herrera-Mendez et al., 2009). There is considerable evidence that the caspases interact with calpains and are able to proteolyse calpastatin (Wang et al., 1998). Thus the current view of post-mortem aging is centered on calpains but involves interactions with several other groups of proteases.

Proteomic approaches have identified several groups of proteins which affect aging. These suggest that the extent of proteolysis is determined by the action of other proteins which protect either the proteases or their substrates (Lana and Zolla, 2016). These include heat shock or chaperone proteins and enzymes involved in metabolism (Gagaoua et al., 2015). There is also a strong correlation of tenderness with proteins which protect against oxidation (Lana and Zolla, 2015) and it is known that calpain is very susceptible to oxidation (Lametsch et al., 2008). The activity of proteases can also be affected by post-translational modification. Calpain 1 activity is changed by phosphorylation either of the protease or its substrates (Li et al., 2016).

The rate and extent of aging is greatly impacted by the changes in temperature, pH and ionic strength within the muscle cells during the hours immediately in following slaughter (Marsh et al., 1987). There is also a relationship with ultimate pH with muscles of intermediate pH 5.8–6.1 being toughest (Purchas, 1990). Many of these changes can be explained by the effect of the conditions on the balance of calpain 1 activity, autolysis and denaturation (Geesink and Koohmaraie, 2000; Mohrhauser et al., 2014).

## Exogenous Proteases and Tenderisation

The highest value muscles on a carcass are those that can be used for grilling but only a small proportion of the muscles become that tender even with optimal aging (Sullivan and Calkins, 2011). The remaining muscles typically have higher levels of connective tissue which cannot be cleaved by the endogenous enzymes. These tough cuts of meat have been successfully tenderised by the use of plant proteases principally papain from papaya, bromelain from pineapple, ficin from figs and actinidin from kiwifruit (Bekhit et al., 2014). The proteases can be infused or injected into the meat or used as a marinade. These plant preparations are often crude extracts with a variety of proteases with low specificity and digest all meat proteins both from the myofibrils and connective tissue. They are very effective at decreasing the shear force of meat but some of them, for example papain, tend to over-digest the meat and leave it with a mushy texture unlike normally aged meat (McKeith et al., 1994). Others, such as actinidin (Lewis and Luh, 1988) and some of the microbial enzymes (Ryder et al., 2015), are more specific and lead to a more controlled tenderness development. The proteolysis following application of infusion of kiwifruit juice to lamb was partly due to activation of calpain 2 (Han et al., 2009).

## Conclusions

Aging or tenderization of meat occurs in refrigerated conditions and is the result of interactions between endogenous proteases and muscle proteins. Calpain 1 is believed to be the main protease responsible for meat tenderization during post-mortem aging, however, there is evidence for the involvement of other proteolytic system either direct or indirectly in the aging process. Calpastatin, the specific calpain inhibitor, modulates the activity of calpain 1. The extent of aging is also affected by other proteins and the temperature and pH of the meat. Aging alone cannot tenderize certain meat cuts and interventions, such as the use of exogenous proteases, could improve the tenderness level.

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## Proteases as Digestive Aids

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### Glossary

AN-PEP *Aspergillus niger*-derived prolyl endoprotease;

CD Celiac disease

Endoprotease Enzymes which catalyze the cleavage of peptide bonds within the polypeptide chain

Exoprotease Enzymes which catalyze the cleavage of the terminal (or the penultimate) peptide bond

EPI Exocrine pancreatic efficiency

GI Gastrointestinal

HLA Human leukocyte antigen

IBD Inflammatory bowel disease

IBS Irritable bowel syndrome

PEP Prolyl endopeptidase;

PERT Pancreatic enzyme replacement therapy

POP Prolyl oligopeptidases

### Overview

Proteases hydrolyze peptide bonds in proteins generating peptide fragments and amino acids. These enzymes have primary roles in protein catabolism. For example, in the small intestine, proteases digest dietary proteins to allow the absorption of amino acids. They are also involved in control of biological processes within the body through highly specific cleavage of substrates (López-Otín and Bond, 2008). Proteases are responsible for maturation of prohormones to generate active proteins or peptides that are intimately involved in thermoregulation, reproduction, appetite and many other critical functions. As such, proteases control the fate and activity of proteins. Proteases have also been implicated with various types of pathophysiological conditions such as cancer, inflammatory, cardiovascular and digestive disorders (Drag and Salvesen, 2010).

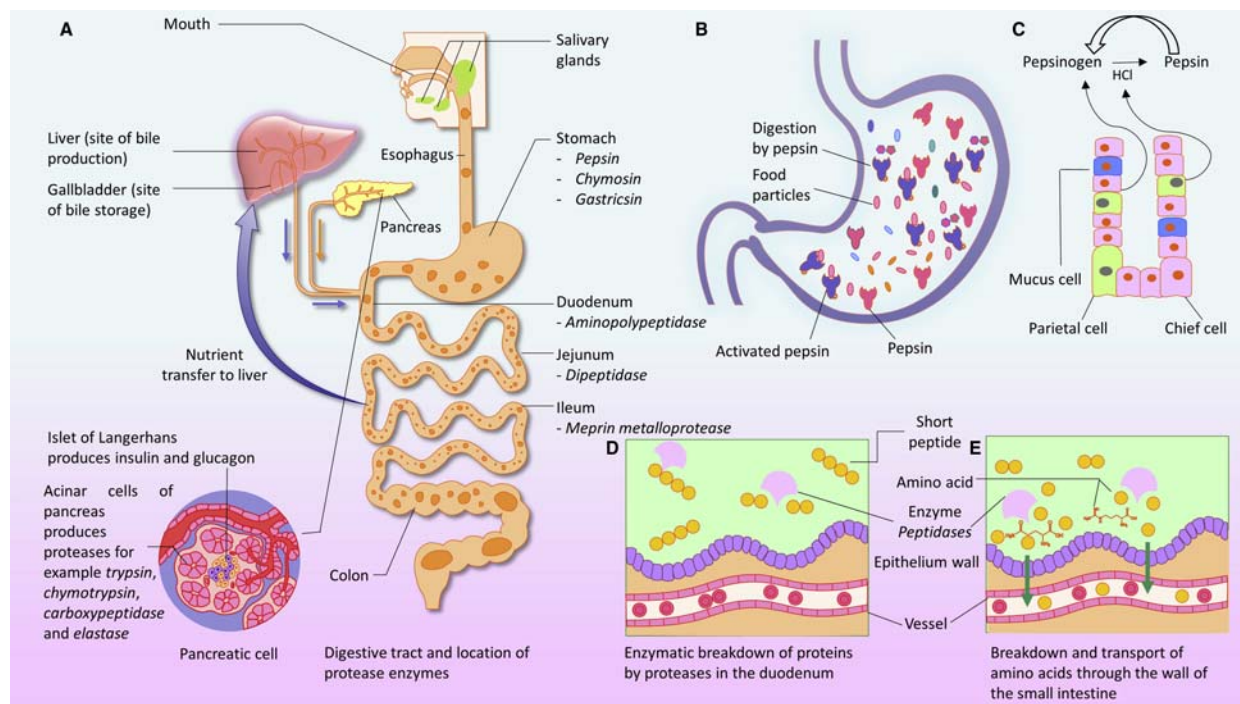
Our understanding of the role of proteases has to a large extent focused on nutrient digestion. The most well-known proteases are the serine protease family of enzymes that comprise pepsin, an enzyme produced in the stomach, as well as trypsin and chymotrypsin, primarily secreted from the pancreas. Together these enzymes are the principal proteases involved in the human digestive system.

The sequencing of genomes from a diverse range of organisms has revealed the structural and catalytic diversity that exists within the protease family. These data have illuminated the critical role that proteases play in the biology and pathology of diseases, especially digestive disorders (Puente et al., 2003). In recent years, the use of proteases as drug therapies has received attention, in particular for treatment of gastrointestinal (GI) disorders (Drag and Salvesen, 2010; Sollid and Khosla, 2005). By regulating peptide and amino acid production, controlling protein–protein interactions, catalyzing, processing and transducing molecular signals in the GI tract, protease enzymes offer a unique opportunity for targeted drug delivery and treatment options for GI disorders (López-Otín and Bond, 2008).

Most proteases are synthesized as inactive zymogens that become activated through a number of mechanisms, including cleavage by upstream proteases, changes in pH, or dimerization. Fig. 1 shows the site of protease production in the human GI tract and their mode of action in the stomach and small intestine. Once the protease is in the active conformation, substrates can enter the active site and be cleaved. However, protease activity can be altered by the presence of endogenous and exogenous protease inhibitors. As a result, keeping the balance between inhibitors and enzymes is a challenge but also represents a path for exploitation in the design of novel drug therapies.

Proteases have differing roles in animals, plants and microbes (Sollid and Khosla, 2005; Sipos, 1995; Nair et al., 1974). In plants, proteases play a vital role in the processing, maturation, or destruction of specific proteins in response to developmental cues or variations in environmental conditions (García-Lorenzo et al., 2006). Additionally, proteases have become important tools in the biotechnology and biopharmaceutical industries because of their efficient digestion of proteins. Examples include the use of alkaline protease to remove hair from hides, the use of pancreatic protease extracts as detergents in leather processing, the use of proteases in rennet from unweaned calves in cheese production and the use of papain for tenderizing meat (Li et al., 2013).

This chapter will provide an overview of the proteases found in humans, the types and their roles in digestion and digestive disorders. In addition to these endogenous enzymes, we will also discuss currently available protease supplements for GI disorders,



**Figure 1** The human digestive system showing the site of protease production and their mechanism of action: (A) location of protease enzymes in digestive tract; (B) initiation of digestion by pepsin in the stomach; (C) interior surface of the stomach; (D) enzymatic action of proteases in the duodenum; and (E) absorption amino acids through the small intestine.

the current status of and prospective for protease-based drug discovery. Finally, we will discuss protease-based drug therapy as an example for two major digestive disorders: celiac disease (CD) and pancreatic insufficiency.

## Types of Proteases

Proteases have been classified based on their specificity and catalytic types (Garcia-Carreón, 1997). Depending upon their specificity, proteases have been divided into two broad groups: exopeptidases and endopeptidases. Exopeptidases break the peptide bonds adjacent to the N- or C-termini trimming the protein or peptide, while endopeptidases act on internal peptide bonds. Based on the exopeptidase site of action or specificity, they can be divided further into subclasses, i.e. the aminopeptidases release the N-terminal amino acid, while the carboxypeptidases hydrolyze peptide bonds at the C-terminus, and di/tri-peptidyl peptidases release a di/tri-peptide from the N-terminus. Unlike exopeptidases, endopeptidases cleave peptide bonds within the protein sequence and cannot break down peptides into monomers i.e. amino acids. In addition to endoproteases, a family of endopeptidases exist whose substrates are size limited. For example, oligopeptidases cleave oligopeptides instead of proteins.

Based on their catalytic mechanism, endoproteases are classified into the following six types: aspartic, cysteine, glutamic, metallo, serine, and threonine proteases (Rawlings et al., 2015; Garcia-Carreón, 1997). The mechanism of peptide bond cleavage involves making the amino acid residue (cysteine and threonine proteases) or a water molecule (aspartic acid, metallo- and acid proteases) nucleophilic so that it can attack the peptide carboxyl group. The generation of a nucleophile can occur via a catalytic triad, where a histidine residue is used to activate serine, cysteine, or threonine as a nucleophile. Endoproteases can be highly specific, for instance the serine protease trypsin cleaves the carboxyl side of Arg and Lys, while chymotrypsin cleaves after the hydrophobic amino acids, Phe, Trp and Tyr, and with lower affinity at Leu and Met.

## Enzyme-Based Therapies

The human diet is typically composed of protein, fat and carbohydrate. Digestive enzymes (proteases, lipases and carbohydrases) act to break these components down aiding in nutrient absorption. Enzyme-based therapies have been successfully applied to the treatment of some metabolic disorders wherein there is an insufficiency in particular class of enzyme. Lactose intolerance arises due to an insufficiency of the enzyme  $\beta$ -galactosidase (lactase). Yeast, fungal and bacterial-derived  $\beta$ -galactosidase enzyme supplementation therapy are commonly used in the management of lactose intolerance (Swallow, 2003). It has been proposed that similar enzyme-based therapies may be used for the treatment of protease-based insufficiencies. For example, combinations of proteases with other digestive enzymes such as lipases, are being used as a treatment option for pancreatic enzyme insufficiency resulting from conditions such as cystic fibrosis and pancreatic cancer (Littlewood et al., 2006).

## Protease Deficiency and Natural Sources of Protease Supplements

Proteases are also known as systemic enzymes because of their vital role in digestion. They are essential in the digestive system as they break down the protein molecules into small pieces and supply the amino acids required by the body for new protein metabolism. Without proteases, the GI tract cannot process proteins leading to serious health problems, including nutrient malabsorption. In addition to digesting food, proteases can help to kill harmful microorganisms in the intestine and break down unwanted waste products such as toxins and cellular debris (Turk and Stoka, 2007). By killing pathogens and degrading waste products, proteases help to reduce the load on the immune system.

Under normal physiological conditions, the body produces a sufficient quantity and diversity of proteases to digest the proteins present in the everyday diet (López-Otín and Bond, 2008). However, protease deficiency may occur due to pancreatic or genetic disorders, illness, injury/trauma, excessive exercise, ageing or a combination, and may require protease supplementation or therapy to restore proper GI function. Protease supplements can be an option to treat or alleviate GI-related disorders. At present, these enzymes are sourced from animals, plants and microbes.

### Animal-Based Protease Enzymes

Animal-derived pancreatic enzyme supplementation has been widely accepted for the treatment of several digestive disorders. For example, trypsin and chymotrypsin either individually or combination, are the most commonly used supplements for pancreatic disorders. These enzymes are extracted from either porcine or bovine pancreas glands. The activity and concentration of these enzymes are determined by multiple factors, including the animal's species, age and sex. The enzyme complement derived from hogs pancreas is most similar to human and primarily contains amylase and lipase enzymes, whereas the bovine pancreas is enriched with proteolytic enzymes such as trypsin and chymotrypsin (Wooldridge et al., 2009). US pharmacopoeia grade trypsin and chymotrypsin are collected from the ox pancreas gland (Ross and Kang, 2008).

### Plant-Based Protease Enzymes

Plant-based proteases have long been used as health supplements in various forms of therapy. Some fruits contain proteases, for instance, actinidin from kiwifruit (Boland, 2013), papain from papaya (Berti and Storer, 1995) and bromelain from pineapple (Maurer, 2001) that can serve an important role in protein digestion.

Actinidin, the main proteolytic enzyme in kiwifruit, can aid the digestive process. Studies have reported that dietary actinidin fed in the form of fresh green kiwifruit increased the rate of gastric emptying and the digestion of several beef muscle proteins in the growing pig (Montoya et al., 2014). The presence of actinidin in a beef muscle protein-based diet significantly enhanced the gastric disappearance of the higher-MW proteins (>32 kDa) during the first three-hours of ingestion, but had little effect on the smaller proteins (<32 kDa) (Montoya et al., 2014).

Papain is a proteolytic enzyme found in green papaya and has been proven to loosen necrotic and encrusted waste material from the intestinal walls (Nair et al., 1974). Traditionally, papain has been used as meat tenderizer, but also in skin burn treatment, as an antiseptic, and as an anti-inflammatory agent (Wagner, 2005). Papain has proposed to be used in controlling dyspepsia and digestive disorders and malfunction in the GI tract (Perry, 2001a). For instance, the commercially available papaya preparation (Caricol®) is used for the maintenance of GI tract physiology and has proven to alleviate various functional disturbances, like symptoms of IBS (Muss et al., 2013). The papaya latex-derived enzyme caricain has also been used to reduce the gliadin content in wheat dough with an aim to develop a bread suitable for celiacs and gluten intolerants (Buddrick et al., 2015). However, the gliadin content in the bread produced was still above 1500 ppm and structure of the bread was poor. A combination therapy of alcalase from *Bacillus licheniformis* and papain has shown a greater efficiency in reduction of gliadin levels in wheat flour in comparison to single enzyme treatment (Li et al., 2016).

Bromelain is an enzyme extract from pineapples that has been noted to have high proteolytic activity (Harrach et al., 1995) and has been reported as a supplementary aid for IBS. Bromelain comprises a number of cysteine proteases that aid in protein digestion and also acts to decrease inflammation (Onken et al., 2008). Studies have reported that this enzyme increases the permeability of the digestive tract, providing better absorption of nutrients (Nair et al., 1974; Perry, 2001b). Several patents have been submitted based on the preparation of bromelain in combination with other enzymes such as papain, trypsin, and chymotrypsin to treat digestive disorders, impaired liver function, cystic fibrosis and as a digestive aid (Gross et al., 1971; Sipos, 1978, 1994, 1995; Ozlen, 1995; Ross and Kang, 2008). Due to its strong proteolytic activities, there has been interest in using it in a diverse range of industries, including baking, meat tenderization, fish protein hydrolysis, alcohol production and so on (Arshad et al., 2014).

The commercial uses and development of proteases in both industry and for scientific research have been reviewed extensively by Li et al. (2013). These findings imply that the afore-mentioned plant-based proteases may assist in gastric digestion of proteins after having a high-meat meal, amino acid absorption in the GI tract and cleaning of the intestinal wall.

### Microbe-Derived Protease Enzymes

Microbial enzymes represent a greater proportion of the worldwide protease enzyme production due to their varied catalytic activities, the ease of genetic manipulation, their high production capacity, inexpensive media for their growth and absence of



time-dependent or seasonal production (Gurung et al., 2013). These enzymes are also more stable than plant and animal enzymes (Singh et al., 2016; Gurung et al., 2013). The production of microbial proteases is considered more convenient and safer, and well suited for industrial-scale production of animal or plant-derived enzymes in the microbial system (Hasan et al., 2006; Luniak et al., 2017).

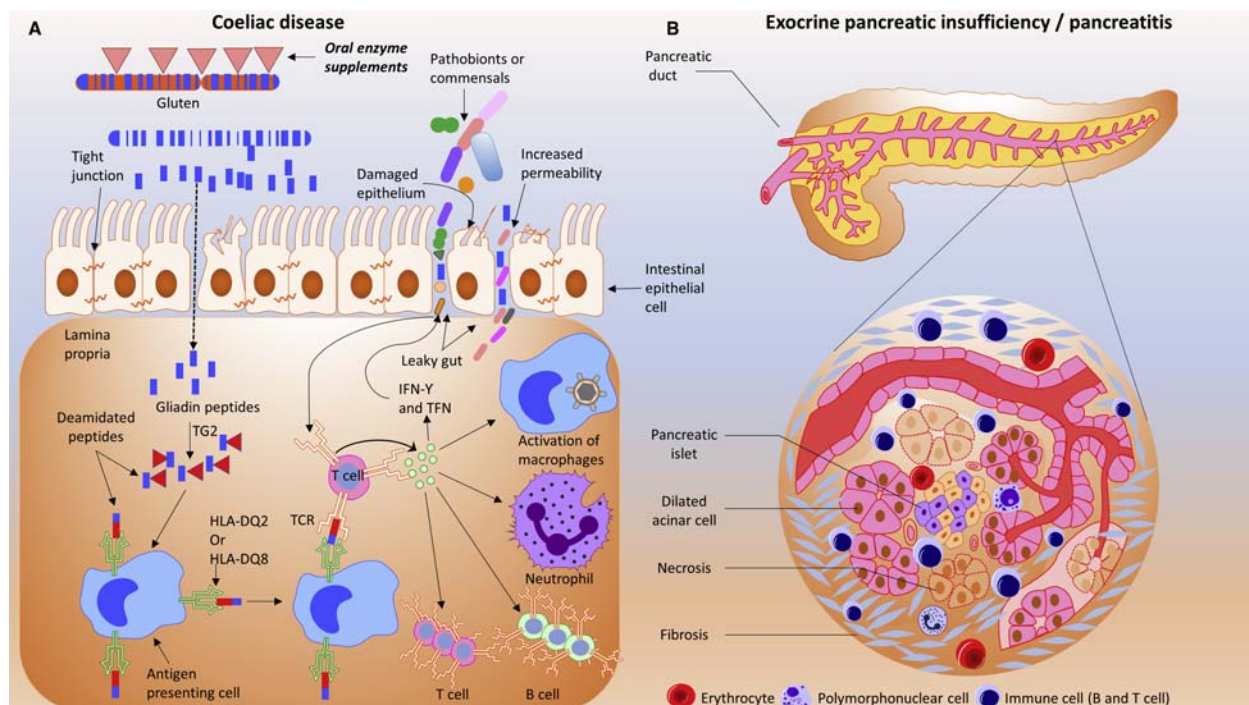
## Uses of Proteases in Treating Digestive Disorders

The gut epithelium controls the digestive, absorptive and secretory functions, and coordinates with the mucosal immune, vascular and nervous systems (Artis, 2008). Proteases regulate the availability and activity of growth factors, cytokines, and extracellular matrix proteins (Antalis et al., 2007). To maintain a proper balance in the intestine, protease enzyme activity is highly regulated along with other digestive enzymes such as amylase and lipase. Uncontrolled proteolysis may cause GI problems as observed in IBS or IBD. Thus understanding how proteases maintain balance and regulatory function in the healthy digestive system and the role they play in the pathophysiology of diseases will facilitate the use of these enzymes as a therapeutic leads for GI disorders. In this section, two disease conditions: CD and pancreatic insufficiency are discussed because protease-based therapy has shown potential as a treatment option.

### Celiac Disease

CD is a lifelong disorder affecting the small intestine that is triggered by the ingestion of gluten proteins present in the wheat, barley and rye (Green and Cellier, 2007). Currently, the only available treatment is complete exclusion of gluten from the diet. There is need for preventative or therapeutic treatment of this condition. In CD (Fig. 2A), people that are genetically predisposed (carry the HLA-DQ 2/8 alleles) may mount an inappropriate immune response to gluten-derived peptides. Gluten refers to a family of proline- and glutamine-rich proteins that are recalcitrant to GI digestion. Gluten proteins also contain fewer basic amino acids (Lys, Arg) which are the sites of cleavage by trypsin. Their unique amino acid composition renders these proteins with a resistance to gastric and intestinal digestion. Thus instead of being degraded to monomers (amino acids) or small oligomers (di-/tripeptides), larger peptides derived from gluten persist after gastrointestinal digestion. These peptides may contain stretches of amino acids, or epitopes that trigger an inflammatory response and are immunotoxic to those with CD (Sollid, 2002).

Aside from hindering digestion, proline plays a crucial role in directing tTG-mediated deamidation of the glutamine residues and thereby increasing the peptides affinity to bind to HLA DQ2 and DQ8 and hence increasing the immunotoxicity (Stoven et al., 2012). Many researchers have investigated the role of transglutaminase as a means for detoxification of  $\alpha$ -gliadin, the main elicitor of CD (Gianfrani et al., 2007; Mazzealla et al., 2012; Mazzeo et al., 2013). These studies have revealed that transamidation of wheat flour resulted in decreased T cell response as evidenced by lower levels of inflammation-related markers. This approach aimed at



**Figure 2** Pathophysiology and treatment targets for coeliac disease (A) and exocrine pancreatic insufficiency/pancreatitis (B).

detoxifying gluten and inhibiting the binding of gluten-derived peptides to tissue transglutaminase represents a complementary approach to oral therapies aimed at gluten digestion (Sollid and Khosla, 2005; Plugis and Khosla, 2015; Sulic et al., 2015).

In the area of CD, microbe-derived proteases can be used indirectly to reduce the toxicity of prolamins epitopes found in the gluten proteins from wheat, barley and rye. For example, the use of selected sourdough lactobacilli-derived proteases has been proposed as a means to minimize the toxicity of gluten-derived peptides without removing the gluten from wheat, barley and rye (De Angelis et al., 2006a,b; Di Cagno et al., 2004; Di Cagno et al., 2002). The use of selected lactic acid bacteria and fungal proteases, which are routinely used in bakery, caused the degradation of gluten and one study reported a concentration of 12 ppm gluten after sourdough fermentation (Rizzello et al., 2007). The lactic acid bacteria *Lactobacillus plantarum* CRL 775 and *Pediococcus pentosaceus* CRL 792 showed proline-specific activity resulting in partial hydrolysis of  $\alpha$ -gliadin substrates (Gerez et al., 2008). As a result, food processing by selected sourdough lactobacilli or fungal proteases may be considered an approach to reduce gluten toxicity prior to ingestion (Mandile et al., 2017). Although, *in vitro* protease treatment shows promise as an option to reduce intact gluten content during sourdough preparation, gluten-derived peptides may remain in the finished product. Moreover, questions remain over the accuracy of the currently used gluten quantitative methods for the analysis of hydrolyzed and/or fermented products (Panda et al., 2015; Slot et al., 2015; Thompson and Méndez, 2008).

Akin to the use of lactase in lactose intolerance, oral therapy by exogenous prolyl endopeptidases (PEPs) that could cleave these proline-rich peptides has been proposed as an alternative therapeutic option (Sollid and Khosla, 2005). Several studies have investigated the possibility of enzymatic defects in the intestinal mucosa of patients with CD and have tried to establish treatment options employing proteases (Bruce et al., 1984; Cornell et al., 2005; Shan et al., 2002). Oral enzyme supplementation is designed to accelerate GI degradation of proline-rich gluten, especially the proteolytically stable antigenic peptides (Cerf-Bensussan et al., 2007). Oral therapy, by exogenous prolyl endopeptidases (PEPs) which act to degrade ingested gluten, was proposed as an alternative treatment to the diet (Monod and Grouzmann, 2010). Shan and co-workers have shown that lack of PEP in the gastric and pancreatic enzymes, and in the human intestinal brush border, prevents efficient digestion of gluten peptides (Shan et al., 2002). Thus, undigested peptides can cross the intestinal barrier and trigger the adaptive immune system, ultimately causing inflammation in the gut. Both *in vitro* and *in vivo* assays by using bacterial prolyl endopeptidase have shown the potential use of PEP in detoxifying immunogenic gluten peptides in CD patients (Shan et al., 2002). Sollid and Khosla showed that addition of PEP in *in vitro* or *in vivo* rat model reduced the ability of toxic peptides to stimulate gliadin-specific T-cells (Sollid and Khosla, 2005). Recently two endopeptidases, KumaMax™ and KumaWT, showed high levels of activity and specificity towards the PQ dipeptide motif present in gluten proteins, with activity retained at pH 4 (Siegel et al., 2017). Although oral-enzyme therapies are currently under development for CD, oral administration of therapeutic enzymes remains challenging due to proteolysis and unfolding (of the therapeutic proteases) in the GI tract.

As discussed above, a range of microorganisms have been investigated as a source of oral enzyme therapies. The ability to degrade gluten has led to these proteases being given the generic name glutenases. The range of glutenases include prolyl oligopeptidases from *Flavobacterium meningosepticum* (FM-POP) (Stepniak et al., 2006) and from *Myxococcus xanthus* (stan-1) (Shan et al., 2002), prolyl endoprotease from *Aspergillus niger* (AN-PEP) and *Sphingomonas capsulata* (SC-PEP) (Stoven et al., 2012), and *A. niger*-derived aspergillopepsin with dipeptidyl peptidase IV (López-Otín and Bond, 2008; Stoven et al., 2012; Bethune et al., 2006). POP is generally a soluble, 76–80 kDa protein (Polgar, 1994) while members of PEPs sub-family are ~75 kDa in size (Gass and Khosla, 2007). AN-PEP has been shown to be active in the conditions found in the GI tract and be capable of degrading intact gluten molecules to remove T cell stimulatory epitopes from gluten and turn them into non-toxic peptides. FM-POP is inactive in the acidic environments, i.e. the stomach, and its structure limits access of larger peptides to the active site, thus FM-POP preferentially cleaves short peptides (Polgár, 1992). As a result oral supplementation of FM-POP is insufficient to breakdown gluten before it reaches the proximal part of duodenum (Matysiak-Budnik et al., 2005).

Plant-derived enzyme supplementation approaches have been used to reduce the peptide size in the key antigenic regions that bind to HLA and initiate inflammation. The glutamine-specific endopeptidase from barley EP-B2 has been shown to degrade gluten (Bethune et al., 2006). A commercial preparation known as ALV003 is a combination of EP-B2, SC-PEP and AN-PEP is currently under Phase 2B clinical trials (Lähdeaho et al., 2014). A recent study has shown that prolyl endoprotease isolated from carnivorous pitcher plant (*Nepenthes* spp.) can increase gliadin solubilization rates which also reduces T cell recognition (Rey et al., 2016).

### Pancreatic Insufficiency

The pancreas produces hormones, for instance, insulin and glucagon from the endocrine gland, and digestive enzymes such as lipases (pancreatic triacylglycerol lipase), proteases (trypsin and chymotrypsin), and amylases ( $\alpha$ -amylases) from the exocrine glands. Exocrine pancreatic efficiency (EPI) may occur as a result of multiple clinical conditions, including cystic fibrosis, chronic pancreatitis, pancreatic cancer, blockage or narrowing of the pancreatic or biliary duct, pancreatic or duodenal tumors and pancreatic surgeries (Littlewood et al., 2006; Nair et al., 1974) (Fig. 2B). EPI can present with varied symptoms such as malabsorption of nutrients, feelings of indigestion, inconsistency in stool production, flatulence, weight loss and more. Pancreatic enzyme replacement therapy (PERT) or enzyme supplementation are used to alleviate the symptoms caused by EPI. In some conditions, people need to take enzyme supplements for life, whereas other conditions may only require a short-term treatment.

In cystic fibrosis, patients suffer from severe intestinal malabsorption that is caused by a deficiency of pancreatic enzymes (Littlewood et al., 2006). High level of bile salts, bicarbonate deficiency and other factors contribute to the problem. Research has found that most patients with cystic fibrosis benefit in terms of nutrient absorption by using PERT that includes a combination



of proteases, lipases, and amylases commercially available as Zenpep® (Eurand) or pancrelipase (Wooldridge et al., 2009). These enzymes can be found in a wide variety of animal, plant, and fungal sources, but all current prescription enzymes are prepared from porcine (pig) sources. There are six FDA approved pancreatic enzymes that are currently available in the market, CREON® capsules, Pancreaze® capsules, Pertyze® capsules, Viokace® capsules, Ultresa® capsules and Zenpep® capsules (FDA).

## Challenges in the Use of Proteases in Digestive Disorders

The therapeutic use of exogenous protease therapies over the past several decades has provided substantial clinical evidence that implicates their use in digestive disorders. However, some challenges need to be overcome before application of these enzymes as a medication for GI-related disorders. For instance, all enzymes must be tested *in vitro* and *in vivo* clinical models to proof their efficacy, safety and determine any potential side effects before patient administration. Investigation of the site-specific action in the digestive tract and efficient pathways for delivering the proteases to the site of action. Moreover, determining the dosage rates and appropriate formulation of proteases and testing their utility in the different food matrices are necessary to ensure their efficacy. The stomach pH is lower than the intestine and pancreas and as such enteric coated enzyme preparations help retain the protease efficacy by preventing its dissolution and disintegration in the gastric environment (Domínguez-Muñoz et al., 2006). Although enteric coated tablets and capsules have been used to treat gastric acidity issues, further work is needed to prepare enteric coated enzyme preparations in the treatment of digestive disorders.

PERT is the standard treatment for EPI-derived malabsorption conditions. To date, this therapy is well tolerated and has satisfactory (>80%) efficacy. However, some challenges remain to be addressed for this therapy: (1) determining the full spectrum of efficacy in a wider group of patients; (2) considering other factors, for instance, disease conditions or genetic differences; (3) oral enzyme therapy development with unprotected enzymes in combination with enteric coated enzymes; and (4) developing a standardized monitoring system for this treatment.

A major challenge for oral protease therapy in GI disorders such as CD is the ability to measure their safety and efficacy in patients across a broad phenotypic spectrum. Because CD is a complex genetic disorder, using a single enzyme or even a cocktail of enzymes may not be effective in treating this disorder. The GI tract represents a complex system, wherein consideration of the pathobionts and commensal bacteria and their role is required. Appropriately designed clinical trials will be required to ensure that protease therapy is suitable for all patients with CD as well as those that suffer from gluten intolerance.

## Conclusions and Future Perspectives

Proteases play a critical role in normal digestion and are often deficient in digestive disorders like IBD, IBS, CD and EPI. The therapeutic use of enzymes over the past five decades has provided clinical results that suggest their utility in GI disorders. As research reveals the pathologies of various GI diseases and finds potential roles for proteases in disease treatment, comprehensive *in vivo* and *in vitro* trials to assess the efficacy and safety of these preparations will be required. Through basic research new candidate proteases from plants or microbes will be revealed, which can be used as a digestive aids in GI disorders. Translational studies, including novel approaches like gene or cell therapies and drug formulation studies are also required to make significant progress in this area (Ellis et al., 2017). Novel enzymes from natural sources and the use of enzyme cocktails combined with probiotics show promise in the treatment of digestive disorders. Oral enzyme therapy may not be a suitable lifelong treatment option, for example, to replace the 'gluten-free diet' for CD, but it might be suitable as a supplemental treatment to avoid accidental ingestion of gluten when dining out. With the advent of gene editing/therapy technology, better-tolerated protease enzyme therapies can be developed by considering the function of the native pancreas to treat EPI. In conclusion, protease enzyme therapies offer promise as digestive aids in the treatment of disorders of the gastrointestinal tract.

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## Protection of Enzymes Against Thermal Degradation

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### Glossary

**Allostery** The behavior of some proteins for which binding of a compound one site of a protein regulates the ability of a substrate to bind at a different site of the same protein. This reveals the conformational dynamics of some enzymes.

**Cofactor** A molecule (such as flavin adenine dinucleotide (FAD)) or an atom (typically a metal such as iron or copper) that an enzyme requires to be active; that is, to be able to catalyze a reaction.

**Electrostriction of water** It is the result of ion–water interactions that causes water molecules oriented around the ion which is mainly due to the high electric field of the ion. This results in the water molecules more densely packed around the ion than in the bulk water, leading to the decrease of the volume of the whole system (Barciszewski et al., 1999).

**Far-UV circular dichroism spectra** Spectra used to understand the secondary structure of proteins, like the fractions of alpha-helix, beta-sheet, or beta-turn conformations.

**Hydrophilicity** It refers to the affinity of a surface to water. A material is considered hydrophilic if a droplet of water dropped on the surface spreads, making wet a large area of the surface. instead of forming a sphere.

**Hyperthermophile** A microorganism, archaea or bacteria, that thrives at elevated temperature in excess of 80 °C.

**K<sub>M</sub>** In enzyme kinetics, it is known as Michaelis–Menten constant and is equal the concentration at which the rate of a simple enzyme-catalyzed reaction is equal to half the maximum rate as defined by the Michaelis–Menten equation:

$$V = \frac{V_{max}C_S}{K_M + C_S}$$

where C<sub>S</sub> represents the substrate concentration.

**Oligonucleotide** Short sequence of nucleotides, that is, fragments of single- or double stranded DNA or RNA that code for the synthesis of one or multiple amino acids

**SDS-PAGE** Sodium dodecyl sulfate polyacrylamide gel electrophoresis. This is a molecule (mostly proteins) separating method depending on the difference of molecular weight.

**V<sub>max</sub>** Is the maximal rate of an enzyme-catalyzed reaction.

### Introduction

Enzymes are sustainable catalysts widely used in the food industry. They have many advantages such as being very specific with respect to the substrate, being biodegradable, being active in relatively low concentrations, mild pH and temperature conditions (Sheldon and Van Pelt, 2013). Moreover, some commonly used enzyme preparations are considered generally recognized as safe (GRAS) by FDA (Simpson et al., 2012). Environmental factors that can destabilize enzymatic activity are temperature, pH and water activity. Stabilization of enzymes is important because they are costly and if they are too labile or have a low activity at the processing conditions (temperature, pH, water activity) then they are not cost effective to be used in food processing. Enzymes have an optimum temperature range at which they display their maximum activity and as temperature increases enzymes become deactivated. Food processes using enzymes operate at 5–20 °C below the temperature with the maximum enzyme activity to have a high activity without inactivating the enzyme before the process is completed (Parkin, 2008). Thermal stability of enzymes is especially important in processes that require the use of relative high temperatures such as production of HFCS. The strategies for thermal stabilization of enzymes covered on this review are mutagenesis, immobilization, chemical modification and high hydrostatic pressure (HHP). Table 1 shows examples of the stabilization strategies mentioned on this review.

### Mutagenesis

Mutagenesis consists of producing mutants by replacing one or multiple oligonucleotides in a gene that codes for a given enzyme. Such mutations alter the sequence of amino acids of the target enzyme for the purpose of altering one of several of its characteristics such as K<sub>M</sub>, V<sub>max</sub>, pH stability, thermal stability, solubility, substrate specificity or the requirement for a cofactor. When the crystal-line structure of the enzyme is not known, it is not possible to rationally hypothesize the exact amino acid substitution that will

**Table 1** Examples of enzyme stabilization studies using different strategies

Enzyme	Source	Application	Stabilization strategy	Mechanism of stabilization	Experimental evidence of stabilization	References
Lipase A	<i>Bacillus subtilis</i>	Interesterifications for production of flavors and structured lipids	Mutagenesis 12-point mutant	Formation of hydrogen bonds, increased rigidity, and stabilization of N-terminal helix	T <sub>m</sub> increased to 77 °C (22 °C greater than the wild type) and ΔG <sub>u</sub> increased to 15.1 kcal mol <sup>-1</sup> (3.7 kcal mol <sup>-1</sup> greater than the wild type)	<a href="#">Singh et al. (2015)</a>
α-amylase	<i>Geobacillus</i> sp.	Starch hydrolysis	Mutagenesis by deletion of two amino acids and replacement of another	Unclear	Half-life of the best mutant was 88 times smaller than the half-life of the wild type enzyme	<a href="#">Gao et al. (2017)</a>
Lipoxygenase	<i>Anabaena</i> sp. PCC 7120	Carotenoid bleaching and rheology improvement in bread making	Site-directed mutagenesis of one amino acid (N305D)	Increased hydrophobicity and hydrogen bonding at the protein surface. Stabilization of the α-helix and hydrophobic side chain	Two-fold increase in half-life relative to the wild type	<a href="#">Guo et al. (2014)</a>
Inulin fructotransferase	<i>Arthrobacter aureus</i>	Difuctose production	HHP	Formation of disulfide bonds and stabilization of hydrogen bonds	Inactivation rate decreased by 55.9% at 200 MPa relative to ambient pressure at 60 °C	<a href="#">Li et al. (2015)</a>
β-glucosidase	Almond	Aroma and flavor development	HHP	Strengthened hydration and stabilization of hydrogen bonds	Inactivation rate of enzyme decreased by 45% at 50 MPa relative to ambient pressure at 70 °C	<a href="#">Terefe et al. (2013)</a>
β-galactosidase	<i>Aspergillus oryzae</i> , <i>E. coli</i>	Dairy processing	HHP	Strengthened hydration	At 250 MPa, temperature leading to 50% activity loss within 30 min increased from 56 to 63 °C for enzyme from <i>A. oryzae</i> , and increased from 49 to 52 °C for enzyme from <i>E. coli</i>	<a href="#">Degraeve et al. (2002)</a>
Glucose dehydrogenase	Thermostable Bacterial strain SM4	Glucose biosensor	Glutaraldehyde crosslinking chemical modification	Prevention of subunit dissociation by Intra-molecular crosslinking of β and α-subunits	Retention of 90% of initial activity after 30 min at 65 °C and one optimum temperature (65 °C) instead of two (45 °C and 75 °C) relative to the native enzyme	<a href="#">Yamazaki et al. (1999)</a>
α-amylase	Not specified	Starch hydrolysis	Macromolecular cross-linked enzyme aggregates (M-CLEAs)	Stabilization of the quaternary structure by chemical cross-linking, retention of conformational flexibility	Two-fold increase in half-life relative to the free enzyme at 50 and 60 °C and 0.5-fold increase at 70 °C	<a href="#">Nadar et al. (2016)</a>
Pectate lyase	Psychrophilic <i>Bacillus subtilis</i> AKPSYP	Pectin degradation	Supplementation with calcium hydroxyapatite nanoparticles and entrapment in lipid functionalized single wall carbon nanotube (SWNT)	Protection of the Integrity of catalytic sites, improved catalytic efficiency by the large surface area of SWNTs	6.5 fold increase in activity with respect to free enzyme at 80 °C	<a href="#">Mukhopadhyay et al. (2015)</a>

result in the desired property change. In that case, random mutations (random mutagenesis) are produced. Each mutant is then tested for the desired property, the best is selected and cloned to mass-produce the enzyme with the desired property. When the crystalline structure is known (determined by X-ray diffraction) it is possible to hypothesize substitutions that result in the desired characteristic (Glick and Pasternak, 1994). Current computational power and software allow modelling such substitutions and predict some of the properties. Inaccuracy of such predictions is decreasing as models and computer power continue to improve. Then, site-directed mutations can be done experimentally by replacing specific nucleotides. The properties of the mutants are then determined and confirmed experimentally. The stability of wild-type and mutant enzymes is typically studied by micro-differential scanning microcalorimetry, which determines the melting temperature ( $T_m$ ) of the protein, which is typically associated to the disruption of the tertiary structure of the enzyme. A limitation of this measurement is that the enzymes can lose their activity upon heating at temperatures below  $T_m$ . Indeed, enzyme activity not only depends on the retention of the tertiary structure but also on the quaternary structure, the dissociation of a cofactor and other factors.

Comparison of enzymes from hyperthermophiles to their analogues from mesophiles has helped elucidating that multiple mechanisms and structural differences that contribute to stability. Enzymes from hyperthermophiles are 40%–85% similar in structure to their corresponding enzymes from mesophiles. Overall, enzymes from hyperthermophiles are more rigid and compact. Hydrophobic interactions in the core of the protein, salt bridges at the surface, alanine or glutamic acid stabilized  $\alpha$ -helices, number and size of loops, and/or hydrogen bonds contribute to the increased stability of enzymes from hyperthermophiles compared to their mesophiles counterparts (Vieille and Zeikus, 1996; Vieira and Degreve, 2009). These observations have contributed to strategically produce stable mutants. Enzymes from hyperthermophilic organisms have been also cloned into mesophilic organisms such as yeasts, *Escherichia coli*, or *Bacillus subtilis* (Schiraldi and De Rosa, 2002). Examples of such enzymes include  $\alpha$ -amylases, lipases, and other hydrolases used in the production of corn syrup from starch. However, because enzymes for hyperthermophiles can typically be cloned and produced in mesophiles, there is typically no need for further protein engineering.

Disulfide bonds typically help stabilizing proteins. Such bonds exist on some wild proteins but can also be formed by site-directed mutagenesis by substituting with cysteine pairs of amino acids that are spatially near each other. The selection of the sites for substitution is often guided by molecular simulations. Typically, such substitutions are done away from the active site of the enzyme to minimize effects on activity. Excessive addition of disulfide bonds can result in complete loss of enzyme activity that can be attributed to the distortion of the peptide backbone or to affecting the allostery of the enzyme, defeating the purpose of stabilization. Asparagine and glutamine can undergo deamidation when heated and destabilize the protein. Replacing these amino acids with others, in some cases increases protein stability (Glick and Pasternak, 1994). Salt bridges have stabilized proteins at high temperature but some reports indicate that they contribute little to stability at room temperature (Elcock, 1998).

Examples of enzymes with current or potential applications in food and feed processing or food analysis that have been stabilized by mutagenesis include lipases, (Singh et al., 2015; Mohammadi et al., 2016) amylases that work at acidic pH (Gao et al., 2017; Priyadharshini and Gunasekaran, 2007), pyranose oxidase (Masuda-Nishimura et al., 1999), lipoxygenase (Guo et al., 2014), and phytase (Shivange et al., 2016; Niu et al., 2017).

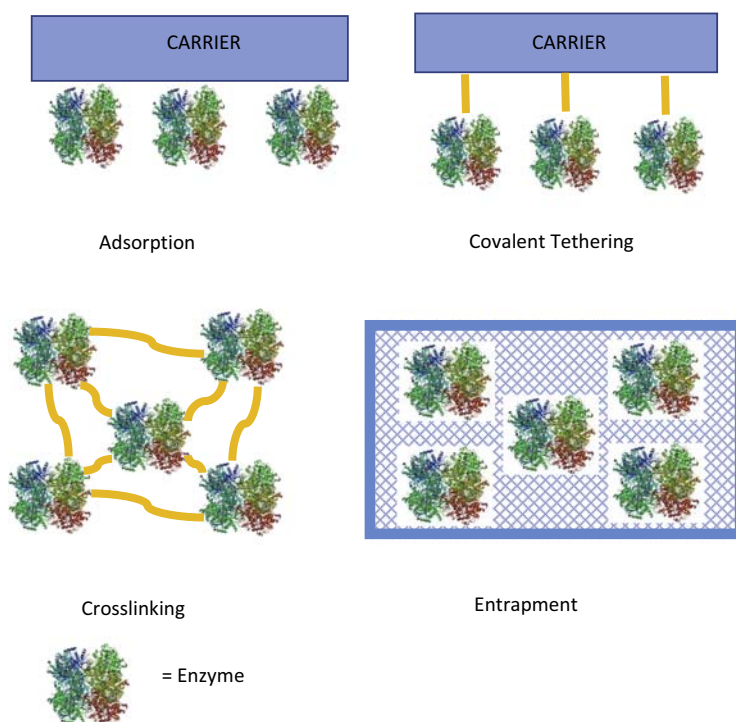
## Immobilization

Immobilization of enzymes consists of attaching an enzyme to a support material (carrier), creating enzyme agglomerates or entrapping enzymes into the support materials. There are two classes of immobilization methods physical and chemical. Physical methods involve weaker, non-covalent interactions such as hydrogen bonds, hydrophobic interactions, van der Waals forces, binding by affinity, ionic binding or mechanical containment. While chemical methods involve covalent bonds through ether, thio-ether, amide or carbamate bonds between the enzyme and support (Brena et al., 2013; Mohamad et al., 2015). Immobilization has been used to improve enzymes activity, stability, substrate specificity, reusability, continuous use, facilitate product separation and be environmentally friendly (Singh et al., 2013; Lee et al., 2017; Sheldon and Van Pelt, 2013). Some disadvantages are that enzyme conformation may change during immobilization or enzyme can be denatured, enzyme activity may be lower than free enzyme and mass transfer can be limited compared to free enzyme (Singh et al., 2013; Mateo et al., 2007). Many books and reviews on enzymes immobilization are available Guisan (2013) is an excellent reference book that describes immobilization protocols in detail that are simple and cost effective. Cao (2005) focuses more on the history, while also covering methods and principles of immobilization. Sheldon and Van Pelt (2013) review the basics of immobilization and its use in reactors. Reviews also have focused on immobilization of specific enzymes that are or have the potential of being used in industrial processes such as  $\alpha$ -amylases (Dey et al., 2016), lipases (Mateo et al., 2007; Shuai et al., 2017), cold adapted enzymes (Lee et al., 2017), thermophilic enzymes (Cowan and Fernandez-Lafuente, 2011). Combination of immobilization with chemical modification or protein engineering has also been covered (Singh et al., 2013; Fagain, 1995; Janecek, 1993). The most common immobilization techniques are adsorption, covalent tethering, crosslinking, and entrapment (Guisan, 2013; Mohamad et al., 2015) (Fig. 1). These four techniques are discussed below in more detail.

## Adsorption

Physical adsorption consists of the reversible immobilization of the enzyme on a solid support (carrier) and is rather random than targeted (Guisan, 2013; Sheldon, 2007; Lee et al., 2017; Mateo et al., 2007). Physical adsorption of lipases in hydrophobic supports





**Figure 1** Common Immobilization techniques. Note: Enzyme structure from the RCSB PDB ([www.rcsb.org](http://www.rcsb.org)) of PDB 5HSA Alcohol oxidase AOX1 from *Pichia Pastoris* (C. Koch, P. Neumann, O. Valerius, I. Feussner, R. Ficner) (2016) Crystal structure of alcohol oxidase from *Pichia pastoris*. PLoS One 11: e0149846-e0149846.

stabilizes their open structure and improves their activity (Guisan, 2013). Optimal reaction temperature of lipase from *Candida rugosa* increased from 35 °C to 50 °C when the enzyme was immobilized on a hydrophobic PVC support (Shaw et al., 1990). Physical adsorption of formaldehyde dehydrogenase (FDH) on mesoporous zirconia material (MPZ) improved thermal stability by preventing changes in the enzyme structure due to a strong interaction with the zirconia. While denaturation temperature of the immobilized enzyme was 78.9 °C compared to 51.8 °C of the native enzyme (Masuda et al., 2014).

### Covalent Tethering

Covalent tethering of enzymes to a solid support can be single or multipoint, or in the case of multimeric enzymes multi-subunit (Mateo et al., 2007; Pedroche et al., 2007; Lee et al., 2017). Multipoint covalent tethering consists of attaching the enzyme through specific residues on the enzyme surface to an activated support via short spacer arms (Guisan, 2013; Mateo et al., 2007) making the enzyme structure more stable by preventing conformational changes. Still the challenge is to achieve the desirable multiple-points configuration (Cowan and Fernandez-Lafuente, 2011; Singh et al., 2013). Multi-subunit covalent tethering has been combined with crosslinking of enzyme subunits to stabilize multimeric enzymes by preventing dissociation of the monomeric units (Mateo et al., 2007; Guisan, 2013). Activation groups commonly used are epoxy, glyoxyl groups or aldehyde (Lopez-Gallego et al., 2005). Glutaraldehyde is a cross-linker that is used to further stabilize enzymes covalently attached to a support via cross-linking with other enzymes or within their structure (Guisan, 2013). The carriers used in supporting bound immobilized enzymes (adsorption and covalent binding) can be synthetic organic polymers, biopolymers or inorganic solids (Sheldon, 2007). Multi-point attachment can increase the optimal temperature of enzymes. Shaw et al. (1990) increased *C. rugosa* optimal temperature from 35 °C to 55 °C using adsorption and multi-point attachment in chitosan using glutaraldehyde. The drawback of the multi-point attached enzyme was that the substrate accessibility was limited and the activity of the immobilized enzyme was lower than the adsorption approach. Lee et al. (2009) attributed the observed thermal stability of porcine pancreas lipase (PPL) immobilized on surface-modified nano-sized magnetite (S-NSM) at pH 7.7 to multipoint attachment or hydrophobic interaction or a combination of both. Activity of the immobilized PPL enzyme at 40 °C – 60 °C ranged from 43% to 133% higher than the free enzyme.

### Crosslinking

Crosslinking of enzyme aggregates or enzyme crystals requires bifunctional or polyfunctional reagents also called cross-linkers to keep enzymes together (Guisan, 2013; Sheldon, 2007) while a support (carrier) is not required (Nadar et al., 2016). Three common crosslinking techniques are cross-linked enzyme aggregates (CLEAS) (Nadar et al., 2016), cross-linked enzyme crystals (CLECs) and

combining 2 or 3 enzymes in a CLEAS (combi-CLEAS) (Sheldon and Van Pelt, 2013). CLEAS preparation involves two main steps: first precipitation of the enzyme in active form using precipitation agents such as water-miscible organic solvents, inorganic salts or non-ionic polymers; second cross-linking with bifunctional cross-linking reagents (Nadar et al., 2016). Glutaraldehyde is the most common cross-linker (Barbosa et al., 2014). Other cross-linkers are formaldehyde, aldehyde dextran, polyethylenimine (PEI) (Rueda et al., 2016). The crystalline matrix of CLECs seems to keep the structure of enzymes stable. CLEC of chloroperoxidase using glutaraldehyde as cross-linker kept 90% of the initial activity after 1 h incubation at pH 6, at 60 and 70 °C. While the soluble enzyme showed <10% of its initial activity. A limitation was that the immobilized enzyme had a lower activity than the soluble enzyme (Ayala et al., 2002). The stabilization mechanism of CLEAS has been attributed to the increase of rigidity of the enzyme caused by the covalent bond formed between enzyme and cross-linkers resulting in inhibition of conformational changes caused by heat. (Nadar et al., 2016). developed dextran and chitosan macromolecular cross-linked enzyme aggregates (M-CLEAs) and glutaraldehyde CLEAS (G-CLEAs) of  $\alpha$ -amylase. Inactivation rate constant of M-CLEAs and G-CLEAS were lower than the free enzyme at 50, 60 and 70 °C, at pH 6. While the activation energy for enzyme deactivation was the highest in M-CLEAs indicating a higher thermal stability than G-CLEAS. Additionally, the mass transfer of M-CLEAs was higher than G-CLEAS.

### Entrapment (Encapsulation)

Entrapment of enzymes is done via inclusion of enzymes within polymeric networks, membranes (Guisan, 2013), micro and nano-carriers (Yadav et al., 2011). To avoid confusion between entrapment and support binding Sheldon and Van Pelt (2013) defined entrapment as the enzyme being present during the synthesis of the polymeric matrix and support binding as the binding of the enzyme to a prefabricated support regardless if enzyme is inside or on the surface of the carrier. Entrapment of enzymes creates an optimal microenvironment for the enzyme and the structure of the enzyme remains stable because the enzyme is not chemically interacting with the material on which is entrapped. The drawback is that the diffusion of the substrate to the enzyme active site is limited (Mohamad et al., 2015; Gorecka and Jastrzebska, 2011) and entrapment is more commonly used to immobilize whole cells rather than enzymes.

### Chemical Modification (hydrophobic/hydrophilic modification)

Chemical modification of enzymes is an inexpensive and rapid way to improve biocatalyst performance and functionality, including thermostability, solubility and specificity. Modification includes attaching hydrophobic or hydrophilic groups, chemical cross-linking, or modifying the amino acid side-chains (Hassani, 2012). Improving enzyme thermostability is often achieved by modifying the hydrophobic/hydrophilic ratio on the enzyme surface (Longo and Combes, 1997, 1999). This approach works because it prevents the heat-induced denaturation caused by the interaction between hydrophobic clusters on the protein surface and the surrounding water. The contact of the non-polar region of enzymes with water is thermodynamically unfavorable and detrimental to the enzyme stability (Hassani, 2012). Chemical modifiers interact with the hydrophobic side chains of enzymes to create a shield protecting the hydrophobic clusters from interacting with water (Longo and Combes, 1997, 1999). Common used modifiers include polysaccharides, polyethylene glycol (PEG) (Longo and Combes, 1999; Liu et al., 2014; Daba et al., 2013), anhydride (Hassani, 2012; Xue et al., 2010), anthraquinone 2-carboxylic acid (AQ) (Mogharrab et al., 2007).

Chemical modification often changes the enzyme conformation by lowering the surface hydrophobicity and increasing hydrogen bond formation (Momeni et al., 2017; Liu et al., 2014). Introducing chemical modifiers, often hydrophilic modifiers, to the protein increases the general enzyme hydrophilicity (affinity to water). The surface hydrophobicity was often reported to decrease after attaching a hydrophilic modifier (Liu et al., 2014; Momeni et al., 2017). But Mogharrab et al. (2007) pointed out that the horseradish peroxidase modified by AQ changed the hydrophobic residues distributions rather than the total number on the enzyme surface. By reducing surface hydrophobicity, the attached hydrophilic groups prevent the hydrophobic regions from interaction with water, and thereby the enzyme thermostability increases. Generally, enzymes have higher thermal stability with introduction of additional hydrophilic groups (Hassani, 2012; Vinogradov et al., 2001). When more hydrophilic groups are introduced to the enzyme, there are less hydrophobic or non-polar groups interacting with water and then the enzyme is more resistant to heat. Introducing hydrophilic groups can also neutralize the positive charges (for example, on lysine residues) and then decrease the charge repulsion within polypeptides, which also contributes to increase the stability (Xue et al., 2010). However, adding too many hydrophilic groups to the enzyme surface can also increase the negative charges, and thereby increase the electrostatic repulsion when the negative charges become predominant. This repulsion prevents the protein refolding when unfolded at high temperatures. The higher the temperature, the less likely refolding will happen. Therefore, greater hydrophilization doesn't necessarily increase the enzyme thermostability (Hassani, 2012). Chemical modification can also increase the rigidity of the enzyme (Longo and Combes, 1999). After modifiers are attached to the enzyme, hydrogen bonds are formed between modifiers and solvent, or between the modifiers and amino acids (Hassani, 2012). A highly hydrogen-bonded structure increases rigidity which improves the conformation stability of the enzyme, decreasing the flexibility of the enzyme in solvent, which promotes thermostability (Liu et al., 2014).

## High Hydrostatic Pressure (HHP)

Though high hydrostatic pressure is typically used to inactivate enzymes, it also has the protective effect on enzymes against high temperature denaturation. This enables enzymes to work at higher temperatures, resulting in faster reaction rates (Li et al., 2015). But this protection effect has mainly been observed at high temperatures (above 50 °C) and relatively low pressures (below 400 MPa) (Terefe et al., 2013; Mozhaev et al., 1996). Pressure–temperature relationship has been comprehensively reviewed and explained (Eisenmenger and Reyes-De-Corcuera, 2009). An elliptical shaped plot (Fig. 2) has been used to show that pressure and temperature have antagonistic effect on the inactivation of enzymes. According to Fig. 2, enzymes in the native state (active) are inside the ellipse and denatured enzymes (inactive) are outside then at high temperature, moderately high pressure favors the native state of enzymes keeping them active (Terefe et al., 2013; Eisenmenger and Reyes-De-Corcuera, 2009; Degraeve et al., 2002).

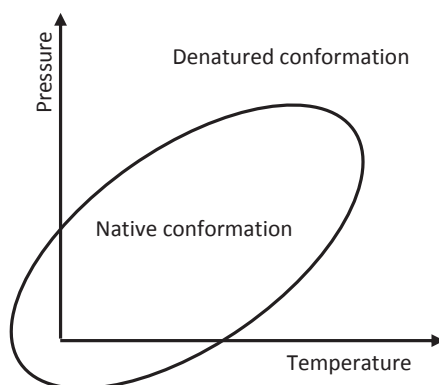
HHP has a different enzyme inactivation mechanism from high temperature. The denaturation is more likely to be caused by penetration of water into the interior enzyme structure rather than completely unfolding. The electrostatic and hydrophobic interactions that support non-specific inter-protein aggregates are weakened by HHP (Degraeve et al., 2002). In many cases, the molar volume of pressure denatured enzyme is smaller than their native counterparts (Kirsch et al., 2013; Mozhaev et al., 1996). The mechanism of stabilizing enzymes by high pressure is not well understood. High pressure can cause protein conformation changes, hydration of charged groups, stabilization of hydrogen bonds, affect the reaction mechanism and the physical properties of solvent or substrate (Eisenmenger and Reyes-De-Corcuera, 2009; Kirsch et al., 2013). Pressure and temperature have opposite effects on the intramolecular interactions, and on the interaction between the protein functional groups and the solvent (Terefe et al., 2013; Eisenmenger and Reyes-De-Corcuera, 2009).

High temperature can cause the disruption of highly ordered structure of electrostricted water, resulting in the essential water loss at the beginning of the protein thermal inactivation, which will lead to structural rearrangement and denaturation. HHP, on the other hand, can strengthen the hydration of charged and non-polar groups since the electrostriction of water around the charged groups decreases the molar volume of water; and hence HHP can compress the protein hydration shell and counteract the process from high temperature (Terefe et al., 2013; Eisenmenger and Reyes-De-Corcuera, 2009; Degraeve et al., 2002; Mozhaev et al., 1996). High temperature weakens the intermolecular O–H interactions, resulting in softening the hydrogen bond network. HHP shortens the distance between the hydrogen bonds and thereby stabilizes them, which strengthens intermolecular O–H interactions (Li et al., 2015; Eisenmenger and Reyes-De-Corcuera, 2009). HHP can also strengthen the ion pairs which further stabilizes the tertiary and quaternary structures of enzymes (Li et al., 2015).

Disulfide bonds are important in the stability of the enzyme conformation. Introducing disulfide bonds into the enzyme is a common way to increase stability. Li et al. (2015) reported that HHP impeded the unfolding of the inulin fructotransferase (IFTase) conformation at high temperatures (70–80 °C). A 200 MPa treatment decreased the IFTase inactivation rate by 55.9% at 80 °C compared to the control enzyme at ambient pressure. Based on Far-UV circular dichroism spectra, fluorescence spectra and SDS-PAGE, the secondary and tertiary structure as well as the molecular weight didn't change. However, intramolecular disulfide bonds increased by 1.8-fold and 2.2-fold when IFTase was hold at 100 MPa and 200 MPa for 15 min at 80 °C. The formation of the intramolecular disulfide bonds was explained as the sulfhydryl-disulfide interchange reactions and the movement of the flexible thiol groups under high pressure; these bonds were related to the enzyme thermostability increase.

## Conclusions

As the structure of enzymes has become better known, the strategies to improve thermal stability have become more precise and effective. Simulation and knowledge of enzymes structure have further developed site-directed mutagenesis which combined



**Figure 2** Theoretical elliptical pressure and temperature diagram with native protein conformation inside and denatured conformation outside.

with tailor-made supports has made immobilization more specific. Hyperthermophile enzymes have been cloned to produce thermally stable mutants or being immobilized to increase reusability. Effectiveness of enzyme immobilization relies on maintaining the enzyme structure stable in the active configuration. Disulfide bonds formation, amino acids replacement, chemical cross-linking and modification of hydrophobic/hydrophilic ratio have been used to modify enzyme structures and make them more stable either through mutagenesis or chemical modification. HHP protective effect of enzymes from thermal denaturation happens at relative high temperatures and low pressures and although not fully understood yet, several mechanisms have been suggested. Combinations of mutagenesis, immobilization, chemical modification and HHP treatment are frequently used to further improve thermal stability of enzymes.

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## Stabilization of Carotenoids in Foods

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### Glossary

**Antioxidants** Substances that inhibit oxidation process

**Bioavailability** The proportion of a substance that enters the circulation when introduced into the body and can have an active effect on the physiological functions

**Biosynthesis** Production of complex molecules within living organisms or cells

**Carotenoids** Class of mainly yellow, orange, or red pigments in fruits and vegetables, which give color to plant parts and have health-promoting effects

**Phytochemicals** Naturally occurring plant substances having protective effects against diseases

### Introduction

Carotenoids are the organic pigments naturally present in plant-based foods. These are mainly comprised of 8 units of isoprene attached with a skeleton of 40 carbon atoms. There are over 600 known types of carotenoids out of which more than 100 types of carotenoids are found in plants. Additionally, these pigments are also present in some photosynthetic algae, fungi and bacteria. The existence of carotenoids has not been reported in animals including humans. The two broad categories of carotenoids are xanthophylls (astaxanthin, lutein and cryptoxanthin etc.) and carotenes ( $\alpha$ -,  $\beta$ -carotenes, lycopene and phytoene etc.). generally, xanthophylls are termed as oxygenated carotenoids whereas carotenes are referred as hydrocarbon carotenoids. In plants, carotenoids are responsible for imparting various colors to different parts of plants such as red, yellow and orange etc (Britton, 1993). In foods, carotenoids also provide attractive colors to the foods whereas in humans, carotenoids are claimed to have important functions (Stommel et al., 2005). For instance, most of the carotenoids are potent antioxidants while some are precursors of vitamin A ( $\beta$ -carotene).

Carotenoids are considered as important dietary bioactive components which are helpful in protecting against several degenerative conditions such as macular degeneration, cancer, diabetes, hypercholesterolemia, cardiovascular ailments, malfunctioning of immune system and cancer (Olson, 1999). Various mechanistic approaches have been reported to describe the health-promoting functionality of carotenoids: modulating the metabolism of carcinogens, ability to quench singlet oxygen, hindering proliferation of cells, scavenging of free radicals, improving the filtration of blue light, enhancing immune response and augmenting differentiation of cells through retinoids.

### Chemistry of Carotenoids

#### Structure and Classification

Carotenoids obtained from different sources have been isolated and characterized by various researchers. Generally, carotenoids are composed of 40 carbon atoms having conjugated double bonds in their structure. They are composed of 8 isoprenoid units which are linked in a way that their arrangement is reversed from the central point so that two methyl groups are arranged in 1,6-position whereas non-terminal methyl groups are positioned in 1,5-relationship. Carotenoid molecules have both cyclic and acyclic arrangements. Furthermore, a myriad of various structures has been seen through different modifications in the structural arrangement of carotenoid molecules which include cyclization, hydrogenation, dehydrogenation, migration of double bonds, extension or shortening of chains, isomerization or different combinations of these phenomena. Presence of conjugated double bonds in the structure of carotenoids is a distinctive property which is responsible for absorbance of light resulting in the development of different colors in various types of foods. Carotenoids are generally present in a stable *trans*-form however minor quantity is found in *cis*-form that are also converted to *trans*-forms during processing. Conventionally, trivial names are given to the carotenoids based on the biological sources from which these substances are derived. Contrarily, scientific names are given in a systematic way which describes their structures in an unambiguous manner. Common and technical names of some naturally-occurring carotenoids are given in Table 1.



**Table 1** Common and technical names of food carotenoids and their major sources

Trivial names	Technical names	Sources
Astaxanthin	3,3'-dihydroxy- $\beta$ , $\beta$ -carotene-4,4'-dione	Algae, shrimp, crabs
$\alpha$ -Carotene	$\beta$ , $\epsilon$ -carotene	Sweet potatoes, dark green vegetables, broccoli, green beans
$\beta$ -Carotene	$\beta$ , $\beta$ -carotene	Carrots, spinach, tomatoes, water squash, broccoli, sweet potatoes
$\alpha$ -Cryptoxanthin	$\beta$ , $\epsilon$ -carotene-3'-ol	Papaya, potatoes, orange rind
$\beta$ -Cryptoxanthin	$\beta$ , $\beta$ -carotene-3-ol	Oranges, persimmons
Lutein	$\beta$ , $\epsilon$ -carotene-3,3'-diol	Kale, spinach, broccoli, peas, lettuce
Lycopene	$\psi$ , $\psi$ -carotene	Tomatoes, watermelon, pink grapefruit, pink guava, papaya, sea-buckthorn, wolfberry
Phytoene	7,8,11,12,7',8',11',12'-octahydro- $\psi$ , $\psi$ -carotene	In majority of fruits and vegetables as precursor of carotenoids
Phytofluene	7,8,11,12,7',8'-hexahydro- $\psi$ , $\psi$ -carotene	In majority of fruits and vegetables as precursor of carotenoids
Zeaxanthin	$\beta$ , $\beta$ -carotene-3,3'-diol	Kale, spinach, broccoli, peas, lettuce

Modified from Rodriguez-Amaya (2001).

### Chemical Properties

The prime feature that provides the carotenoids their particular molecular shape, light-absorbing properties and chemical reactivity is the presence of alternate single and double bonds in their polyene chains. Mostly, carotenoids are lipophilic in nature having no or a very minute solubility in water and good solubility in different organic solvents. This property restricts them to the hydrophobic regions of the cell (inner core of cell membranes). However, sometimes carotenoids come in contact with the aqueous environment due to their interactions with proteins. *Trans*-form of carotenoids has linear structures and are found as rigid molecules due to the presence of strong conjugated double-bond system. However, *cis*-isomers greatly differ from the *trans*-configurations in their shape which significantly alters their reactivity. Additionally, the capability of *cis*-carotenoids to readily aggregate or crystalize also increases their solubility, absorbance and transportation.

Generally, carotenoids are unsaturated chemical compounds and are readily subjected to oxidation and isomerization. Interaction of active surface of the molecules with light, heat or acids results in isomerization of *trans*-configured carotenoids into *cis*-forms which results in loss of provitamin A activity and color. Oxidative degradation of carotenoids is the prime causative agent for loss of carotenoid activity and is usually stimulated by the presence of light, oxygen, metal ions and enzymes. The initial step in the oxidative denaturation of carotenoids is the generation of apo-carotenoids and epoxides. These molecules have shortened skeletons of carbon atoms and this fragmentation also leads to the formation of other smaller substances having low molecular weights like fatty acid oxidation process. The final consequence of this multi-step process is the complete loss of color and biological activity of carotenoids. Therefore, retention and stability of natural or added carotenoids in different food products is very important to preserve their biological activities.

### Biosynthesis of Carotenoids

Carotenoids are bio-synthesized in the chromoplasts through isoprenoid pathway. The biosynthesis of carotenoids is initialized with the condensation process in which pyruvate and glyceraldehyde 3-phosphate are converted to 2-C methyl-D-erythritol 4-phosphate. This compound is further metabolized to isopentenyl diphosphate via a series of different reactions after passing through the stages of reduction, phosphorylation and dehydration (Lichtenthaler, 1999). During the biosynthesis of carotenoids, C5 units are successively added to the C5 primer which results in the elongation of chain and formation of successive C10, C15 and C20 compounds. After that prenyl transferase enzyme builds up isoprenoid chain which is transformed to Ggps (geranylgeranyl diphosphate). Later, the two molecules of Ggps are linked (tail-to-tail combination) together to form phytoene. Phytoene is a colorless substance and undergoes a succession of chemical reactions leading to the development of new double bonds which extend the chromophore by two conjugating double bonds. The addition of double bonds on both sides of phytoene results in the formation of phytofluene. The end-product of the reaction is lycopene and carotenoids are claimed as the derivatives of lycopene.

The composition of carotenoids synthesized by plants is enriched by the presence of various biosynthetic precursors and derivatives of the main substances. Although, carotenoids are considered as plant pigments, these substances can also be encountered in animal-based food products. Animals are not capable of producing carotenoids so their carotenoids are usually derived from plant-based food/feed which they consume. These dietary carotenoids are accumulated in the animal tissues as unchanged pigments or transformed into animal carotenoids after slight modifications. Carotenoids can also be used as biomarkers to determine the quality of fruit-based food products or to detect the adulteration (Kurz et al., 2008).

### Sources of Carotenoids

As mentioned previously, carotenoids are majorly obtained from the plant sources. The other possible sources of carotenoids are bacteria, yeasts and algae. This section will mainly focus on the plants as prime sources of carotenoids.

### Fruits and Vegetables as Major Sources of Carotenoids

About 70%–90% of the totally consumed carotenoids is obtained from the fruit and vegetable origins. Plant-based foods are generally classified in three broad categories based on the presence of various types of carotenoids in them (Khachik et al., 1991).

1. Green vegetables generally contain different types of carotenes and xanthophylls. Examples include broccoli, green beans and spinach.
2. Red and yellow fruits and vegetables mainly possess carotenes. This group has more diversity of carotenoids as compared to the previous one. Plums, tomatoes, melons and carrots are included in this group.
3. Orange and yellow-colored fruits primarily have xanthophyll esters. This group generally consists of oranges (citrus group), pumpkins and peaches.

**Leafy vegetables** are considered as excellent source of carotenoids. They majorly contain lutein and  $\beta$ -carotene (80%) and traces of  $\alpha$ -carotene. In leafy vegetables,  $\beta$ -carotene is considered as the major source of vitamin A (Takyi, 2001). Studies have revealed that amongst various leafy vegetables, Moringa has  $\beta$ -carotene contents of 1400  $\mu\text{g}/100\text{ g}$  while lettuce has (19700  $\mu\text{g}/100\text{ g}$ )  $\beta$ -carotene contents (Bhaskarachary et al., 1995). Additionally, *Brassica oleracea* also contains significant quantities of lutein and  $\beta$ -carotene (Kopsell et al., 2004). Usually, lutein contents in *B. oleracea* are ranged from 4840–13430  $\mu\text{g}/100\text{ g}$  whereas  $\beta$ -carotene contents are present in the range from 3820–10000  $\mu\text{g}/100\text{ g}$ . Similarly, **roots** of carrots and sweet potatoes are also reported as imported sources of various dietary carotenoids. In addition to  $\beta$ -carotene,  $\alpha$ -carotene contents of carrots are also high. Generally, carrots possess 6500 to 8840  $\mu\text{g}/100\text{ g}$  of  $\beta$ -carotene (Muller, 1997; Murkovic et al., 2000).  $\beta$ -carotene contents of sweet potato ranged from 20  $\mu\text{g}/100\text{ g}$  (white-fleshed sweet potato) to 21800  $\mu\text{g}/100\text{ g}$  (yellow-fleshed sweet potato) in different cultivars sweet potato (Rodriguez-Amaya, 1997). Among **fruits**, pumpkin is one of the excellent sources of vitamin A and carotenoids.  $\beta$ -carotene concentration in different cultivars of pumpkin is varied from 60–7400  $\mu\text{g}/100\text{ g}$  while  $\alpha$ -carotene contents are ranged from 0–7500  $\mu\text{g}/100\text{ g}$  (Murkovic et al., 2000). Other important sources of carotenoids include mango, banana, watermelon, papaya and guava etc (Wall, 2006; Charoensiri et al., 2009). **Table 2** presents the quantities of various carotenoids in different fruits and vegetables.

### Health Benefits of Carotenoids

Carotenoids are present in plant tissues, algae and different microorganisms and majorly responsible for imparting color to different fruits and vegetables. Additionally, they also give color to the feathers and flesh of some birds and fish. Moreover, carotenoids also play an important role to improve the health of plants and animals. The major role of these phytochemicals is as antioxidants which is helpful in protecting tissue degradation due to oxygen and light (Britton et al., 1995). Because of increased awareness regarding the health-promoting functionalities of carotenoids, studies have been focused to develop functional food products having carotenoids as active ingredients. Dietary Carotenoids are claimed to play a vital role in upholding physical functions

**Table 2** Concentrations of different carotenoids in fruits and vegetables

Fruits/Vegetables	Carotenoid contents on fresh weight basis ( $\mu\text{g}/100\text{ g}$ )				
	Lutein	$\beta$ -Cryptoxanthin	Lycopene	$\alpha$ -Carotene	$\beta$ -Carotene
Apple	100–840	–	–	30	–
Apricot	0–141	28–231	0.5	0–37	140–6939
Avocados	–	36	–	28	53
Banana	0–37	–	–	0–157	0–92
Blackberry	270	–	–	9	100
Blueberry	230	–	–	–	49
Broccoli	830–4300	0	–	1	414–2760
Carrot	110–2097	–	–	530–35833	1161–64350
Grapefruit (red)	–	12	–	5	603
Guava	270	–	769–1816	–	102–2669
Lettuce	73–4537	–	–	–	48–3120
Mango	100	0–1640	–	–	300–4200
Orange	64–350	14–1395	–	0–400	0–500
Papaya	20–820	60–1483	2080–4750	0–60	71–1210
Peach	9–120	12–510	–	0–9	30–1480
Spinach	2047–20300	–	–	–	840–24070
Tomato	40–1300	–	21–62273	–	36–2232
Watermelon	0–40	62–457	2300–7200	0–1	44–324

Modified from Yahia and Ornelas-Paz (2010).

and preventing several diseases.  $\beta$ -carotene, lutein, lycopene and zeaxanthin are considered as the prominent carotenoids having numerous health benefits.

$\beta$ -carotene is helpful in maintaining the eye health, proper functionality of immune system, development of embryo and epithelial function (National Academy of Sciences et al., 2001). It is also important in reducing the risks of lung cancer and cardiovascular diseases. Similarly, consumption of lycopene is associated with the reduced risks of heart diseases (Wilcox et al., 2003) and various types of cancers viz. prostate (Miller et al., 1996), colon, skin, pancreas, esophagus, stomach (Clinton, 1998) and breast (Shi, 2000). Likewise, lutein and zeaxanthin prevent muscular degradation by delaying the aging process. Besides, lutein also reduces the incidences of cardiovascular disorders and cancer (Alves-Rodrigues and Shao, 2004).

## Factors Affecting Stability and Concentrations of Carotenoids in Foods

Various factors determine the quantity and stability of carotenoids in different food commodities. The most important contributors in this regard include variety of fruits or vegetables, type of product, stage of maturity or ripening, post-harvest handling, type of processing and storage conditions (Yahia et al., 2007). This section will provide an overview about the above-mentioned factors that influence concentrations of carotenoids and their stability.

### Fruit Maturity and Ripening Stage

Ripening or maturity stage of fruits and vegetables is regarded as the prime factor that affects carotenoid contents. Carotenoid contents are reported to increase during the ripening process due to metabolic action of ethylene (Carrillo-Lopez and Yahia, 2009). For example, lutein, carotene, xanthophyll and lycopene contents are increased at the stage of maturity in different fruits like mango, apricot, pepper and tomato. However, in some fruits carotenoid concentrations are decreased after ripening. For instance, studies have revealed that ripen dates have lower carotenoid contents as compared to the *Khalal* stage (Boudries et al., 2007).

During the ripening process, degradation of chlorophylls and chloroplasts occurs and chromoplasts are produced which lead to the increase in carotenoid contents. Additionally, several genes are switched-on during the ripening of fruits that enhance the production and accumulation of carotenoids in ripened fruits. Furthermore, several enzymes have also been detected that are responsible to increase the synthesis of carotenoids during the ripening process. An example is ripening-specific phytoene synthase which is activated in tomatoes at the stage of maturity and responsible for production of carotenoids (Fray and Grierson, 1993).

### Genotype

Genotype of fruits and vegetables is another important factor that influences the carotenoid contents in the final produce. A study confirms variations in carotenoid contents of various mango cultivars (Ornelas-Paz et al., 2007). Previous works have also demonstrated differences in the carotenoid concentrations in different varieties of other fruits such as tomato, apricot, cassava and sweet potato etc. Another investigation was based on determining the carotenoid contents in 52 different cultivars of lettuce (crisphead, butterhead, red leaf, Latin, romaine, stem lettuce, primitive and wild species) and proposed variations in carotenoid contents based on their genotypes (Beiquan, 2005). The study concluded that wild species had higher  $\beta$ -carotene contents as compared to cultivated varieties. Amongst cultivated varieties, the order of the carotenoid contents was; romaine > red leaf > butterhead > crisphead.

### Geographical Origin

Geographical origin of fruits and vegetables is also another important contributor that affects carotenoid contents in these commodities based on climatic conditions. For example, carotenoid contents of apricot fruit are reported to be higher in Mediterranean region as compared to the other geographical zones (Dragovic-Uzelac et al., 2007). Similarly, differences were observed in the carotenoid contents of same mango variety cultivated in two different regions of Brazil (Mercadante and Rodriguez-Amaya, 1998). These variations might be attributed to the differences in intensity, pattern and exposure time to sun light because these parameters also influence the stability of carotenoids.

### Structure of Fruits and Vegetables

Generally, carotenoid contents differ in their concentration in various parts of the same fruit or vegetable. Usually, peels of fruits or vegetables contain higher amounts of carotenoid contents as compared to pulp (Carrillo-Lopez and Yahia, 2009). This pattern is observed in different fruits such as tomato, kumquat, muskmelon, papaya, persimmon and mandarin hybrid. However, exceptions exist in nature. For example, red pomelo and pin-fleshed guava have shown higher carotenoid contents (particularly  $\beta$ -carotene) in pulp portion as compared to peels.

### Effect of Storage on Stability of Carotenoids

Storage conditions of fruits and vegetables also have considerable influence on the stability of carotenoids. It is generally observed that losses of carotenoids during the storage of mature fruits and vegetables are less in comparison with the ripened fruits and vegetables. Inappropriate storage conditions lead to the rapid loss of carotenoids during the storage period. Carotenoid losses are increased in the stored commodity due to the exposure of sunlight or oxygen during the storage or because of favorable conditions for wilting improper temperature. The interaction between temperature and maturity also influences the carotenoid stability during the storage period (Prono-Widayat et al., 2003). It is reported that mature and pre-mature pepino fruits showed significant increase in  $\beta$ -carotene contents at 18 °C as compared to 5 °C. It is also observed that storage of fruits at temperature range of 7 to 20 °C for 16 to 43 days causes a decrease in total carotenoid contents. Additionally,  $\alpha$ - and  $\beta$ -carotene contents are lost in carrots at storage temperature above 4 °C (Melendez-Martinez et al., 2004). On the other hand, lycopene contents in some fruits and vegetables are increased at about 25 °C (Lurie et al., 1996). Moreover, in low-temperature sensitive fruits (tomato) carotenoid contents are decreased if the temperature falls below chilling point (Yahia et al., 2007). It is also revealed that production of carotenoids is limited at storage temperature of 30 °C or above due to inhibition of enzymatic activity and ethylene production (Lurie et al., 1996).

### Effect of Processing on Stability of Carotenoids

Processing of fruits and vegetables also influences the stability and concentrations of carotenoids. The alteration in the carotenoid contents and stability may be influenced in a positive or negative way depending upon the processing type and conditions. Homogenization and chopping processes are reported to decrease carotenoid contents due to tissue disruption. For example, after maceration of green leaves, about 30% of  $\beta$ -carotene is lost in the first few minutes. Several carotenoids are also thought to be stable during the thermal processing. It is observed that during heat treatment, *cis*-lycopene is increased while *trans*-forms are decreased in guava juice. Additionally,  $\beta$ -carotene contents in mango are also retained during thermal processing.

### Strategies to Improve Stability of Carotenoids

Carotenoids have been extensively used in the food industry for imparting color to various food products or to enhance their storage stability (Britton and Khachik, 2009). Additionally, they have also been utilized as antioxidants to prevent oxidative degradation of emulsions such as in fat spreads, dairy products, salad dressings and mayonnaise etc (Lee and Choe, 2013). However, food processing or improper storage, transportation and handling of foods may cause decrease in the bioavailability and stability of carotenoids (Soukoulis and Bohn, 2018). These major factors include excessive storage period, exposure to light or oxygen and severe thermal treatments (Wang and Bohn, 2012). Hence, it is important to maintain the bioavailability and retention of carotenoid contents in various processed foods. Accordingly, this section will provide a brief description of various strategies to augment the stability of carotenoids during processing and storage of carotenoid-containing food products.

#### Encapsulation Techniques

Encapsulation is a technique which is used to provide a protection casing to the active compounds to enhance their stability. Encapsulation is aimed to provide protection to the active compounds against harsh environmental conditions (Augustin and Sanguansri, 2008). Selection of suitable carrier material for encapsulation is of prime importance. Important characteristics for encapsulating materials are inertness, safety (GRAS), versatility in organoleptic properties, cost-effectiveness, emulsifying & water-binding properties and controlled & targeted delivery (Connet et al., 2010). In case of carotenoids, some additional properties of wall-materials should also be kept under consideration which include solubility of the material in different solvents, crystallinity, changes in the droplet size during incorporation and structural modifications as these features directly affect the bioavailability, functionality and stability of carotenoids (Ribeiro et al., 2010). The most commonly employed encapsulating materials for carotenoids are soybean polysaccharides, mesquite gum, gellan gum, gum arabic and gelatin (Hojjati et al., 2011).

Various strategies have been adopted for encapsulating carotenoids to reap full benefits from these pigments in terms of their bioactive functionalities. The most extensively used techniques in this regard include freeze-drying, spray drying, formation of liposomes, use of inclusion complexes, coacervation and extrusion technology. Approaches to elevate the stability of carotenoids against several unfavorable environmental factors during processing and storage and to prevent the undesired chemical reactions with different food substances include micro- and nano-encapsulation techniques include use of nano-structured lipid carriers, solid-lipid nanoparticles, self-assembled protein nanomaterials (Soukoulis and Bohn, 2018).

#### Thermal and Non-thermal Treatments to Maintain Carotenoid Stability during Storage

Use of hot-air drying, freezing, pasteurization and freeze-drying etc. have been reported to maintain the stability of encapsulated carotenoids. In addition, these techniques have also shown their potential to reduce the losses of carotenoids during the storage of fruits and vegetables. For instance, spinach preserved through freezing or dehydration techniques did not show significant losses in lutein, zeaxanthin and violaxanthin contents during the storage (Rodriguez-Amaya, 1999). It is also reported that dehydration of mango fruits facilitates conversion of *trans*-forms of  $\beta$ -carotene to *cis*-forms (Pott et al., 2003). Similarly,  $\beta$ -carotene contents of

*Lycium barbarum* are also reported to increase up to 2–22 times during dehydration process (Wen-ping et al., 2008). Likewise, pasteurization of juices also results in improvement in the red color and carotenoid contents (Odriozola-Serrano et al., 2009).

Studies have also revealed less cooking time at low temperature also facilitates retention of carotenoids in fruits and vegetables. Additionally, scalding of carrots for 15 min. at different temperatures (50, 70, 90 °C) is also useful in maintain the lycopene contents (Meléndez-Martínez et al., 2004). Moreover, canning process is helpful in improving the stability of carotenoids during storage period. Studies have revealed that canning significantly increases the carotenoid contents in various fruits and vegetables such as 39% increase in sweet potatoes, 33% in carrots, 20% in tomato juice, 19% in collards, 13% in spinach and 10% in peaches (Yahia and Ornelas-Paz, 2010).

In addition to thermal processing techniques, some non-thermal processing approaches have also shown their potential in improving the stability of carotenoids. In this regard, pulsed electric field processing (Cortés et al., 2006), high pressure processing technique, osmotic dehydration (Tonon et al., 2007) and irradiation (Hajare et al., 2007) are reported to induce non-significant losses and improved stability of carotenoids in intact fruits and vegetables and their purees and juices. This discussion concludes that use of low-temperature processing methods, less processing time, prevention from exposure to oxygen and employing non-thermal processing methods can significantly prevent carotenoid losses and improve the stability of carotenoids during processing and subsequent storage.

## Conclusions

The present chapter concludes that carotenoids are quite beneficial for improving the health status of consumers. Food containing carotenoids are needed to preserve or process in a way so that their carotenoid contents are not lost during the processing and subsequent storage. In this regard several approaches including encapsulation technologies, freeze-drying, low-temperature storage, canning, pasteurization, scalding, dehydration, pulsed electric field technology, high hydrostatic pressure processing, osmotic dehydration and irradiation are reported to have positive effects in retaining the stability of carotenoids in various food commodities. Hence, it is recommended that use of these processing techniques and other related methods (Cryogenic freezing, ultra-freezing techniques, ultrasonication etc.) should be encouraged to maintain or enhance carotenoid contents in stored food products for maximum bioavailability of these beneficial phytochemicals.

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# The Role of Bioinformatics in the Discovery of Bioactive Peptides

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## Glossary

**Allergens** substance that causes an allergic reaction by stimulating an abnormal response by the immune system.

**Bioactive peptides** protein hydrolysates which, upon entry and absorption into the body, have the ability to induce certain desirable and physiologically measurable activities such as immunomodulatory, cytomodulatory, antihypertensive, and antimicrobial activities.

**Bioinformatics** an integration of methods, software and statistical tools for studying and analysing complex biological data.

**Chemoinformatics** the collection, analysis and storage of biochemical data.

**Chemometrics** the use of mathematical and statistical tools to analyze biochemical data and develop models for better understanding a biological or chemical system.

**In silico** scientific experiments or research conducted or produced by means of modelling or simulation on a computer.

**Quantitative structure-activity relationship (QSAR)** the application of bioinformatics to identify relationships between chemical structure (or structural-related properties) and biological activities (or target property) of compounds.

**Tastant** A taste-provoking chemical compound.

## Definition, Health Functions, and Applications of Bioactive Peptides

By definition, the bioactive peptides discussed in this paper are short amino acid chains derived from proteins by the proteolytic actions of exogenous or endogenous enzymes, or microorganisms used for fermentation (Agyei and Danquah, 2012; Udenigwe and Aluko, 2012). These peptides are also known as cryptic peptides, to indicate that they are inactive or hidden within the parent protein structure; therefore, enzymatic release is necessary for the peptides to exhibit their biological activities (Udenigwe, 2014). As briefly discussed in this section, food-derived peptides have several biological roles. These activities mostly stem from the fact that the peptides are structurally similar to endogenous peptide ligands, which bind their physiological receptors to trigger signalling processes in cells. Therefore, some food peptides can mimic these endogenous peptides in regulating cellular functions, especially in preventing and managing aberrant health processes and conditions. The diverse structural features of the peptides also broaden these possibilities. For instance, the range of peptide molecular size, net charge and hydrophobicity, increases the chances of their binding to many biological targets.

Most food-derived bioactive peptide reported to date downregulate blood pressure increases during hypertension. This biological function involves structure-dependent binding and can be achieved through various mechanisms including inhibition of angiotensin converting enzyme (ACE) and renin activities, upregulation of ACE2 expression, production of vasorelaxation factors, and angiotensin II receptor and calcium channel blocking effects (Majumder and Wu, 2014; Udenigwe and Mohan, 2014). Moreover, some amino acid residues act as electron donors, prooxidant metal chelators, radical trapping agents, and activators of cellular redox enzymes, which make them to become antioxidative and useful in combating cellular oxidative stress (Samaranayaka and Li-Chan, 2011). Other notable biological activities reported for food peptides include antimicrobial activity (due to peptide amphipathicity) (Haque and Chand, 2008), and hypolipidemic activity (due to their roles in binding bile acids, disrupting intestinal lipid micelles, regulating lipogenic genes/proteins in hepatocytes and adipocytes) (Howard and Udenigwe, 2013). Furthermore, some food peptides possess anticancer properties (Rajendran et al., 2017) and anti-diabetic activity (due to inhibition of dipeptidyl peptidase [DPP-IV] and incretins) (Lacroix and Li-Chan, 2014; Nongonierma and Fitzgerald, 2016). These bioactivities form the basis of application of peptides in the food and health sectors as nutraceuticals and functional ingredients for health product and cosmetics formulations.

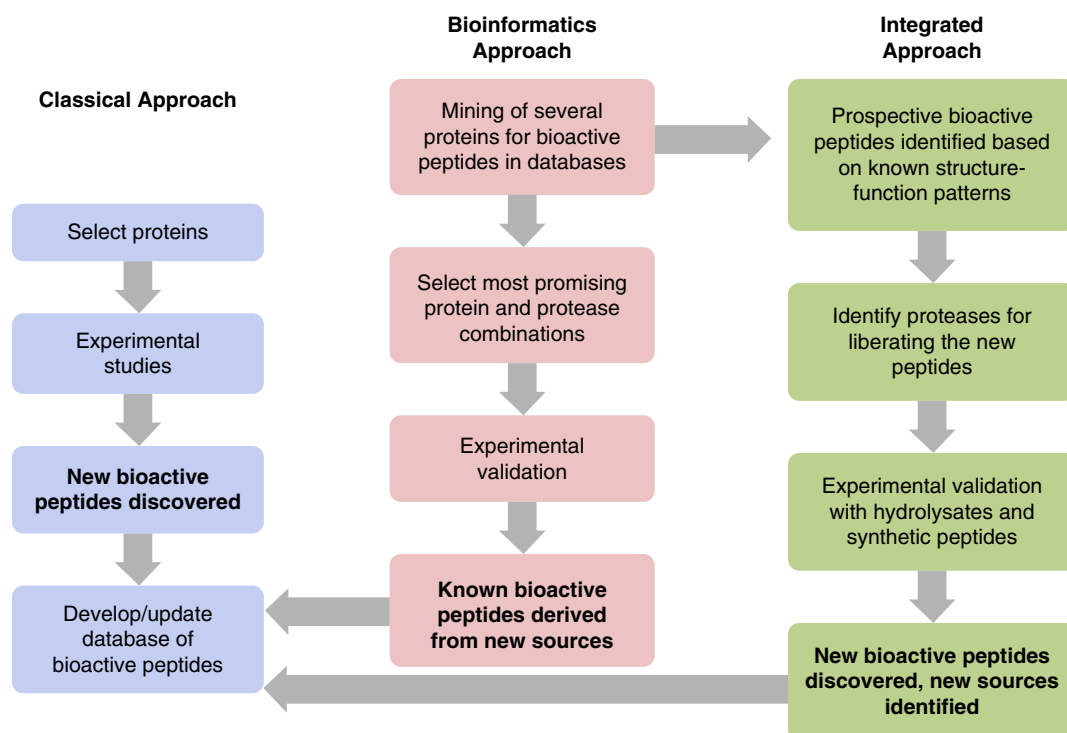
## Prospects of Bioinformatics in the Production of Bioactive Peptides

The bioactivity of a given peptide ligand is heavily dependent on its chemical structure. Therefore, the success of peptide discovery research lies on having a solid knowledge of peptide chemistry and processing techniques for releasing and fractionating the peptides of interest. The traditional approach used to discover bioactive peptides from food proteins could be laborious when evaluating many food proteins and proteases. Typically, a few starting materials are selected for bioactive peptide discovery, which may lead to limited positive leads for further exploration. Moreover, the process cannot easily capture the diverse structural features of peptides or recover high yields of the peptides when there is limited knowledge of the chemistry of the starting materials. For

instance, dipeptides with N-terminal bulky amino acid and C-terminal aliphatic amino acid would likely inhibit ACE and DPP-IV activities whereas a combination of net cationic charge and hydrophobicity of peptides is typical for antimicrobial peptides. Likewise, antioxidant peptides often need to have the sulfhydryl group of cysteine or free radical trapping moiety of phenylalanine in their structures. Traditional method of protein/protease selection and bioassay-guided fractionation for food peptide discovery does not particularly rely on these important structure-function relationships. Consequently, the use of bioinformatics facilitates the strategic screening of combinations of protein sources, hundreds of protein sequences and tens of proteases with defined specificities, in order to find the best matches that can yield the desired bioactive peptides. An integrated approach to the discovery of bioactive peptides combines the predictive powers of bioinformatics and experimental validation with using synthetic or pure peptides and hydrolysates. A comparison of the various discovery strategies are captured in Fig. 1.

Bioinformatics has been successfully used in analysing bioactive peptides in food proteins (Holton et al., 2013b; Iwaniak et al., 2015; Udenigwe, 2014). The *in silico* process can allow for a closer look at the important structural information needed for the desired activity and application, and the design of tailored processing methods for the release and fractionation of peptides with such properties from their parent proteins (Agyei et al., 2016). Furthermore, bioinformatics tools can help in identifying negative attributes during bioactive peptide production. For instance, it is possible to detect the presence of known or potential allergenic epitopes in food proteins using *in silico* tools (Hayes et al., 2015). This information can help in developing appropriate measures to avoid their presence, limit their amounts, or remove them entirely from the peptide preparations prior to experimental studies or functional food product formulation.

Besides bioactivity, peptides have a wide range of taste including bitterness (Temussi, 2012), which would likely influence quality and consumer acceptability of the products containing them (Udenigwe, 2014). Peptide bitterness is often evaluated after their production, using trained human tasters or computerized approaches e.g. electronic tongue. As discussed below, *in silico* tools also provide information on the bitterness potential of peptides based on the presence of bitter-tasting hydrophobic amino acid residues e.g. phenylalanine and proline (Iwaniak et al., 2016a). This prediction helps in identifying and eliminating peptide bitterness early in the discovery phase prior to product development. Furthermore, many peptides need to be transported across cells and be bioavailable in order to exhibit their bioactivities. Bioinformatics tools are being developed to predict the cell penetrating properties of peptides (Holton et al., 2013a), which can provide important information on their potential bioavailability. Therefore, the *in silico* approach has the potential to enhance various aspects of food peptide research, especially when complemented with wet lab analyses and *in vivo* studies.



**Figure 1** Various approaches for the discovery and production of bioactive peptides from food proteins. Reprinted from Udenigwe, C.C., 2014. Bioinformatics approaches, prospects and challenges of food bioactive peptide research. Trends Food Sci. Technol. 36 (2), 137–143. Copyright (2014), with permission from Elsevier.

## Bioinformatics Tools in the Discovery of Bioactive Peptides

Bioinformatics use computer software, mathematical algorithms, and statistical tools to archive, retrieve, and analyze biological data (Aamer Mehmood, 2014). Due to their ability to analyze huge amount of biological data quickly and cost-effectively, bioinformatics tools are vastly used in studies of bioactive peptides to characterise the structure and properties of these peptides, thereby assisting in the prediction of their potential impacts on human health and in food quality estimation (Iwaniak et al., 2015; Udenigwe, 2014). In this context, several databases exist for purposes such as generating peptides from protein sequences in silico, characterising the biological (including allergenicity and toxicity) and sensorial properties of the peptides generated. The web addresses of some of these databases and in silico tools are shown in Tables 1–4, and are freely available for access and use.

### Databases for In Silico Digestion and Bioactivity Prediction

The first step is to select food source and/or proteins and retrieve their amino acid sequence from databases such as UniProt, Pubmed, and BIOPEP. BLAST (Basic Local Alignment Search Tool) and ExPasy ProtParam tools are often used to compare and to calculate significant areas of commonality between protein sequences in order to avoid repetition of data. The selected proteins are then subjected to in silico proteolysis or “enzyme action” by using popular in silico proteolysis tools such as BIOPEP and ExPASy Peptide Cutter. Suitable enzyme are selected based on the goal of the, i.e. whether the aim is to generate novel peptides from a known food protein or to generate peptides from newly discovered proteolytic enzymes. These databases also allow the selection of multiple enzymes to act sequentially; and this makes it possible to predict the release of peptides following a physiologic process such as simulated gastric digestion. Known and unknown peptides generated in silico are identified by using databases such as BIOPEP, CAMP and PeptideDB. These databases also help identify which of the derived peptides are bioactive, by comparing with published bioactive peptide sequences. To determine the peptide rank and prospect of bioactivity based on established structure–function patterns, peptides from each protease/subunit combination can be entered into Peptide Ranker in the Bioware webserver (Lafarga et al., 2014, 2015a; Udenigwe, 2014; Udenigwe et al., 2013). The best protein-protease combinations are then used in follow-up studies to experimentally confirm under laboratory conditions, the production and bioactivities of the peptides generated in silico. This approach to the discovery of bioactive peptides has been used to predict the release of various bioactive sequences from a range of different food sources (Nongonierma and Fitzgerald, 2014), including dipeptidyl peptidase IV (DPP-IV) inhibitory peptides from camel milk (Nongonierma et al., 2018), soy and lupin proteins (Lammi et al., 2016), and silver carp (Zhang et al., 2016); prolyl endopeptidase inhibitors from meat proteins (Lafarga et al., 2015a); antihypertensive peptides from Rubisco cereal proteins (Udenigwe et al., 2013); and multifunctional (DPP-IV, renin and ACE inhibitory) peptides from bovine serum albumin (Lafarga et al., 2014) and bovine fibrinogen (Lafarga et al., 2015b). Tables 1 and 2 shows a list of protein/peptide sequence databases, and databases for in silico digestion/bioactivity prediction respectively.

### Databases for Chemometric Characterisation of In Silico Peptides

While chemometrics involves the use of mathematical and statistical tools to analyze biochemical data and develop models for better understanding a bio/chemical system (Bertacchini et al., 2013); the collection, analysis and storage of biochemical data is known as chemoinformatics (Wishart, 2007). In chemometrics, analysis of molecules are done to predict biological activity of interested molecules, and the molecular activity, and the properties of biomolecules are defined as endogenous and exogenous variables respectively. To explain the effects of endogenous and exogenous variables on bioactivity, several mathematical models based on regression analysis are used. Artificial neural networks (ANNs), principal component analysis (PCA) and partial least squares (PLS) regression are examples of the most widely used chemometric models. Chemometrics is made possible by the fact that proteins, peptides and amino acids under certain conditions have a defined 3D conformation which exhibit a high degree of structure–activity relationships. The principle for predicting biological properties of designing processes for separation of the peptides may be informed by mechanistic modelling of the quantitative structure–activity relationships (QSAR), based on molecular properties such as size, charge, and polarity or hydrophobicity (Agyei et al., 2016; Li-Chan, 2015). Some databases that hold chemical information, properties and structures of molecules are PubChem, eMolecules, DrugBank, ChemSpider, and AAindex (Iwaniak et al., 2015).

The role of chemometrics in the discovery and characterisation of bioactive peptide has been demonstrated in a number of studies. The amino acid concentration of selected proteins can be determined using ProtParam, an in silico analysis program which computes the physicochemical properties of a peptide or protein from its amino acid sequence (Lafarga et al., 2015a). Chemometric analysis of the amino acid has also been done using PLS regression method to investigate antioxidant activities of food protein hydrolysates (Udenigwe and Aluko, 2011). QSAR studies have been used to identify the structural properties of peptides that is responsible for the biological properties of peptides. For example, the factors that determine the antioxidant activities of tuna protein hydrolysate fractions have been identified to be short peptide sequence (between tripeptides and hexapeptides), low molecular weight, the presence of hydrophobic and aromatic amino acid residues, and a peculiar order of amino acid sequences (Chi et al., 2015). A list of databases for chemometric characterisation of peptides is shown in Table 3.

**Table 1** Some databases of protein and peptide sequences

<i>Name of database/in silico tool</i>	<i>Description available on homepage</i>
BIOPEP <a href="http://www.uwm.edu.pl/biochemia/">http://www.uwm.edu.pl/biochemia/</a>	Database of protein and bioactive peptide sequences, allergenic peptides with their epitopes, and sensory peptides and amino acids
PepBank <a href="http://pepbank.mgh.harvard.edu/">http://pepbank.mgh.harvard.edu/</a>	Database of publicly available peptides (less or equal to 20 amino acids)
ACEpepDB <a href="https://www.re3data.org/repository/r3d100010972">https://www.re3data.org/repository/r3d100010972</a>	Database of antihypertensive peptides
AHTpin <a href="http://crdd.osdd.net/raghava/ahtpin/">http://crdd.osdd.net/raghava/ahtpin/</a>	Database for predicting and designing antihypertensive peptides
BioPD <a href="http://biopd.bjmu.edu.cn/">http://biopd.bjmu.edu.cn/</a>	Databases of mammalian (human, mouse and rat) bioactive proteins and peptide sequences
SwePep <a href="http://www.swepep.org/">http://www.swepep.org/</a>	Database of endogenous peptides from rat and mice
EROP-Moscow <a href="http://erop.inbi.ras.ru/">http://erop.inbi.ras.ru/</a>	Data bank of Endogenous Regulatory OligoPeptides (EROPs) of between 2 and 50 amino acid residues
MilkAMP <a href="http://milkampdb.org/">http://milkampdb.org/</a>	Database of microbiological and physicochemical data on milk-derived antimicrobial peptides
UniProtKB <a href="http://www.uniprot.org/">http://www.uniprot.org/</a>	Database of protein sequence and functional information
NCBI Protein database <a href="https://www.ncbi.nlm.nih.gov/protein/">https://www.ncbi.nlm.nih.gov/protein/</a>	Collection of protein sequences that determines biological structure and function
PeptideDB <a href="http://www.peptides.be/">http://www.peptides.be/</a>	Database of naturally occurring signalling peptides from animal sources
AMPer <a href="http://marray.cmdr.ubc.ca/cgi-bin/amp.pl">http://marray.cmdr.ubc.ca/cgi-bin/amp.pl</a>	Database and discovery tool for antimicrobial peptides
AHTPDB <a href="http://crdd.osdd.net/raghava/ahtpdb/">http://crdd.osdd.net/raghava/ahtpdb/</a>	Database of antihypertensive peptides
THPdb <a href="http://crdd.osdd.net/raghava/thpdb/index.html">http://crdd.osdd.net/raghava/thpdb/index.html</a>	Database of therapeutic peptides

**Table 2** Some databases for predicting protease specificity and biological property, as well as performing in silico digestion

<i>Name of database/in silico tool</i>	<i>Description available on homepage</i>
BIOPEP <a href="http://www.uwm.edu.pl/biochemia/index.php/en/biopep">http://www.uwm.edu.pl/biochemia/index.php/en/biopep</a>	Database of protein and bioactive peptide sequences, allergenic peptides with their epitopes, and sensory peptides and amino acids
PeptideCutter <a href="http://web.expasy.org/peptide_cutter/">http://web.expasy.org/peptide_cutter/</a>	In silico tool for predicting potential cleavage sites of proteases or chemicals in a protein sequence
POPS <a href="http://pops.csse.monash.edu.au/pops-cgi/index.php">http://pops.csse.monash.edu.au/pops-cgi/index.php</a>	In silico tool for modelling and profiling protease specificity and predicting substrate cleavage
NeuroPred <a href="http://neuroproteomics.scs.illinois.edu/neuropred.html">http://neuroproteomics.scs.illinois.edu/neuropred.html</a>	Database for predicting the cleavage sites and mass from neuropeptide precursors
PeptideRanker <a href="http://bioware.ucd.ie/~compass/biowareweb/">http://bioware.ucd.ie/~compass/biowareweb/</a>	Predicts the likelihood that a peptide will be bioactive
BIOPEP <a href="http://www.uwm.edu.pl/biochemia/index.php/en/biopep">http://www.uwm.edu.pl/biochemia/index.php/en/biopep</a>	Database of protein and bioactive peptide sequences, allergenic peptides with their epitopes, and sensory peptides and amino acids
AntiBP2 <a href="http://www.imtech.res.in/raghava/antibp2/">http://www.imtech.res.in/raghava/antibp2/</a>	Server for predicting antibacterial peptides
TumorHoPe <a href="http://crdd.osdd.net/raghava/tumorhope/">http://crdd.osdd.net/raghava/tumorhope/</a>	A database of tumor homing peptides
CPPpred <a href="http://bioware.ucd.ie/~compass/biowareweb/Server_pages/cpppred.php">http://bioware.ucd.ie/~compass/biowareweb/Server_pages/cpppred.php</a>	A server for predicting cell penetrating peptides
SYFPEITHI <a href="http://www.syfpeithi.de/">http://www.syfpeithi.de/</a>	A database of peptide sequences known to bind class I and class II MHC (major histocompatibility complex) molecules

**Table 3** Some databases or in silico tools for chemometric characterisation of peptides

<i>Name of database/in silico tool</i>	<i>Description available on homepage</i>
PubChem <a href="https://pubchem.ncbi.nlm.nih.gov/">https://pubchem.ncbi.nlm.nih.gov/</a>	Database of chemical molecules and their activities against biological assays
ProtParam <a href="https://web.expasy.org/protparam/">https://web.expasy.org/protparam/</a>	An in silico tool which allows the computation of various physical and chemical parameters for a given protein stored in Swiss-Prot or TrEMBL or for a user entered protein sequence
ChemSpider <a href="http://www.chemspider.com/">http://www.chemspider.com/</a>	Database of chemical structures
AAindex <a href="http://www.genome.jp/aaindex/">http://www.genome.jp/aaindex/</a>	Database of numerical indices representing various physicochemical and biochemical properties of amino acids and pairs of amino acids
eMolecules <a href="https://www.emolecules.com/">https://www.emolecules.com/</a>	A free search engine of chemical structures
DrugBank <a href="https://www.drugbank.ca/">https://www.drugbank.ca/</a>	Database that provides bioinformatics and cheminformatics information on compounds (i.e. drugs and their target molecules)
BindingDB <a href="http://www.bindingdb.org/bind/index.jsp">http://www.bindingdb.org/bind/index.jsp</a>	Database of binding affinities between protein/peptide-based drug targets and small drug-like molecules
Common Chemistry <a href="http://commonchemistry.org/">http://commonchemistry.org/</a>	Database of common chemical structures
ChemSynthesis <a href="http://www.chemsynthesis.com/">http://www.chemsynthesis.com/</a>	Freely accessible database of chemicals
Human Metabolome Database (HMDB) <a href="http://www.hmdb.ca/">http://www.hmdb.ca/</a>	A database of information about small molecule metabolites found in the human body
ChemAxon <a href="https://www.chemaxon.com/">https://www.chemaxon.com/</a>	Platform for cheminformatics software, applications and services
ZINC15 <a href="http://zinc15.docking.org/">http://zinc15.docking.org/</a>	Free database of commercially-available compounds for virtual screening
FoodDB <a href="http://foodb.ca/">http://foodb.ca/</a>	A comprehensive online resource on food constituents, chemistry and biology
Chemical Entities of Biological Interest (ChEBI) <a href="https://www.ebi.ac.uk/chebi/">https://www.ebi.ac.uk/chebi/</a>	Electronic dictionary of molecular entities focused on small chemical compounds

### Databases for Predicting Tastant Peptides

The basic taste sensations associated with food components are sweet, sour, salty, bitter and umami. Among these, sweet, bitter, and umami have been linked to the peptide components of some foods (Iwaniak et al., 2016a). Taste-provoking peptides are called peptide tastants, and a number of research studies have shown that most bioactive peptides reported in the literature exhibit one or more of the aforementioned tastes (Cheung and Li-Chan, 2010; Cheung et al., 2015; Li-Chan, 2015). But bitterness is often distinctively avoided and thought to be a protective mechanism against consuming toxics (Breslin, 2013). Many clinical drugs cause bitter tastes which presents a major compliance problem for pediatric drugs (Bahia et al., 2017; Dagan-Wiener et al., 2017). Prediction of bitterness is important in food industry because, for the most part, consumers avoid bitter foods no matter how healthy or rich in bioactive components (Drewnowski and Gomez-Carneros, 2000). Prediction of bitterness is also important as it assists in the development of bitterness masking products and procedures (Dagan-Wiener et al., 2017). Bitterness is recognized in human by 25 G-protein-coupled receptors of hTAS2R gene family. These receptors are expressed in the oral cavity, the gastrointestinal tract, the upper airways, and the heart and in other tissues (Bahia et al., 2017; Dagan-Wiener et al., 2017; Li-Chan, 2015; Wiener et al., 2012). Efficient tools for predicting, measuring and masking bitterness of ingredient is often sought after by pharmaceutical and food industries. The chemical diversity of bitter compounds, and occurrence of minor differences between bitter and non-bitter compounds are the main challenge in computational bitterness prediction. Ligand-based methods, structure-based methods, and machine learning techniques are few models used in bitterness prediction. Consequently, most of the predictive models focus on specific families of bitter compounds such as cynaropicrin and grosheimin derivatives, and benzenesulfamates (Dagan-Wiener et al., 2017). However, previous studies have proposed a correlation between hydrophobicity and bitterness. For example, bitterness of the soy peptides is predominantly associated with molecular mass (Wiener et al., 2012). Chemical structure-based computational prediction approach were used in BitterDB, a database of bitter compounds. It includes structurally diverse bitter compounds such as ions, peptides, alkaloids, polyphenols, glucosinolates (Dagan-Wiener et al., 2017). The BIOPEP database also has a built-in function that gives information about the sensory properties of peptides (Iwaniak et al., 2016b). This function is accessible by clicking the "Sensory Peptides and amino acids" link on the database homepage. Other databases for predicting taste-evoking properties of peptides is given in Table 4.

**Table 4** Some databases or in silico tools for predicting tastant properties of peptides and amino acids

<i>Tastant prediction</i>	
BIOPEP <a href="http://www.uwm.edu.pl/biochemia/index.php/en/biopep">http://www.uwm.edu.pl/biochemia/index.php/en/biopep</a>	Database of protein and bioactive peptide sequences, allergenic peptides with their epitopes, and sensory peptides and amino acids
BitterDB <a href="http://bitterdb.agri.huji.ac.il/dbbitter.php">http://bitterdb.agri.huji.ac.il/dbbitter.php</a>	A database of bitter compounds
BitterPredit <a href="http://bitterdb.agri.huji.ac.il/dbbitter.php#BitterPredit">http://bitterdb.agri.huji.ac.il/dbbitter.php#BitterPredit</a>	A platform for the structure-based prediction of bitterness of compounds
BitterX <a href="http://mdl.shsmu.edu.cn/BitterX/">http://mdl.shsmu.edu.cn/BitterX/</a>	A web server on bitter compound, their identification, and prediction (especially in the case of small molecule compounds)

### Databases for Allergenicity and Toxicity Prediction

Food allergies are a major health concern in the development of food products, and recent studies show that cases of food allergies in developed countries are on the rise (Prescott et al., 2013; Tang and Mullins, 2017). The detection and elimination of food-derived allergens is therefore of prior concern to the food technologists. Since almost all allergenic compounds in food are protein in nature (Verhoeckx et al., 2015), it is important to study the allergenic potential of bioactive peptides, or use peptides and protein hydrolysates as a molecular marker for the detection of food allergens. Further, being able to predict the potential toxicity of peptides saves time, money and effort in the development of peptide-based functional foods and pharmaceuticals. The importance of this is highlighted by the fact that the WHO and FAO have proposed guidelines for the studying and predicting the allergenic potential of food proteins (FAO/WHO, 2001).

Predicting whether a food peptides could be toxic or allergenic is made possible because the sequences and structures of several allergenic or toxic proteins is already known. The bioinformatics approach for predicting toxic or allergenic peptides relies on identifying structural similarities and sequence identity of the peptide with those of known allergenic protein/peptide epitopes or toxic regions in protein sequences (Dziuba et al., 2013; Gupta et al., 2013; Schein et al., 2007). These in silico tools allow the prediction of allergenic peptides by one or more of the following approaches: mapping IgE epitopes; searching for allergenic motifs using motif-based sequence analysis tools such as MAST (motif alignment and search tool) and Multiple EM for Motif Elicitation (MEME); support vector machine modelling of the composition of amino acids and (di)peptides; and Basic Local Alignment Search Tool (BLAST) searches of allergen-representative peptides (ARPs). The accuracy of prediction is enhanced by using a hybrid approach which combines one or more of the above approaches.

In silico prediction of allergenic peptides has been studied in a number of food products, including a tryptic digests of the marine bivalve *Ruditapes philippinarum* using AlgPred. Out of 966 peptides generated, 510 and 31 peptides were identified to be allergens or potential allergens respectively (Yu et al., 2017). Further, the toxicity of the meat-derived prolyl endopeptidase inhibitors has been studied with ToxinPred (Lafarga et al., 2015a). Table 5 gives a list of other databases or in silico tools for predicting toxicity and allergenicity in peptides.

### Challenges With the Use of Bioinformatics in Bioactive Peptides Discovery

Despite the prospects discussed above, there are some limitations to the use of bioinformatics in analysing food proteins towards bioactive peptide discovery. First, during in silico analysis, it is assumed that the structure of the protein substrate is linear (primary structure), and that the selected protease will cleave every possible peptide bond that it can to release a repertoire of peptides, some of which can be bioactive. This approach does not consider the 3-dimensional structure of proteins, stabilized by covalent and non-covalent bonds, which would create a sort of hindrance that limits protease accessibility to peptide bonds in the protein core. We found that this issue led to the generation of more peptides in silico than experimentally during potato protein hydrolysis (Rajendran et al., 2016). Moreover, the effect of food processing on the structural integrity of the protein prior to hydrolysis is unaccounted for during in silico digestion. For instance, protein aggregation can occur during heating and high-pressure treatments, and this can perturb protein-protease interactions and limit protein hydrolysis. Our peptidomics study also noted the occurrence of post-translational modifications (PTMs) in peptides generated by protein hydrolysis (Rajendran et al., 2016). The PTMs include methionine oxidation and glutamine/asparagine deamidation. Based on fundamental chemistry, the peptides are also susceptible to undergo other PTMs e.g. dehydration, acetylation, pyro-glutamate formation, trimethylation, etc. This factor is important because the PTMs can alter the structure, function/bioactivity, and bioavailability of the peptides. While it is possible to map the amino acid residues susceptible to PTMs in silico, currently available bioinformatics tools cannot predict their occurrence during protein hydrolysis. This makes it challenging to predict accurately the nature of the released peptides using bioinformatics. Finally, while comprehensive databases exist for some protein sources (e.g. bovine milk), proteins from non-traditional sources such as microalgae and



**Table 5** Some databases or in silico tools for predicting toxicity and allergenicity in peptides

<i>Name of database/in silico tool</i>	<i>Description available on homepage</i>
ToxinPred <a href="http://www.imtech.res.in/raghava/toxinpred/">http://www.imtech.res.in/raghava/toxinpred/</a>	In silico tool to predict and design toxic/non-toxic peptides
BIOPEP <a href="http://www.uwm.edu.pl/biochemia/index.php/en/biopep">http://www.uwm.edu.pl/biochemia/index.php/en/biopep</a>	Database of protein and bioactive peptide sequences, allergenic peptides with their epitopes, and sensory peptides and amino acids
AlgPred <a href="http://www.imtech.res.in/raghava/algpred/">http://www.imtech.res.in/raghava/algpred/</a>	Plat-form for predicting allergenic proteins and peptides and mapping immunoglobulin E epitopes
Allerdictor <a href="http://allerdictor.vbi.vt.edu/">http://allerdictor.vbi.vt.edu/</a>	A sequence-based allergen prediction tool
SORTALLER <a href="http://sortaller.gzhmu.edu.cn/">http://sortaller.gzhmu.edu.cn/</a>	An online allergen classifier
ProPepper™ <a href="https://propepper.net/">https://propepper.net/</a>	Database of cereal prolamin epitopes, peptides and proteins
IEDB <a href="http://www.iedb.org/">http://www.iedb.org/</a>	Database characterizing antibody and T cell epitopes studied in humans, non-human primates, and other animal species
SDAP <a href="http://fermi.utmb.edu/SDAP/">http://fermi.utmb.edu/SDAP/</a>	Web server or database of allergenic proteins
WebAllergen <a href="http://weballergen.bii.a-star.edu.sg/">http://weballergen.bii.a-star.edu.sg/</a>	Web server for predicting allergenic proteins
AllerTOP <a href="http://www.ddg-pharmfac.net/AllerTOP/">http://www.ddg-pharmfac.net/AllerTOP/</a>	Bioinformatics tool for allergenicity prediction
PREAL <a href="http://gmobl.sjtu.edu.cn/PREAL/index.php">http://gmobl.sjtu.edu.cn/PREAL/index.php</a>	Web application for predicting allergenic proteins
EVALLER <a href="http://bioinformatics.bmc.uu.se/evaler.html">http://bioinformatics.bmc.uu.se/evaler.html</a>	A web server for in silico assessment of potential allergenicity in proteins

edible insects are yet to be completely identified, and many of their sequences are currently not available in protein databases. This limits the exploration of such proteins, commonly regarded as sustainable, as precursors of bioactive peptides, which would potentially leading to missed opportunities.

## Concluding Remarks

The traditional means of bioactive peptide discovery for food applications is labour intensive and often involves working with a few protein-protease sample sets at a time, with limited knowledge of structure-function relationships. Bioinformatics offers a unique approach that can rapidly screen many proteins for bioactive motifs, with detailed information on their structural properties. Although with limitations, the ability to predict undesirable features, such as peptide bitterness and allergenic epitopes, and rank a wide range of food proteins using the in silico approach facilitates the selection of top candidates for bioactive peptide discovery. Furthermore, the bioinformatics method can result in the rapid discovery of new bioactive peptides that would otherwise require lengthy procedures to accomplish. Future studies need to address the challenges discussed in this paper in order to strengthen the use of bioinformatics in food-derived bioactive peptide research.

## Relevant Websites

For list of relevant websites see **Tables 1–5**.

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# Thermal Analysis for Lipid Decomposition by DSC and TGA

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## Introduction

Lipids are one of the macronutrients in food and play an important role in the food system such as texture, flavour and taste. Lipids can be in the solid (fat) or liquid (oil) forms at ambient temperature, soluble in the organic solvents and insoluble in water. The major component of lipids is triglycerides that consist of glycerol and fatty acids. Previous studies have shown that omega-3 fatty acids are beneficial to human health such as reducing the risk of cardiovascular disease, cancers, inflammatory disorders, cognitive decline and Alzheimer's disease (Holub, 2009; Calder, 2006; Mandal et al., 2010; Cockbain, 2012; Morris et al., 2005). Fish oil and omega-3 fatty acids concentrates have been incorporated into the food products and marketed as functional foods. However, these foods are more susceptible to oxidation due to the high polyunsaturated fatty acids content in them.

Lipid oxidation can occur in the presence of oxygen, light, temperature, moisture and trace metals. Lipid oxidation will decrease the food quality, stability, safety and nutritive value of the food (Luther et al., 2007) as it produces unpleasant off-flavours and unhealthy compounds such as free radicals and reactive aldehydes (Jacobsen et al., 2008). It also gives a negative impact on sensory and customer acceptance to the food products fortified with fish oil (Sullivan et al., 2011; Dekker et al., 2012). Humans have a low sensory threshold for volatile off-flavours resulting from oxidation of omega-3 fatty acids, therefore oxidised food products are less acceptable to the consumers (Frankel, 2005). Furthermore, consumption of high amounts of oxidation products can cause oxidative stress in the human body, which may increase the risk of prevalence of cancer, coronary heart disease and other diseases (Frankel, 2005).

Oxidative stability is an important quality control parameter for the manufacturers and users of many different types of commercial materials including edible fats and oils (Hassel, 1976). Fish oil is susceptible to oxidation due its high polyunsaturated fatty acid (PUFA) content. Thus, oxidative and thermal stability of fish oil are important parameters in the processing and production of fish oil and fish oil-fortified food products (Tengku-Rozaina and Birch, 2016). Autoxidation is the most common oxidation that leads to oxidative deterioration of lipids in the presence of oxygen (Shahidi and Zhong, 2005). Oxidation of lipids at high temperature is known as thermal oxidation.

## Lipid Oxidation (Autoxidation)

Lipid oxidation (autoxidation) is a free radical chain reaction and occurs in three steps: initiation, propagation and termination (Fig. 1). According to Shahidi and Wanasundara (2002), initiation of lipid oxidation begins with abstraction of a hydrogen atom adjacent to a double bond in a fatty acid (RH), to form an alkyl free radical (R<sup>•</sup>). The free radical will then react with atmospheric oxygen to produce a peroxy radical (ROO<sup>•</sup>) and sequester a hydrogen atom from another unsaturated fatty acid (RH) to form a hydroperoxide (ROOH) and a new alkyl radical (R<sup>•</sup>). Further oxidation is initiated by the new alkyl radical that contributes to the chain reaction (propagation). The chain reaction will be terminated when two radical species are combined to produce non-radical products.

Hydroperoxides are the primary oxidation products produced at the beginning of oxidation. These products are stable at room temperature but decompose at elevated temperatures, or in the presence of metals, or when exposed to light (Frankel, 2005; Choe

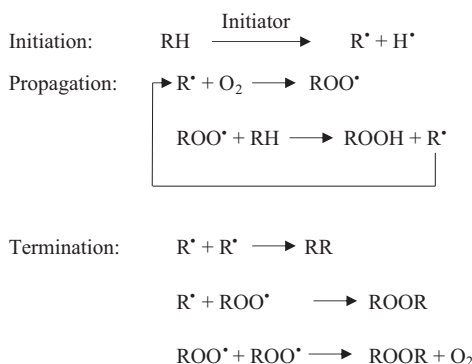


Figure 1 Mechanism of lipid oxidation.

and Min, 2006). The breakdown or decomposition of hydroperoxides leads to the formation of secondary oxidation products such as aldehydes, ketones, acids, esters, alcohols and short chain hydrocarbons (Choe and Min, 2006).

### Conventional (Physical and Chemical) Methods of Lipid Oxidation Measurement

Analysis of lipid oxidation can be carried out through chemical, physical, instrumental and sensory methods. Among the common chemical methods are peroxide value (PV), p-anisidine value (p-AnV), free fatty acid (FFA), total polar compounds (TPC) and total oxidation (TOTOX) value. PV and p-AnV are two common oxidation methods that have been used to measure the oxidative stability of oils. PV is a measurement for primary oxidation compounds (hydroperoxides) in the oil and can be carried out by an iodometry technique. This technique is based on the reduction of the hydroperoxide group (ROOH) with iodide ion ( $I^-$ ). The concentration of peroxide is proportional to the liberated iodine ( $I_2$ ) from potassium iodide (Shahidi and Wanasundara, 2002). Since hydroperoxides are produced in the beginning of oxidation and then decomposed to produce secondary oxidation products, the PV may not represent the actual oxidation stage of the oil.

p-AnV is a measurement for secondary oxidation products produced during oxidative degradation of oil. Aldehydes, will react with p-anisidine reagent under acid conditions and produce yellowish products (Shahidi and Wanasundara, 2002). p-AnV is useful in determination of the quality of crude oils and the efficiency of the processing procedure (O'Brien, 2004). The TOTOX value has been carried out extensively to estimate oxidative deterioration of food lipids (Rossell, 1983) as it combines the evidence about the past history (as reflected in the p-AnV) and the present state (as evidenced in the PV) of an oil (Shahidi and Wanasundara, 2002).

Physical methods of lipid rancidity are refractometry, specific density, weight gain, dielectric constant, viscosity and others (Shahidi and Zhong, 2005). Refractive index is a measurement of the change in unsaturation of fat or oil during hydrogenation (O'Brien, 2004) or onset of rancidity (Arya et al., 1969). The value for refractive index increases during autoxidation possibly due to conjugation and polymerisation of partially oxidised oils and fats (Arya et al., 1969).

Analysis of weight gain can be carried out by heating the oil in an oven. The oil is then taken out from the oven, cooled and weighed. This method can be used to measure the induction period of the oil sample and prediction of shelf life (Shahidi and Zhong, 2005). However, this method has several disadvantages since the heating is not continuous and the results may not be reproducible, require intensive labour and is time consuming (Shahidi and Zhong, 2005). Taking this limitation into consideration, Antolovich et al. (2002) suggested the instrumental techniques, namely Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA) as replacement for the conventional weight gain method.

### Instrumental Methods of Lipid Oxidation

The instrumental methods are less tedious and hazardous compared to the conventional physical and chemical methods. The commercial instruments used to measure oxidative stability of fats and oils are Rancimat and Oxidative Stability Instrument (OSI) (Tan et al., 2002). Other instrumental methods in assessing lipid oxidation in fats and oils are by Nuclear Magnetic Resonance (NMR), Fourier Transform Infrared (FTIR), Active Oxygen Method (AOM) and gas chromatography (GC). Nowadays, Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA) are two thermal analysis methods that have gained popularity due to their simplicity and rapid operation, requirement of a small sample size and no chemical or solvent use.

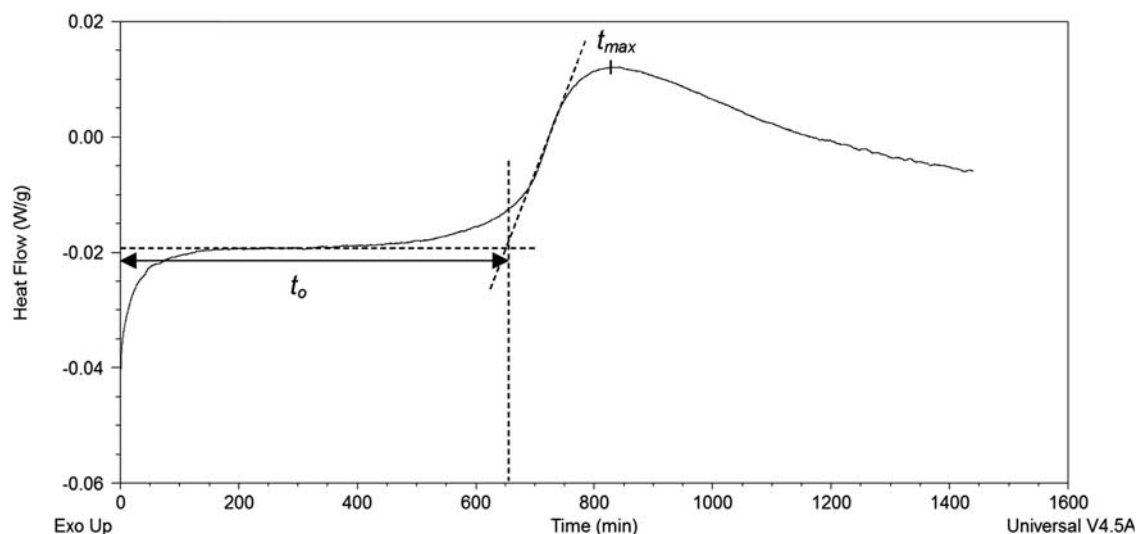
Oxidative stability of fats and oils measurement by DSC and TGA can be carried out by isothermal or non-isothermal operation. DSC and TGA have been used in previous studies on oxidative stability analysis of vegetable oils (Cross, 1970; Mikula and Khayat, 1985; Tan and Che Man, 2002; Arain et al., 2009; Ostrowska-Ligeza et al., 2010; Pardaul et al., 2011) and fish oils (Tengku-Rozaina and Birch, 2016; Sathivel et al., 2003; Sathivel, 2005; Huang and Sathivel, 2008; Araujo et al., 2011). Previous studies on oxidative stability of vegetable oils showed a good correlation between DSC and AOM (Cross, 1970), Rancimat (Arain et al., 2009; Ostrowska-Ligeza et al., 2010) and OSI (Pardaul et al., 2011) methods. DSC (Shahidi and Zhong, 2005; Arain et al., 2009; Ostrowska-Ligeza et al., 2010; Pardaul et al., 2011; Tan et al., 2001; Litwinienko, 2005) and TGA (Mikula and Khayat, 1985) are reported as rapid methods for analysis of lipid oxidation.

### Thermal Analysis of Lipid Oxidation

#### Oxidative Stability by DSC and TGA (Isothermal)

DSC is the most frequently (70%) used instrument for thermal analysis (Thomas and Schmidt, 2017). Oxidative stability and shelf life analysis by the DSC technique can be carried out in a short time (a day) instead of a few months by other methods (Cipriotti and Chiavaro, 2015). Furthermore, Hassel (1976) stated that measurement of oxidative stability of oil samples can be carried out in less than 4 hours by DSC compared to 14 days by the Active Oxygen Method (AOM).

The DSC technique measures the heat flows during a sample phase transition (Tan et al., 2002) or thermal changes during oxidation reactions (Shahidi and Zhong, 2005), as a function of time and temperature. Measurement of oxidative stability of fats and oils by the DSC technique is based on the thermal release of oxidation products associated with temperature and time (Tan and Che

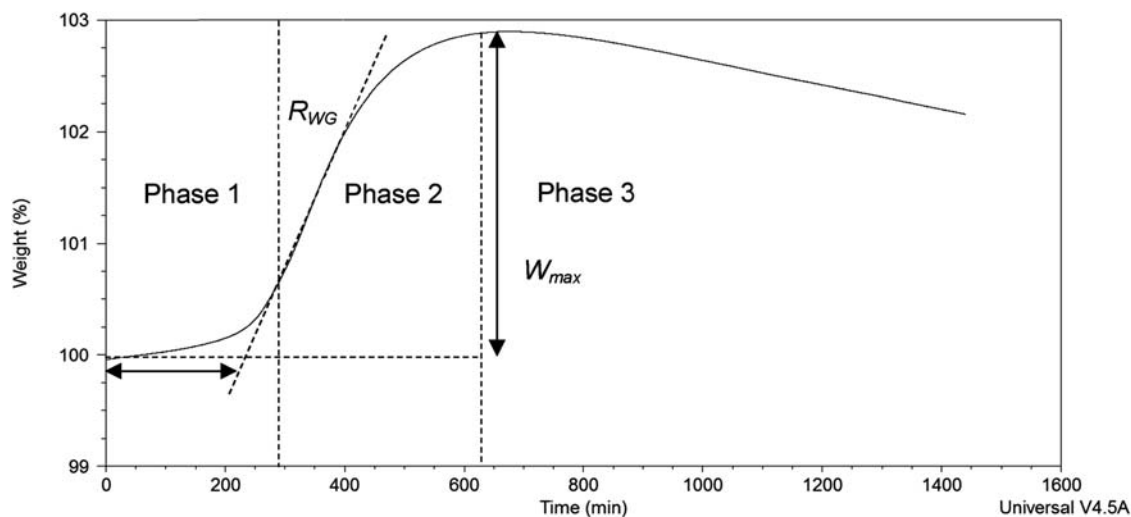


**Figure 2** DSC curve of hoki oil during isothermal heating.

Man, 2002). Oxygen (purge gas) or air atmosphere are used with a calorimeter to measure the heat flow into (endothermic) or out of (exothermic) an oil sample undergoing oxygen changes. On the other hand, TGA measures the weight changes of a sample as a function of temperature and time during heating. Therefore, it can be used to measure the oxidation process through weight gain of the sample or oxygen uptake during decomposition (Hassel, 1976).

The onset time or induction period measured by TGA and DSC indicates the beginning of lipid oxidation and change in oil quality, respectively (Wesolowski and Erecinska, 1998). In the DSC oxidative analysis, the onset time for oxidation ( $t_o$ ) can be measured by intersection of extrapolated baseline and tangent line (leading edge) of the exotherm (Tan et al., 2002) as shown in Fig. 2. Time for maximum reaction (heat flow) was recorded as  $t_{max}$  (Tengku-Rozaina and Birch, 2016). Meanwhile, the onset time for oxidation analysis by TGA can be measured from the extrapolation of the baseline and upward portion of the curve (Mikula and Khayat, 1985) as shown in Fig. 3. The maximum rate of weight gain ( $R_{WG}$ ) of the TGA was determined from the slope of an upward portion of the curve (Mikula and Khayat, 1985). The onset time is related to the stability of the sample. In both DSC and TGA, a sample with a higher onset time is more stable compared to a sample with a lower onset time.

Oxidation of the oil occurs in three stages as illustrated by the TGA curve (Fig. 3). Phase 1 is the beginning of oxidation and the quality of the oil is measured by the induction period (IP) or onset time for oxidation ( $t_o$ ), where the oil is resistant to oxidation (Mikula and Khayat, 1985). Oil that has a higher IP is more resistant to oxidation. In phase 1, a weight change is minimal. Phase 2 is an autocatalytic oxidation phase where the sample gains weight due to formation of hydroperoxides as indicated by PV analysis. The final stage of oxidation occurs in phase 3 where weight loss is observed. In this phase, hydroperoxides start to decompose and volatile compounds such as aldehydes, ketones and alcohols are produced.



**Figure 3** TGA curve of hoki oil during isothermal heating.

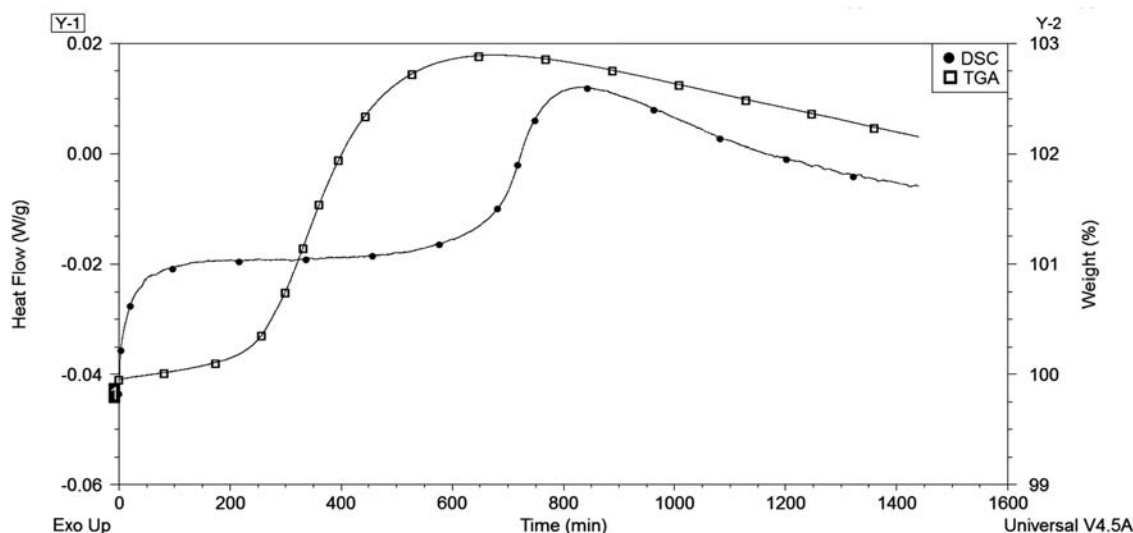


Figure 4 Oxidative stability of hoki oil by DSC and TGA.

The trend for time ( $t_{max}$ ) for maximum thermal decomposition (DSC) and weight gained (TGA) of oil was similar to that of its onset time ( $t_o$ ), where the  $t_{max}$  for TGA was earlier than the  $t_{max}$  for DSC (Fig. 4).

#### Effects of Changing the Isothermal Temperature on Oxidative Stability of Oil

DSC and TGA can also be used to measure effects of a change in isothermal temperature on oxidative stability of the oil. Tengku-Rozaina and Birch (2016) studied the effects of oxidative stability of hoki oil by DSC (Fig. 5). They (Tengku-Rozaina and Birch, 2016) found that the onset time for oxidation ( $t_o$ ), time for maximum weight gained ( $t_{max}$ ) and time for maximum rate of thermal decomposition ( $t_{mdr}$ ) of hoki oil were inversely correlated with heating temperature. A similar finding was reported in the previous studies on vegetable oils by DSC (Tan et al., 2001, 2002; Pardaul et al., 2011). Cross (1970) reported a good correlation coefficient ( $r = 0.974$ ) between a conventional AOM and DSC, indicating that DSC is a rapid method to measure oils stability. Arain et al. (2009) also reported a good correlation ( $r > 0.99$ ) on the oxidative stability of plant oils measured by DSC and Rancimat.

Effects of temperature on the oxidative stability of hoki oil by TGA were carried out by Tengku-Rozaina and Birch (2016) as shown in Fig. 6. They (Tengku-Rozaina and Birch, 2016) found that the times recorded for  $t_o$ ,  $t_{max}$ ,  $t_{mdr}$ , and maximum weight gained ( $W_{max}$ ) of hoki oil were inversely correlated with heating temperature. Their findings are in agreement with Frankel (2005) who suggested that the oxidation rate of lipid is exponentially associated with temperature. The hydroperoxide formation and the decomposition of hoki oil reached an equilibrium level faster as the temperature increased (Tengku-Rozaina and Birch, 2016) and in accordance with a PV plot against time at different heating temperatures for linseed oil (Hess and O'Hare, 1950). On the

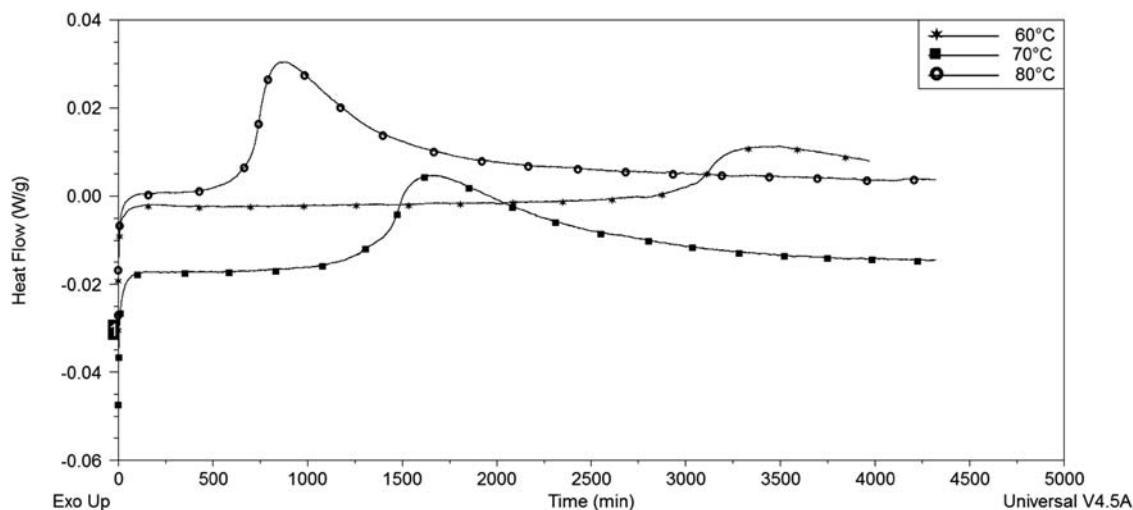
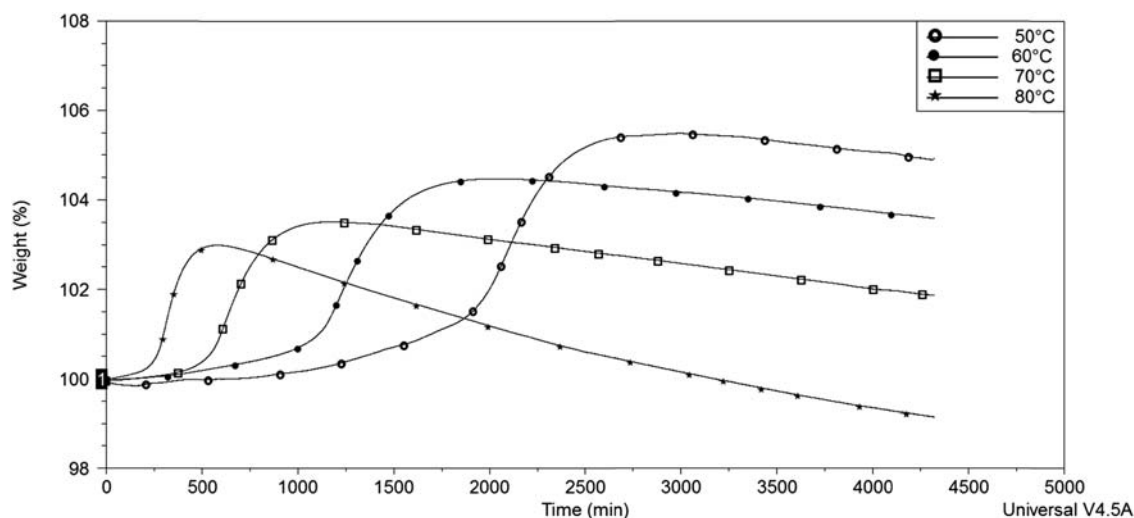


Figure 5 Oxidative stability of hoki oil at different temperatures measured by DSC.





**Figure 6** Oxidative stability of hoki oil at different temperatures measured by TGA.

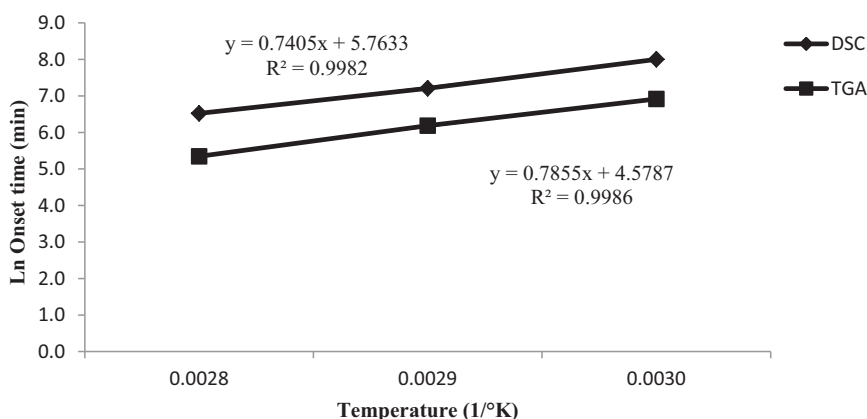
other hand, the maximum rate of weight gained ( $R_{WG}$ ) increased with increasing temperature. The results of the effect of temperature on oxidative stability of hoki oil in [Tengku-Rozaina and Birch \(2016\)](#) are in accordance with [Mikula and Khayat \(1985\)](#) findings on soybean oil.

The maximum weight gained ( $W_{max}$ ) for hoki oil decreased with heating temperature since hydroperoxides do not accumulate but rapidly decompose at high temperatures. This finding is supported by an earlier  $t_{max}$  of hoki oil at a higher heating temperature compared to the lower temperature. The hydroperoxides started to decompose after they reached the  $t_{max}$  and the secondary oxidation products such as aldehydes, ketones and alcohol were produced ([Tengku-Rozaina and Birch, 2016](#)).

Both DSC and TGA data in [Tengku-Rozaina and Birch \(2016\)](#) study on the effects of temperature on oxidative stability of hoki oil follow the  $Q_{10}$  law on the relationship between temperature and rate of chemical reaction. The  $t_o$ ,  $t_{max}$  and  $t_{mdr}$  of hoki oil were reduced by approximately half with an increase of 10 °C. A similar finding on reduction of onset time for oxidation ( $t_o$ ) of vegetable oils measured by DSC was reported in previous studies ([Tan et al., 2002](#); [Arain et al., 2009](#); [Pardaul et al., 2011](#)).

### Prediction of Shelf Life of Oil by DSC and TGA

DSC and TGA can also be used as accelerated heating methods to replace conventional heating methods to predict the shelf life of the oils. Prediction of shelf life of the oils is possible by an Arrhenius extrapolation from elevated isothermal DSC and TGA analyses. The data of onset time for oxidation ( $t_o$ ) or induction period are used in plotting an Arrhenius plot to predict shelf life of the oil. [Tengku-Rozaina and Birch \(2016\)](#) predicted the shelf life of hoki oil at lower temperature using onset time for oxidation data obtained via DSC and TGA by construction of Arrhenius plots ([Fig. 7](#)) according to equations ([SETARAM, 2010](#)) as follows:



**Figure 7** The Arrhenius plot for oxidation and decomposition onset times of hoki oil by DSC and TGA.

$$\ln \text{ Onset} = A(1/T) + B$$

$$E_a = A \times R$$

where:  $T$  = temperature ( $^{\circ}\text{K}$ );  $A$  = slope of an Arrhenius plot;  $E_a$  = activation energy;  $R$  = gas constant ( $8.314 \text{ J/mol}^{\circ}\text{K}$ ).

Activation energy ( $E_a$ ) of the oil also can be measured by DSC and TGA through the Arrhenius equation. The  $E_a$  value is one of the important parameters in characterisation of oils since it indicates the delay of the initial oxidation process (Farhoosh et al., 2008). The  $E_a$  for oil that has a high polyunsaturated fatty acid content is lower than the oil with a higher saturated fatty acid content (Adhvaryu et al., 2000).

### Thermal Decomposition of Oil by TGA (Non-isothermal)

Studies on the effects of programmed heating (non-isothermal) on fats and oils can provide information on thermal behaviour and stability of the fats and oils during processing and the processing method employed. Thermal stability of the oils is related to their fatty acid analysis and antioxidants present (Santos et al., 2002). Previous researchers have studied the thermal behaviour of vegetable (Santos et al., 2002, 2004; Dweck and Sampaio, 2004; Szabo et al., 2012) and fish oils (Tengku-Rozaina and Birch, 2016; Araujo et al., 2011) during non-isothermal heating by TGA.

Fig. 8 illustrates the weight loss and the rate of weight loss during thermal decomposition process for hoki oil heated from  $25^{\circ}$  to  $700^{\circ}\text{C}$  by TGA in Tengku-Rozaina and Birch (2016) study. Data on the percentage of weight changes were obtained from the TA Universal Analysis and further analysed using a Microsoft Excel spread sheet. A slight increase (0.1%) in the initial sample weight was observed in hoki oil at temperature between  $100^{\circ}\text{C}$  and  $150^{\circ}\text{C}$  which showed the beginning of oxidation as the oil gained weight due to oxygen uptake, and formation of hydroperoxides (Tengku-Rozaina and Birch, 2016). An increasing weight loss of hoki oil due to temperature increase was observed after this temperature range. A similar finding was observed in previous works on unrefined tuna oil (Tengku-Rozaina and Birch, 2016), pink and red salmon oils (Sathivel, 2005), unrefined salmon oil (Huang and Sathivel, 2008) and unrefined pollock oil (Sathivel et al., 2008).

The oil with a high saturated fatty acid content is more resistant to heat and formation or build up of polymer (Tengku-Rozaina and Birch, 2016). Furthermore, the length and degree of unsaturation and intermolecular dispersion forces influenced heat decomposition of fatty acids (Sathivel et al., 2003). Fatty acids with a longer carbon chain are more stable toward thermal decomposition than a short chain fatty acid due to an increase of intermolecular dispersion forces (Nawar, 1996).

Thermal decomposition of hoki oil occurred in three stages or temperature ranges, possibly related to degradation of polyunsaturated fatty acids, monounsaturated fatty acids, saturated fatty acids, followed by volatilization of polymerisation and pyrolysis products (Tengku-Rozaina and Birch, 2016). The onset time for thermal decomposition was extrapolated from the thermal decomposition curve (Dweck and Sampaio, 2004). A higher value of the initial decomposition temperature implies better quality of oil (Wesolowski and Erecinska, 1998).

Thermal decomposition of oils is related to their fatty acid contents and positional distribution. For example, thermal decomposition of tuna oil occurred at lower temperature compared to hoki oil as observed in Tengku-Rozaina and Birch (2016). This is due to tuna oil having a higher PUFA content which makes it more susceptible to oxidation compared to hoki oil (Tengku-Rozaina and Birch, 2014). However, tuna oil was more stable at higher temperature due to its higher saturated fatty acid content, and

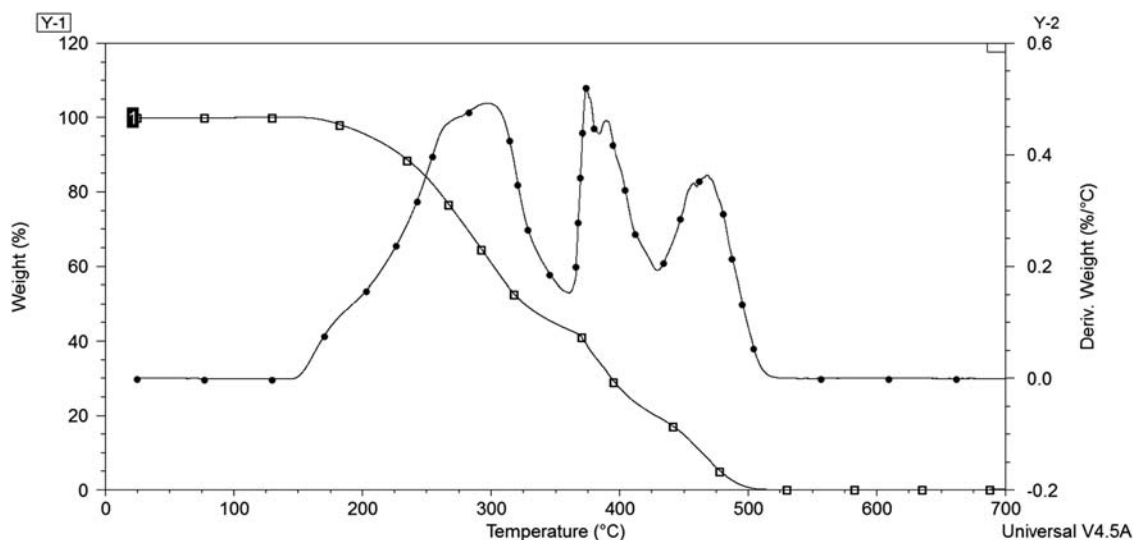


Figure 8 Thermal decomposition of hoki oil.

positional distribution of docosahexaenoic acid (DHA). Tuna has more DHA in the *sn*-2 compared to *sn*-1,3 positional distribution (Tengku-Rozaina and Birch, 2014) which makes it more stable to oxidation. This is in accordance with Wijesundera et al. (2008) who stated that PUFA are more stable to oxidation when located a *sn*-2 position compared to *sn*-1,3 position.

## Conclusion

DSC and TGA can be used as rapid methods to assess oxidative stability of oils and to predict shelf life of the oil using Arrhenius extrapolation from elevated isothermal analyses. Both DSC and TGA elevated isothermal analyses data showed the effects of temperature on oxidative stability follow the  $Q_{10}$  law on the relationship between temperature and rate of chemical reaction. DSC data on the oxidative stability of oils correlated well with other instrumental methods such as Active Oxygen Method, Rancimat, and Oxidative Stability Instrument (OSI). The DSC and TGA methods are faster, require less sample size and no chemicals or solvents compared to other conventional and modern oxidative stability methods and conventional shelf life estimation.

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# Pyrazines in Thermally Treated Foods

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## Introduction

Pyrazines is a family of volatile compounds with a common chemical structure that includes a monocyclic aromatic ring with two nitrogen atoms in para position. Since different groups (alkyl, methoxy or sulfur-containing chains) may be incorporated at 2-, 3-, 5- and 6- positions, a wide range of pyrazines may be present in foodstuff. The main source of food pyrazines is their formation during thermal treatments of raw foods. However, some pyrazines appear in nature as products of secondary plant metabolism and as metabolites of different microorganism (Gronquist and Schroeder, 2010). Generally, these compounds are methoxypyrazines which are very stable and, although they are masked by the formation of other volatile during food processing, they may cause serious problems in the quality of the final products. The role in food of methoxypyrazines is not discussed in the present work.

Pyrazines usually present a low vapor pressure and low values of odor thresholds (concentration at which they are detected). They largely impact flavor profile and give foods a very specific organoleptic characteristics mainly associated to roasted and nutty aromatic notes, although some of them also can give unpleasant green and burnt notes (Wang et al., 1968, 2017).

The first found papers about pyrazines in foods were published in the 1960s. They mainly focused on the effect of pyrazines on the flavor of different food matrices as cocoa, coffee and peanuts among others (Mason et al., 1966; Rizzi, 1967; Bondarovich et al., 1967; Wang et al., 1968). From these years, papers dealing with food pyrazines have been continuously published, although with variable ranges among years. In a basic search using "food pyrazines" as topic into the Web of Science data base, 1371 works published from 1965 to 2017 are found. Probably, the correlation of pyrazines with sensory properties of foods has been the most extensively studied topic. In this way, basic search using "pyrazines" and "food flavor" indicated 635 papers; using "pyrazines" and "food aroma" 521 papers, and 401 papers were found using "pyrazines" and "food quality". According to these results, many papers showed information about analytical methods (sample treatment, extraction and isolation techniques, etc.) and about parameters that influence the synthesis or formation of pyrazines in foods.

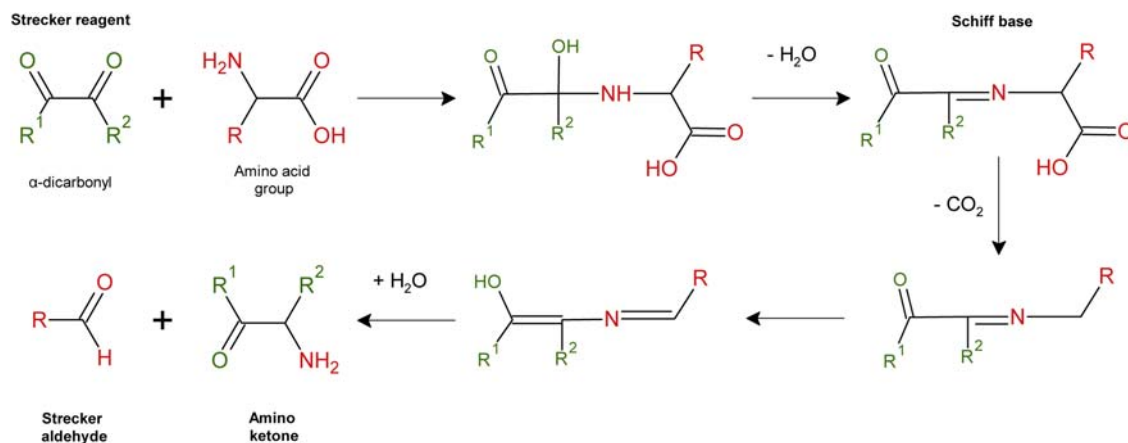
The first review about pyrazines in food was published by Maga and Sizer (1973) and Maga and Katz (1982) published an updated version citing 173 references 9 years later. These reviews discussed the occurrence of pyrazines in food, their organoleptic properties, as well as the current theories and proofs for their formation in foods. In similar date, Flament (1981) published other compiling work about "naturally-occurring pyrazines" which were isolated from raw, cooked or roasted foods. This author pointed out the potential and unique organoleptic properties of pyrazines, their analytical properties, reactive capacities and different formation phases.

A relatively large information about pyrazines of different foods as barley, malt, beer, bread, cocoa, coffee, roasted seed and nuts, dairy products, fish, poultry and other meat products, mainly beef meat, was able at 1980s. However, most of the studies about food pyrazines were carried in the ulterior decades, specially from the beginning of the 21st century. During the last 17 years, the 66% of the global 1371 previously indicated papers have been published, and according to the Web of Science data base, 2014 was the most prolific year (74 papers). In the last decades, papers studying healthy properties of pyrazines have increased notably. In the past century, pyrazines were related to some negative healthy effects mainly associated to their mutagenic potential (Spingarn and Garvie, 1979). However, nowadays, it is accepted that the negative effects could be caused by other products formed during food heating and cooking, such as acrylamide, rather than by pyrazines (Ehling and Shibamoto, 2005; Low et al., 2006). Currently, they are considered safe and can be used as GRAS flavor ingredients (Adams et al., 2002) since pyrazine metabolism produce innocuous products (Mattia et al., 2006). Furthermore, some positives effects have been described for such as antimicrobial (Seitz et al., 2002; Premkumar and Govindarajan, 2005) chemopreventive (Kim and Kim, 1999) and vascular protective properties (Jiang et al., 2015). The potential benefits of pyrazine is corroborated by the large number of patents submitted in the last decade to their application in food industry as reviewed by Ferreira and Kaiser (2012).

## General Notes About Pyrazine Formation in Thermally Treated Foods

Generally, pyrazine formation takes place in high-temperature processing (usually above 100 °C). The chemical mechanism behind pyrazine formation is closely related to the so-called Maillard reactions. The main route of pyrazine formation is the condensation of  $\alpha$ -dicarbonyl with aminoacids rendering diverse types of pyrazines. Three main stages can be described in pyrazine formation: Strecker degradation, condensation of aminoketones and incorporation of alkyl chains.

Strecker degradation can be generally defined as the decarboxylation and deamination of an aminoacid group produced by a Strecker reagent (usually a carbonyl group). The reaction between the carbonyl and aminoacid groups is followed by dehydration producing the so-called Schiff base that is very unstable and is rapidly decarboxylated and hydrolyzed forming the so-called Strecker aldehyde and an aminoketone (Fig. 1). The most commonly source of Strecker reagents is the reducing sugars, although other alternative pathways have been described in the literature such as ascorbic acid or quinones (Adams and Kimpe, 2009). Other compounds derived from lipid oxidation such as 2,4-decadienal may also enter the reaction as Strecker reagents which explain the correlation between lipid oxidation and pyrazine formation (Hidalgo and Zamora, 2016). Regarding the source of amino group



**Figure 1** Strecker degradation between an  $\alpha$ -dicarbonyl and an amino acid group.

required for Strecker degradation, it might be amino groups from amino acids, peptides or proteins. Ammonia is also able to participate in the Strecker degradation leading to the formation of aminoketones (Rizzi, 1999).

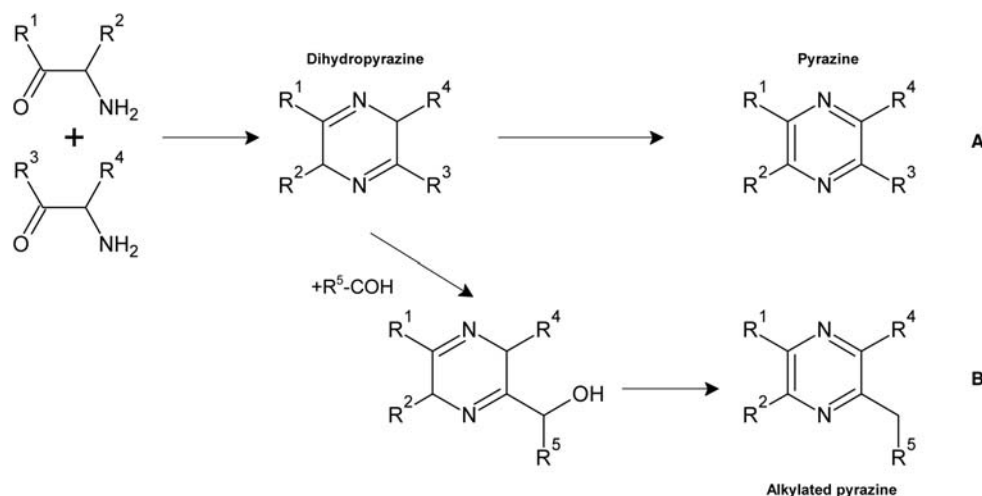
Aminoketones formed as consequence of the Strecker degradation may undergo further condensation with other aminoketones produced by similar reactions resulting in diverse dihydropyrazine structures (Fig. 2A), which are also very unstable and may rapidly be oxidized to form different pyrazines. Dihydropyrazine can also react to another carbonyl or aldehyde group present in the medium leading to the formation of alkylpyrazines (Fig. 2B).

These reactions play a key role in the wide diversity of pyrazines that may be formed during thermal processing since many different compounds can be formed depending on the compounds that are incorporated into the pyrazine structure. According to that, the next paragraphs show a summary of the most frequent pyrazine structures formed in thermally treated foods.

The simplest structure is the unsubstituted pyrazine (without any group attached in the aromatic ring). It is usually present in a wide range of products, but its relevance is limited since it is usually masked by other pyrazines (Maga, 1992).

The most frequent formed pyrazines are alkylpyrazines (Maga and Katz, 1982; Maga and Sizer, 1973), which include those with a chain in the 2-, 3-, 5- or 6- substituent of only carbon and hydrogen atoms. There has been found more than 70 types of alkylpyrazines with different substituents, including methyl, ethyl and propyl chains (Table 1).

Generally, alkylpyrazines present nutty and roasty aromas which are highly appreciated in cooked products. However, some of them present earthy or potato-like odors, which may present negative connotations. Pyrazines with long alkyl-substituted chains present green or burdock-like odors (Mihara and Masuda, 1988). Methylpyrazines are those pyrazines produced at the largest concentrations, being the most frequent in food systems 2-methylpyrazine followed by 2,6-dimethylpyrazine and 2,5-dimethylpyrazine. The relatively high odor threshold of these pyrazines (higher than 1 ppm) limits their relevance in food systems. In contrast, those pyrazines with an ethyl group that are formed in a lower proportion, present a higher relevance due to their lower threshold values (Table 1).



**Figure 2** Condensation of aminoketones to form a dihydropyrazine followed by the formation of a pyrazine (A), or a new alkylated pyrazine (B).



**Table 1** Daily intakes and odor thresholds of the most relevant pyrazine in foods

Pyrazines substituents	Daily intake ( $\mu\text{g}/\text{person and day}$ )	Odor threshold (ppb)	Aroma
Unsubstituted	0.2	500,000	Pungent, corn-like, hazelnut
2-Methyl	7	60,000	Popcorn, burnt, nutty, roasted
2,3-Dimethyl	4	2500	Green, nutty, caramel, pungent
2,5-Dimethyl	8	800	Roasted, butter, fried, nutty, musty, earthy
2,6-Dimethyl	2	200	Roasted, coffee, nutty, peanuts, chocolate flavor, potato aroma.
Trimethyl	46	400	Earthy, nutty
Tetramethyl	19	10	Pungent, flowery
2-Ethyl	6	6000	Musty, nutty, buttery, roasted
2-Ethyl-3-methyl	9	0.4	Burnt, cereal, nutty, roasted, earthy
2-Ethyl-5-methyl	1	100	Nutty, roasted.
2-Ethyl-6-methyl	0.4	40	Roast, sweet.
2-Ethyl-3,5-dimethyl	9	1	Nutty, burnt
2-Ethyl-3,6-dimethyl	9	0.4	Nutty, burnt
(2 or 5 or 6)-Methoxy-3-methyl	14	3	Odor reminiscent of roasted peanuts, hazelnuts, almond
2-Ethyl-(3 or 5 or 6)-methoxy	1	0.4	Roasted nut character; hazelnut, earthy
2-isoButyl-3-methoxy	1	0.002	Hot paprika, earthy
Acetyl-	122	62	Roasted corn
(3 or 5 or 6)-(Methylthio)-2-methyl	13	1–4	Toasted, roasted, nutty, caramel corn-chip

Modified from Mihara and Masuda (1988), Xu et al. (2017), Adams et al. (2002), and Maga and Sizer, 1973.

Regarding the relation between chemical structure and odor threshold, this seems to be correlated to the polarity of the molecule and the polarity of their substituents. Increasing lipophilicity leads to lower odor threshold (Mihara and Masuda, 1988). In fact, Guadagni et al. (1972) found that pyrazine threshold may be between 2 and 60 times higher in oil matrix than in aqueous.

Alkoxy-pyrazines include those compounds with an oxygenated functional group in the aliphatic side chain. The most studied alkoxy-pyrazines are those with undesired pea and potato-like odors causing negative impacts on wine, coffee and cocoa. These pyrazines are usually correlated to microbial metabolism rather than with thermal formation. Others alkoxy-pyrazines present similar olfactory notes than alkylpyrazines (nutty, roasted and burnt notes) (Table 1). For instance, acetyl-pyrazines are associated with popcorn notes odors. They are formed usually at lower levels than alkylpyrazines, but they may have odor threshold as low as 0.01 ppb as in the case of 2-methoxy-3-methyl-5-(2-methylbutyl)-pyrazine (Shibamoto, 1986).

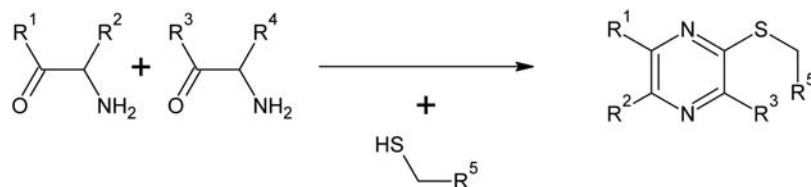
Thiopyrazines include the pyrazines with a sulfur substituent in the aliphatic side chain of the pyrazine ring (Fig. 3). According to published data, the compounds formed at the largest concentration are 3 or 5 or 6-(methyl-thiol)-2-methylpyrazine with odor thresholds ranging between 1 and  $7 \times 10^{-17}$  ppb (Brown, 2003; Mihara and Masuda, 1988).

## Main Factors Influencing Pyrazine Formation in Thermally Treated Foods

### Temperature and Heating Time

Pyrazine formation under 100 °C is negligible and start being constant at temperatures between 120 and 130 °C. Generally, papers indicated that the formation of pyrazines in thermally treated food increased with the temperature, time and heating ranges (Neill et al., 2012; Potočník and Košir, 2017; Maga and Sizer, 1973).

Huang et al. (1989) observed, in an arginine-glucose model, a linear relationship between pyrazine formation and temperature, with higher formation at higher temperature. Regarding heating time, pyrazine stop forming when pyrazine concentration reaches an equilibrium. Several studies have reported that the activation energy is similar to all the pyrazines (between 13.09 and


**Figure 3** Formation of thiopyrazines through the condensation of two aminoketones and a sulfur-containing chain.

18.84 kcal/mol), which suggests that the pathway formation is similar to all of them (Huang et al., 1989). However, the pathway of pyrazine formation may be different at different temperatures, mainly because at high temperatures sugars are transformed into many small fragments of  $\alpha$ -dicarbonyl groups which can go into the Strecker reaction, fact that is not possible at temperatures lower than 110 °C (Koehler and Odell, 1970; Jusino et al., 1997).

The type of pyrazine formed is also affected by the range of heating temperature. For instance, dimethylpyrazine formation is promoted over methylpyrazines in the range of 110–130 °C (Koehler and Odell, 1970). The increase of heating times also promotes pyrazine formation, however this fact could have lower relevance due to higher formation of other volatiles which could mask pyrazines (Macleod and Coppock, 1976). The correlation between temperature–time and pyrazine formation was also studied by Liu et al. (2015a). They concluded that high temperatures (over 100 °C) applied to model system with chicken peptides and xylose, increase the formation of meaty aroma that was correlated with the pyrazines formed, whereas lower temperature and longer heating times tended to generate a broth-like taste.

### Type of Aminoacids and Sugars

The studies carried out in model systems allowed to obtain interesting information about the effect of sugars and aminoacids on the formation of pyrazines. It is known that pentoses produce higher levels of pyrazines than hexoses (Burton and Mcweeny, 1963) and promote the formation of methylpyrazine over dimethylpyrazines (Koehler and Odell, 1970). Martin and Ames (2001) showed that asparagine produced the highest formation of pyrazines in a fried potato model with glucose, followed by glutamine and phenylalanine. Ten times less formation of pyrazines was observed using leucine, isoleucine and methionine. Chun and Ho (1997) observed similar results in the total amount of pyrazines, but asparagine produced larger amount of ethylpyrazine and methylpyrazine than glutamine, which increased the formation furylpyrazines.

Leahy and Reineccius (1989) observed the highest rate of pyrazine formation in systems with lysine-ribose followed by lysine-glucose, whereas the lowest rate was found for asparagine-fructose. Differences between pyrazine proportion was also found. The unsubstituted pyrazine was the major compound formed in the reaction between lysine-glucose, whereas 2-methyl-pyrazine was the predominant pyrazine with the combination of lysine-fructose, lysine-ribose and asparagine-glucose. The combination of asparagine-glucose produced very similar proportions of 2-methyl pyrazine, 2,5-dimethyl and 2,6-dimethyl pyrazine. It seems that the presence of fructose, instead of glucose, promotes the formation of 2,5(6)-dimethylpyrazine, 2-ethyl-6-methylpyrazine and 3-ethyl-2,5- dimethylpyrazine, and decreases the formation of pyrazine, ethylpyrazine, vinylpyrazine, and 2-vinyl-6-methylpyrazine (Leahy and Reineccius, 1989; Martin and Ames, 2001).

The ratio sugar:aminoacid also impacts on the final yield of pyrazines formed, since an excess of sugar in a ratio 3:1 strongly inhibited the final pyrazine yield in comparison to a ratio 1:1. In contrast, the excess of aminoacid levels did not produce relevant effects (Koehler and Odell, 1970).

In contrast with the effect of aminoacids, the role of peptides in the formation of pyrazines may have been underestimated and it has been less studied. Scalone et al. (2015) indicated that the presence of oligopeptides from hydrolyzed whey protein contributed notably to pyrazine formation; while free aminoacids, formed by the total hydrolysis of proteins, had a much lower contribution. Similar results were observed with chicken peptides that caused an increase in pyrazine content, imparting a nutty and roasted aroma (Liu et al., 2015a,c). The combination of chicken peptides-xylose-leucine was more effective, regarding pyrazine formation, than models with peptides-xylose or xylose-leucine (Liu et al., 2015a). The low-molecular-weight peptide (<500 Da) was considered as the main contributor to pyrazine formation (Liu et al., 2015c).

### pH and Water Activity

Some works showed a promoting effect of higher pH on the formation of pyrazines, most likely due to the increased reactivity of the amino group as well as the rearrangement and fragmentation of sugars (Meynier and Mottram, 1995; Müller and Rappert, 2010). In agreement with this comment, Liu et al. (2015c) noted that pH lower than 5.0 inhibited the generation of pyrazines in a model system with chicken peptides. Regarding water activity, lower values are usually linked to increases in the pyrazine formation (Scalone et al., 2015).

### Presence of Reducing Compounds (Other Than Sugars)

Recently, there has been a growing literature concerning the effect of natural antioxidant (such as polyphenols) on the formation of pyrazines and other Maillard-reaction products, obtaining contradictory results. For instance, Porter et al. (2006) noted that ellagic acid strongly promoted pyrazine whereas gallic acid inhibited almost completely the reaction. A total inhibitory effect of pyrazine formation was also described to rosmarinic acid and epigallocatechin-3-gallate in model system, bakery rolls and applesauce (Favreau-Farhadi et al., 2015). However, Wilker et al. (2015) observed that pure polyphenols, specially gallic acid, were able to promote the Maillard reaction. In contrast, Misnawi Jinap et al. (2004) showed that samples with higher phenolic content produced lower levels of pyrazines during roasting process and modified pyrazine profile, affecting specially the formation of 2,3-dimethylpyrazine. The inhibitory effect was ascribed to the radical scavenging activity of phenols, while the promoting effect was correlated with the capacity of phenols to promote the formation of  $\alpha$ -dicarbonyls derived from carbohydrates (Wilker

et al., 2015; García-Lomillo et al., 2016). Furthermore polyphenols, in their quinone state, can also be modified as a Strecker reagent, promoting the formation of aminoketones in the Strecker degradation (Rizzi, 2006).

### Storage Time

Pyrazines are relatively stable volatile compounds and the concentration remains very stable over storage in contrast to other volatile compounds such as 2-methyl-3-furanthiol or pyrrolines (Kaneko et al., 2013). In terms of pyrazine relevance, it may be reduced due to the formation of new volatiles derived from lipid oxidation which may mask them. Furthermore, some slight pyrazine degradation may be caused by free radicals and hydroperoxides derived from lipid oxidation (Williams et al., 2006).

## Pyrazines in Foods

Probably, cocoa, coffee, nuts and roasted seeds, cereals and meat products are the most common foods in which the formation and levels of pyrazines have been studied. For that reason, the next paragraphs try to summarize the available information about pyrazines in these foods, including also some brief recently notes about other food matrices. In advance, the authors want to indicate that by logistic reasons it has been impossible to include all the published references and they want to apologize for those references not cited in this work. Furthermore, authors recommend consulting the reviews previously cited in this article.

### Coffee

Green coffee beans present a flavor profile very different from the final beverage. The variety and extension of coffee flavor is developed mainly during roasting process. Due to the relevance in the economy of coffee industries and the high relevance of pyrazines in the final quality of its aroma, coffee was probably one of the first food matrix in which pyrazines were evaluated (Bondarovich et al., 1967; Goldman et al., 1967). More than 200 different pyrazines has been identified in coffee (Maga, 1992), however it is widely recognized that the key odorant are alkylpyrazines with ethyl groups as 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine (Belitz and Grosch, 1999).

Several factors may impact the concentration of the different pyrazines in the final beverage. For instance, Robusta varieties produced larger amounts of pyrazines than Arabica varieties (Hashim and Chaveron, 1995), and roasting parameters and brewing steps also produce very important differences. Strongly roasted coffees are usually linked to higher pyrazine concentration (de Toledo et al., 2016). Hashim and Chaveron (1995) observed an intense increase in pyrazine formation for roasting at 205 °C and 10 min, whereas higher temperatures or longer roasting times produced lower amounts of pyrazines. The ratios 2-methyl-/2,5-dimethyl- or 2-methyl-/2,6-dimethyl- pyrazines were proposed as indicators of roasting conditions, and authors noted significant correlation between consumers' preference and coffees with ratio value of 2.5. In contrast, Yang et al. (2016) did not find significant differences when the roasting temperature decrease from standard (210 °C) to 135 °C. Interestingly, lower temperatures and longer roasting times (160 °C, 30 min) seem to reduce acrylamide formation and catechin degradation with an adequate pyrazine formation (Mizukami et al., 2008). Recently, the important role of pyrazines as markers of roasting degree of coffee and then as quality markers of roasting process has been once again pointed out. Different pyrazines were selected by discriminant analysis as roasting markers (de Toledo et al., 2016, 2017), being the pyrazine family the most abundant among the volatile selected markers.

As it was described in the introduction, some microorganism can produce methoxypyrazines, this fact is of paramount importance in the case of coffee since infected beans usually showed significant levels of these pyrazines, revealing important defects of aroma (Cruz-López et al., 2016). However, methoxypyrazines are not the unique pyrazines considered as low-quality markers. Toci and Farah (2014) suggested as potential defective seed's markers the presence of 2-butyl-3,5-dimethylpyrazine, 2,3,5,6-tetramethylpyrazine and 2-butyl-3-methylpyrazine.

### Cocoa

Green cocoa beans, as green coffee beans, have a very simple aroma profile compared with the flavor of final cocoa products. The characteristic aroma profile of cocoa is developed during different steps of cocoa bean processing, being fermentation, drying and roasting the most significant steps. Roasting intensely affect the development of the cocoa flavor, being one of the most important operation in the cocoa-based industries.

The pyrazine content in cocoa beans and cocoa products have been intensively studied, and the first studies appeared in similar data than for coffee (van Praag et al., 1968; Rizzi, 1967). Some similarities regarding coffee and cocoa pyrazines have been pointed out along the time. For instance, 2-ethyl-3,6-dimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine are also recognized as the key pyrazines of cocoa roasted beans, and some methoxypyrazines present in green bean or developed during fermentation may also negatively impact the final quality of the cocoa products (Belitz and Grosch, 1999; Aprotosoai et al., 2016).

Among other studies, Serra Bonvehí and Ventura Coll (2002) pointed out that pyrazine concentration rapidly increased after 3 minutes of roasting and then reaches a stable concentration. Roasting at 135 °C increased total pyrazine formation and specially 2,3,5-trimethylpyrazine; and bean alkalization before roasting, which produced oxidative deamination of aminoacids, induced a drastic reduction in the final pyrazine formation. Roasting at different temperatures (150–250 °C) and times (10–50 min), using

superheat steam, produced significant differences in pyrazines formation. Although pyrazines were formed in all the treatment assayed, the highest total quantity was observed in treatment of 200 °C and 10 min (Zzaman et al., 2017).

A relatively recent work by Rodriguez-Campos et al. (2012) studied the effect of drying conditions on pyrazines formation and no significant differences were observed between traditional drying processes (sun-dried) and beans dried at 70 and 80 °C.

Six pyrazines were selected among the 32 major aroma-active compounds of chocolate, and analyses indicated that dark chocolate usually content more quantity of pyrazines than milk chocolate, and this fact was well correlated with the sensory properties of each product (Liu et al., 2015b).

### Roasted Nuts and Seeds

Traditionally, consumers appreciate the typical roasted nut flavor which are closely linked to pyrazine. This explains that the presence of pyrazines in roasted nuts has been largely studied and the first studies also appears in the 1960s (Mason et al., 1966). More than 70 pyrazines have been found in roasted nuts which provide them with their excellent organoleptic properties and notably contribute to their flavor profile (Maga, 1992).

Regarding the pyrazine type, formed quantity and their relation to the typical roasted flavor, bibliography shows some apparently contradictory data. Buckholz et al. (1980) described that 2-methyl and 2,6-dimethyl pyrazines were the compounds formed at the largest concentration during the roasting of peanuts, but they indicated that 2-ethyl-6-methyl pyrazine and 2-ethyl-3,6-dimethyl pyrazine had the biggest effect on the consumer preference. In contrast, Wang et al. (2017) have pointed out that 2,5-dimethyl pyrazine and 3-ethyl-2,5-dimethyl pyrazine were formed at the largest concentration, whereas 2-ethyl-6-methyl pyrazine had the largest impact on roasted liking flavor. Kaneko et al. (2013) found that 2-ethyl-5-methylpyrazine and 2,5-dimethyl-3-ethylpyrazine were the main compounds with roasted notes in-shell peanuts. These differences and other could be attributed to different factors, such as the composition of each nuts and the roasting parameters (time, temperature, type of oven, etc.). In this sense, 2,3-Diethyl-5-methylpyrazine and 3,5-dimethyl-2-ethylpyrazine, together with some pyrroline derivatives, were also pointed out as appropriate marker odorants to differentiate the various nut aromas (Kiefl and Schieberle, 2013). These authors indicated that pyrazine level must not be excessive to avoid an over-roasted smell, and the desired aroma can be obtained for the different nut cultivars, applying specific roasting times, temperatures, and roasting techniques adapted to each cultivar characteristics. Increasing of roasting time usually promotes the total pyrazine formation, although some exception has been reported for specific pyrazines, mainly due to the volatilization of some compounds during roasting (Buckholz et al., 1980).

Pyrazines were detected in significant quantities after roasting almonds but not in fried almonds (Valdés et al., 2015). Xiao et al. (2014) reported similar results after roasting of almonds although they indicated that 2,5-dimethylpyrazine was also detected in raw almonds.

One of the main issue concerning roasted nuts is the development of “flavor fade” which is a defect related to the development of lipid oxidation products (mainly aldehydes) that mask positive notes from pyrazines. In fact, the formation of lipid-oxidation-derived products presented the largest and negative impact on consumer liking rather than pyrazine content (Wang et al., 2017). This would explain the decrease in liking score despite the relatively high stability of pyrazines during storage. Authors indicated that pyrazines remained in the product during storage with only slight losses, being 3,5-dimethyl-2-vinylpyrazine the compound with the largest loss among pyrazines (only 36%, after 6 months). Slight decreased of pyrazines during storage of roasted peanuts was also indicated by Jiao et al. (2016).

Relatively recent studies showed the effect of roasting on flavor and pyrazines content of different products derived from roasted rich-oil seeds and kernels. The roasting of palm kernels improved the aroma of palm kernel oil; and roasting treatment and enzymatic kernel pretreatment before roasting induced significant differences. Tenfold higher quantities of pyrazines were detected in palm oil derived from the enzymatic pre-treated medium roasted kernels (Zhang et al., 2016). This may be interesting to increase the flavoring properties of oil, with nutty notes, that may be applied in bakery and confectionary industries but also to widen the scope of its applications (Zhang et al., 2016). Similar results were observed in pepper seed oil after roasting the pepper seeds applying temperatures above 140 °C, and maximum content of 2, 3, 5, 6-methyl pyrazine was reached increasing the roasting time until 20 min (Lu et al., 2017). The roasting treatment of pumpkin seeds over 110 °C until 200 °C increased the content of pyrazines in the respective oil. However, this increase might be considered as negative since consumers prefer oils with green and fresh notes which disappear to higher temperatures (Potočník and Košir, 2017). Formation of pyrazines, mainly alkyl derivatives, were also reported during roasting of kirilowii seeds by Wu et al. (2014).

The content of pyrazines was increased after prolonged roasting time of sesame seeds (Zhao et al., 2016). Furthermore, the effect of “new” conservation methodologies on pyrazine formation has also been studied. For instance, the relative content of pyrazines in sesame paste treated with high-pressure thermal sterilization process (combined 400 MPa and 45 °C) has been shown to be 2.73 times higher than those in fresh paste, fact that was associated to the increment of aromatic intensity and quality of the sesame paste (Ren et al., 2016).

### Cereals and Cereal Products

The contribution of pyrazines to the highly appreciated roasted flavor of cereals products has been also the main reason to study their formation in this type of food matrix. In this case, many of the first published papers focused on the pyrazines formed during roasted barley and malts due to their important role in the flavor of beers (Wang et al., 1968). Harding et al. (1978) described 21

pyrazines isolated from roasted barley, six of them described in roasted barley by the first time. Methylpyrazine was the most abundant, followed by 2,3-, 2,5- and 2,6- dimethylpyrazines and by trimethylpyrazine. Authors indicated that pyrazines contributed to the flavor of beer brewed with roasted barley.

Furthermore, pyrazines strongly contribute to the flavor of crust bread aroma. 2-Acetyl pyrazine and 2-methyl-3-ethyl pyrazine were described as some of the compounds responsible for bread crust aroma (Fadel and Hegazy, 1993). Chang et al. (1995) described 2-ethyl-3-methyl pyrazine and methylpyrazine as the two most abundant pyrazines (among 13 detected) in the crust of different wheat breads contributing notably to their pleasant flavor.

Heat-treatment of flours has gained popularity in the recent years due to the technological benefits exerted by this technology such as: increasing microbial safety, improve rheological properties and development of positive odors (Neill et al., 2012). Xu et al. (2017) noted that the positive odor effect depends on the heating severity. Low-temperature treatments (100–110 °C) have a positive effect by elimination of “rancidity” component, but without inducing the formation of additional positive roasted notes. Treatments at 120 °C or higher temperatures induced the formations of pyrazines, furans and others, giving roasting flavor. Authors indicated that, considering organoleptic properties, heating at 140 °C was the most optimum treatment. Furthermore, Bettge et al. (2000) indicated that waxy flour was prone to the formation of active odor components. The higher free reducing sugar content of this flour and the higher level of damage of starch could be explain this fact. The positive effect on bread aroma have been also described to oatmeal. Heat treatments applied before baking produced significant increase of pyrazine formation, resulting a strong flavor associated with baking (Gu et al., 2015). A recent review showed the effect of sourdough on volatile compounds of bread, including data about 11 pyrazines (Pétel et al., 2017).

The lack of flavor of gluten-free bread has been correlated to the low quantities of pyrazines detected in this sort of bread. This negative effect may be mitigated by the addition of aminoacids and sugars before baking. The tandem proline-glucose allowed to obtain gluten-free bread more desirable, to celiac patients, than control one (Pacyński et al., 2015).

The possible negative effect of some Maillard-reactions products such as acrylamide, whose formation is coupled to pyrazine formation in bread crust, cookies and other cereals products is one of the main concern of baking, pastry and confectionery industries. In this sense, different studies showed the natural antioxidants as phenolic compounds may be able to block of acrylamide formation (Hedegaard et al., 2008; Jin et al., 2013), although this positive effect may also affect roasting flavor due to the accompanying reduction of pyrazines formation, which can reach until 81% of reduction in bread (Mildner-Szkudlarz et al., 2017). Similar results were found in potatoes chips using vacuum frying, with up to 300 times reduction of pyrazine formation (Belkova et al., 2018). Furthermore, together with the block of pyrazines other negative effect as the formation of unpleasant flavor components have been described when feruloylated oligosaccharides were used in different dough model systems (Zhao et al., 2017).

## Meat Products

The main role of pyrazines in meat products is also related to their flavor notes, mainly due to their contribution to roasted and cooked aromatic notes. There are several families of odorants that have been associated with the quality of cooked meat, being among them pyrazines, which are solely formed during heat treatments of meat products. Pyrazines have been widely detected and accepted as key odor components in roasted chicken, beef, pig, turkey and lamb products (Shahidi, 1994). However, meat flavor is a rather complex issue, in which many factors may impact the quality of the final product, beginning with the state and composition of the raw material (Shahidi et al., 1986) and followed by those related to the cooking procedure. Roasting is the cooking technique that induces the largest formation of pyrazines, and much less pyrazines (and in less quantities) were detected in boiled and microwaved cooked meat (Macleod and Coppock, 1976). Furthermore, significative differences in the pyrazine profile were observed in meat cooked by different techniques: 2-ethyl-6-methylpyrazine and 2,3,5-trimethylpyrazine were predominant in boiled meat, whereas methylpyrazines and 2,3-dimethyl-5-ethylpyrazine and 2,5-dimethyl-3-ethylpyrazine were more relevant in microwaved meat products. Authors ascribed this fact to a rapid surface dehydration induced by microwave treatment, so the low water content would may promote pyrazine-formation reactions. Yang et al. (2017) reported that pyrazines presented much higher relevance (approximately 80% of total volatile concentration) in electric oven-cooked pork loin, whereas the relevance in air fryer or oven was much lower. The difference in the type of cooking was ascribed to the different temperature profile for each method. The pyrazine level was higher in pork neck cutlets marinated with and aqueous solution with 1% of glucose compared to those marinated without glucose, being pyrazines some of the discriminant volatile compounds proposed to justify the differences among samples (Biller et al., 2016).

## Miscellaneous

Roasting stem tea has a characteristic flavor derived from the roasting process of the tea stems. This flavor has been associated to higher pyrazine levels of roasted stem than roasted leaf. This fact may have an important impact on the development of new tea products (Sasaki et al., 2017). Previous paper indicated the presence of different pyrazines (methyl-, ethyl- and 2-ethyl-5-methyl- derivatives) in Kangra orthodox black teas, and ethylpyrazine was pointed out as one of the odor active components of this type of tea (Joshi and Gulati, 2015).

Recently, three pyrazines have been indicated as important discriminant markers and responsible of the different aroma of cane brown sugars (Asikin et al., 2017). Previously, Asikin et al. (2016) described the relation of 5-methyl-2pyrazinylmethanol and 2,5-



dimethylpyrazine with the nutty-roasted note of the cane brown sugar. Authors also indicated that pyrazines were mainly formed when drying solidification process were carried out in open pan against horizontal or vertical thin-film dryers.

Recent papers indicated the role of some pyrazines as 2,5- and 2,6- dimethylpyrazines on the flavor of different fish products. These pyrazines seem to be useful to characterize grass carp soup (Li et al., 2017). Similar results were described to seaweed (López-Pérez et al., 2017), although in this case 2,6 dimethylpyrazines and tetramethylpyrazine were the selected markers. Working with fish powder hydrolysate, Peinado et al. (2016) indicated that EPA, DHA and fish oil promoted formation of pyrazine and methylpyrazine but reduced the formation of 2,5-dimethylpyrazine. Pyrazines were also present in ready-to eat-fried loach but not in fresh loach (Lv and Zhao, 2016).

## Conclusion and Future Trends

It seems that there will be an increase in the pyrazine studies and applications in the following years since their flavor is highly appreciated by consumers, they are natural compounds and do not present any hazard to population health. Although they have been historically studied, food scientists will face new challenges in the field:

- Improve the detection techniques of those pyrazines produced at low concentration but with extremely low thresholds
- Increase the understanding of pyrazine profile in complex matrix (synergism with different volatiles), not only according to their concentration but also according to their relevance.
- Optimization of the conditions (temperature, time, pH) in thermal-heating process to obtain the desired pyrazine profile according to the consumer's liking preference.
- Reduction the presence of carcinogenic compounds whose formation is also coupled to Maillard-type reaction (acrylamide, heterocyclic amines) without modifying pyrazine flavor profile.

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# Formation of Selected Heterocyclic Flavor Chemicals in Beverages

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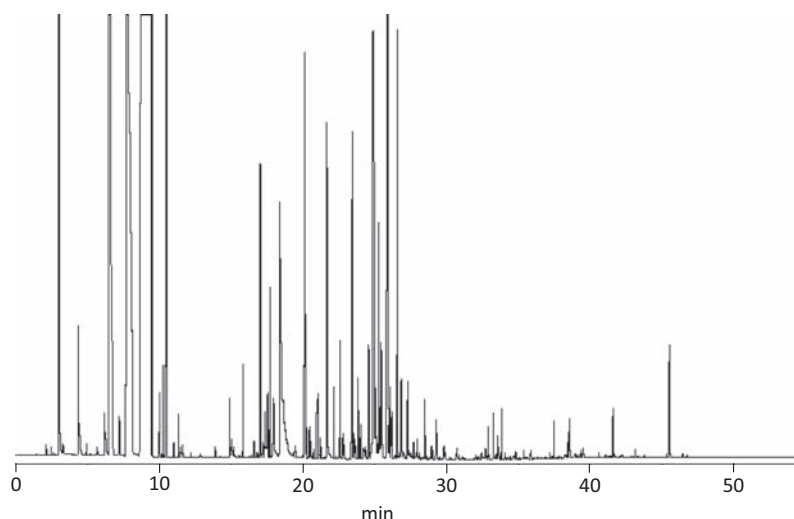
## Introduction

Heterocyclic compounds are aromatic chemicals with heteroatom, such as oxygen, nitrogen and sulfur, in their rings. One striking quality of heterocyclic compounds is that they possess unique and strong characteristic odors. Since the mid-1950th, in particular after the development of gas chromatography, relatively low molecular heterocyclic compounds have been identified in beverages (wine, coffee, and cola) and have come to receive considerable attention by flavor chemists as compounds formed by the Maillard reaction. Formation of these heterocyclic flavor chemicals has been studied intensively using Maillard reaction model systems consisting a sugar and an amino acid (Hodge, 1953; Shibamoto, 1983; Ledl et al., 1989; Martinez et al., 2001). Fig. 1 shows a typical gas chromatogram of a dichloromethane extract of a Maillard reaction solution prepared with a *D*-glucose/cysteine Maillard model system. As the chromatogram shows, there are over 1000 peaks, suggesting that a simple Maillard reaction model system produces tremendous numbers of volatile chemicals. These volatile chemicals are mainly heterocyclic chemicals. Fig. 2 shows the approximate number of volatile heterocyclic compounds reported up to the present (prepared based on Shibamoto, 1983; Whitfield, 1992). It should be noted that these numbers are increasing because many studies on the formation of these chemicals are currently underway around the world. Volatile heterocyclic compounds play an important role in heat-treated foods and beverages with preferable flavors because these chemicals are mainly formed through Maillard reaction (Yamaguchi et al., 1979; Shibamoto, 1980, 1989; Fors, 1983; Mottram, 1998).

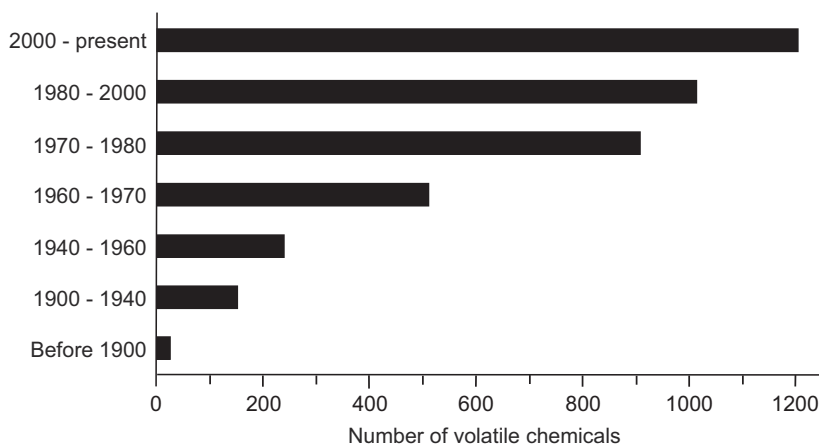
In 1912, French chemist L. C. Maillard hypothesized the reaction that accounts for the browning pigments and polymers produced from the reaction of the amino group of an amino acid and the carbonyl group of a sugar as shown Fig. 3. The Maillard reaction is also called the Non-enzymatic browning reaction, and it occurs readily upon heat treatment in any matrix containing amines and carbonyls. Therefore, Maillard reaction products include many different types of chemicals including brown pigments, flavor chemicals, pro- and anti-oxidants, pro-and anti-mutagens and carcinogens. It is obvious that many heterocyclic compounds form in foods and beverages by heat-treatment. There have been numerous reports on the formation of these products in heated (cooked) foods because these volatile heterocyclic compounds possess a characteristic roasted or toasted flavor.

However, some Maillard reaction products are not preferable because they damage the freshness of products, such as fruit and vegetable juices, beer and wine. On the other hand, they play an important role in heat-treated beverages, such as tea and coffee. Fig. 4 shows typical flavor characteristics of basic heterocyclic compounds along with their structures. There are many flavor chemicals related to these simple five- and six-ring compounds.

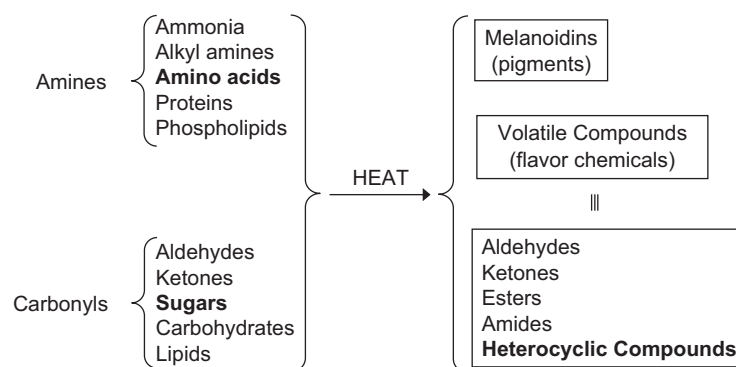
The presence of heterocyclic compounds in fruit and vegetables juices is due to the incidental heat-treatment during preparations, such as in sterilization. On the other hand, coffee and tea go through intentional heat-treatment in preparation, suggesting that they contain many of these chemicals. Therefore, beverages are divided into two kinds of product: one is prepared without heat-treatment like fruit and vegetable juices as well as alcoholic beverages, such as wine and beer; the other is prepared with heat-treatment and includes tea and coffee.



**Figure 1** Typical gas chromatogram of a volatile extract from a *D*-glucose/cysteine Maillard model system. A 60 m × 0.25 mm × 0.25 μm DBWAX bonded phase fused silica capillary column was used. The oven temperature was held at 35 °C for 5 min and then programmed to 250 °C at 2 °C/min. The linear carrier gas flowrate was 33 cm/s at split ratio of 1:40.



**Figure 2** Approximate number of volatile heterocyclic compounds reported up to the present.



**Figure 3** General scheme of Maillard reactions and their products.


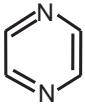
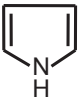




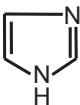
## Furans

Furans are the most abundant heterocyclic compounds found in foods and beverages. There are many review articles summarizing formation pathways of furans in Maillard reaction systems. Their major proposed pathways involve carbonyl compounds formed from lipids or sugars and amine compounds as precursors (Yaylayan, 2006; Paine et al., 2008; Owezarek-Fendor et al., 2011; Shibamoto, 2014). It should be noted that furans do not contain nitrogen atoms but their formation is catalyzed by amine compounds. However, as mentioned above, most heterocyclic compounds form from heat-treatment *via* Maillard reaction. They are recognized as artifacts rather than preferable components in fresh juices. In fact, fresh juice manufacturers are trying to avoid formation of these compounds as much as possible during juice preparation.

## In Fruit and Vegetable Juices

Only a few reports on the presence of furan and related compounds in fruit and vegetable juices are available. Kim et al. (2016) reported  $0.59\text{--}27.39\text{ ng mL}^{-1}$  of furan in 18 commercial orange juices. Their values were significantly higher than the juice obtained from a fresh orange directly by squeezing, suggesting that some heat-treatments, such as sterilization, cause furan formation. On the other hand, Wongfhun et al. (2010) reported that more 2-pentylfuran was found in fresh pennywort juice ( $87.9 \pm 23.7\text{ ng L}^{-1}$ ) than in sterilized pennywort juice ( $34.2 \pm 19.9\text{ ng L}^{-1}$ ). Another study found that furan in carrot and prune juices ranged from zero to  $33\text{ ng g}^{-1}$  and from  $0.7$  to  $14.2\text{ ng g}^{-1}$ , respectively (Wegener and Lopez-Sanchez, 2010).

Furfural and 5-hydroxymethylfurfural form naturally from pentose and hexose, respectively, and are found in various foods and beverages. However, only a few reports showed their presence in fresh fruit and vegetable juices. Abu-Bakar et al. (2016) found furfural ranging from  $0$  to  $16.2 \pm 0.14\text{ ng L}^{-1}$  (mango), 2-furoic acid ranging from  $0$  to  $8.72 \pm 0.38\text{ ng L}^{-1}$  (mango) and 5-hydroxymethyl furfural ranging from  $19.5 \pm 0.49\text{ ng L}^{-1}$  in 17 fresh fruit (mango, date, roselle, orange, pomegranate, mangsteen, soursop) juices. Two orange juice samples contained furfural at levels of  $8.5\text{ }\mu\text{g g}^{-1}$  and  $6.3\text{ }\mu\text{g g}^{-1}$  (Terixidó et al., 2006). Furfural was also found in 5 orange juice samples at levels ranging from less than LOD to  $106 \pm 1.7\text{ mg kg}^{-1}$  (Terixidó et al., 2011). Furfural and 5-hydroxymethylfurfural were found in an apple juice at the level of  $0.3$  and  $0.2$ , respectively (Gaspar and Lucena, 2009). Terixidó

Name		Structure	Typical substituents	Sensory description
Common	UPAC			
Furan	Oxole		CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub> , CH <sub>2</sub> OH, CHO, Acetyl	Sweet caramel-like, slightly cinnamon-like
Pyrazine	Pyrazine		CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub> , OCH <sub>3</sub>	Roasted, toasted, cooked flavor
Pyrrole	1H-Pyrrole		CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub> , CHO, Acetyl	Sweet, ethereal, burnt-nauseating odor
Thiophene	Thiophene		CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub> , CHO, SH, Acetyl	Onion-like, slightly-sulfurous odor
Oxazole	1,3-Oxazole		CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub> , CHO	Sweet, roasted
Thiazole	1,3-Thiazole		CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub> , CH <sub>2</sub> OH, CHO, SH, Acetyl	Cooked-meat, sulfurous-odor, characteristic foul-like odor
Pyridine	Pyridine		CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub> , CHO	Pungent, smokey fishy-odor
Imidazole	1,3-Imidazole		CH <sub>3</sub> , C <sub>2</sub> H <sub>5</sub>	Pungent off-flavor

**Figure 4** Typical flavor characteristics of basic heterocyclic compounds along with their structures.

et al. (2011) reported furfural in five apple juice samples at the levels ranging from less than LOD to  $3.51 \pm 1.0 \mu\text{g kg}^{-1}$ . 5-Hydroxymethylfurfural was also found in apple juices ( $0.36 \pm 0.021$ – $3.05 \pm 0.23 \mu\text{g g}^{-1}$ ) and pear juices ( $0.09 \pm 0.001$ – $1.04 \pm 0.28 \mu\text{g g}^{-1}$ ) (Marsol-Vall et al., 2016). Wongfhun et al. (2010) reported the formation of tetrahydrofuran in a pennywort juice ( $71.5 \pm 58.1 \text{ ng L}^{-1}$ ) prepared by high pressure processing. Same authors also found 2-pentylfuran in pennywort juices:  $87.9 \pm 23.7 \text{ ng L}^{-1}$  in fresh juice, trace amounts in high pressure processed and  $13.7 \pm 12.3 \text{ ng L}^{-1}$  in pasteurized samples. Furfural was found in 12 commercial tomato juices in amounts ranging from  $0.27 \pm 0.02 \text{ mg kg}^{-1}$  to  $4.20 \pm 0.18 \text{ mg kg}^{-1}$  (Vallverdu-Queral et al., 2013). Formation of furfural by heat-treatment was strongly suggested by its presence in cooked tomato (5.91 ppb), whereas furfural was not detected in fresh tomato essence (Zhu et al., 2017).

## In Beer

Only a few furans are reported in alcoholic beverage beer. Their levels in beer are relatively low. Their formation in alcoholic beverages is proposed to form from sugars *via* fermentation processes. In fact, furfural was found in an alcoholic beverage produced by whey fermentation at the level of  $101.1 \mu\text{g L}^{-1}$  (Dragone et al., 2009).

Vesely et al. (2003) found furfural in beer when they were analyzing aldehydes in beer. The furfural levels ranged from 28.8 ppb stored at  $0^\circ\text{C}$  to 458.3 ppb stored at  $30^\circ\text{C}$  for 12 weeks, suggesting that furfural forms during storage at high temperatures. Wort from beer also contained furfural at the levels of 4–400  $\text{ng L}^{-1}$  (de Schutter et al., 2008a). When wort was boiled, furfural, furfuryl alcohol and acetylfuran increased with time, suggesting that furfural and related compounds are formed by the Maillard reaction (de Schutter et al., 2008b). Vanderhaegen et al. (2004) detected furfuryl alcohol ( $1.741 \text{ mg L}^{-1}$ ), furfuryl ethyl ether ( $0.006 \text{ mg L}^{-1}$ ), furfural ( $0.045$ ), 3-methylfural ( $0.007 \text{ mg L}^{-1}$ ), 3-acetylfuran ( $0.020 \text{ mg L}^{-1}$ ) and 5-methyl-2-furfural ( $0.008 \text{ mg L}^{-1}$ ) in fresh beer. Among these, furfuryl ethyl ether was associated with a solvent-like stale flavor. They also found that addition of deoxyribose increased furfuryl alcohol significantly when beer was stored at  $60^\circ\text{C}$  for 7 days, suggested that the solvent-like off flavor was due to the formation of furfuryl alcohol. 3-Hydroxymethyl furfural formed in malt during roasting and then it was reduced during fermentation upon transferring to 5-hydroxymethyl furfuryl alcohol (Akilhoglu et al., 2011). When a fresh Pilsner type beer was forced to age under deaerated condition, the levels of furfural and 5-hydroxymethylfurfural increased from  $0.26 \pm 0.001 \mu\text{M}$  to  $4.2 \pm 0.2 \mu\text{M}$  and  $19.3 \pm 0.3 \mu\text{M}$ , respectively. On the other hand, the levels of furfural and 5-hydroxymethylfurfural increased from  $0.26 \pm 0.001 \mu\text{M}$  to  $5.5 \pm 0.2 \mu\text{M}$  and  $21 \pm 2 \mu\text{M}$ , respectively, under aerated conditions (Rakete et al., 2014). Rico-Yuste et al. (2016) determined furfural levels using a colorimetric method in beer during storage. They found a linear correlation between the storage time and the furfural concentration. The furfural concentrations were  $205 \pm 13 \mu\text{g L}^{-1}$  at 49 days storage,  $330 \pm 14 \mu\text{g L}^{-1}$  at 187 days storage,  $366 \pm 26 \mu\text{g L}^{-1}$  at 284 days storage and  $687 \pm 46 \mu\text{g L}^{-1}$  at 627 days storage. Moreiz et al. (2013) analyzed 10 beer samples using an automated headspace solid-phase microextraction (HS-SPME) combined with gas chromatography and ion trap mass spectrometry detection (GC-IT/MS). They found furfural ( $1.68 \mu\text{g L}^{-1}$  –  $32.7 \mu\text{g L}^{-1}$ ), 5-methylfurfural ( $5.82 \mu\text{g L}^{-1}$  –  $6.55 \mu\text{g L}^{-1}$ ) and 5-hydroxymethylfurfural (not detected –  $2019 \mu\text{g L}^{-1}$ ).

## In Wine

Ubeda et al. (2016) reported furfural ( $0.14$ – $1.03 \mu\text{g L}^{-1}$ ), 5-methyl-2-furfural ( $3.45 \mu\text{g L}^{-1}$ ), 5-hydroxymethyl furfural ( $1.83$ – $3.45 \mu\text{g L}^{-1}$ ) and furfuryl alcohol ( $0.16$ – $0.66 \mu\text{g L}^{-1}$ ) in Chilean sparkling wines. Various furfural and related compounds were also found in Zalema white wines: furfural ( $60.4 \pm 33.1 \mu\text{g L}^{-1}$ ), 5-methylfurfural ( $8.9 \pm 2.0 \mu\text{g L}^{-1}$ ), 5-hydroxymethylfurfural ( $90.7 \pm 49.6 \mu\text{g L}^{-1}$ ), ethylfuroate ( $20.8 \pm 12.9 \mu\text{g L}^{-1}$ ) and furfuryl alcohol ( $10.90 \pm 3.3 \mu\text{g L}^{-1}$ ) (Gómez-Míguez et al., 2007). Ma et al. (2017) reported furaneol ( $175.2 \pm 3.3 \mu\text{g L}^{-1}$ ) as a furan compound which had significant effects on ice wine character. Formation of some toxic chemicals in wine, including furan and furfural, has been reported to be associated with the ripeness and maceration of the grapes (Lago et al., 2017). Burin et al. (2013) analyzed heterocyclic compounds in 29 French wine samples of different origins, types and vintages. They found that 5-methylfurfural ranged from  $1.5 \mu\text{g L}^{-1}$  (Bordeaux red wine) to  $250 \mu\text{g L}^{-1}$  (Bordeaux white wine), 3-acetyl-2,5-dimethylfuran ranged from 0 (Bordeaux red wine and Bourgogne red wine) to  $0.9 \mu\text{g L}^{-1}$  (Bordeaux red wine, Bourgogne red wine and Bordeaux white wine), 2,3-dihydrobenzofuran ranged from 0 (Bordeaux red wine and Alsace white wine) to  $0.67 \mu\text{g L}^{-1}$  (Bourgogne red wine) and acetylfuran ranged from  $1 \mu\text{g L}^{-1}$  (Alsace white wine) to  $50.2 \mu\text{g L}^{-1}$  (Bordeaux red wine).

## Pyrazines






Pyrazines are most abundant heterocyclic flavor compounds formed by the Maillard reaction. There have been numerous reports on their presence in heat-treated foods and beverages (Maga and Sizer, 1973; Muller and Rappert, 2010). Therefore, many heterocyclic flavor compounds found in beverages which are not treated by heat have been considered as off-flavor chemicals. On the other hand, only a few pyrazines (mainly methoxypyrazines) found in non-heat processed beverages are reported. Methoxypyrazines are known to be present in natural plants, insects, fungi and bacteria (Lei et al., 2017). Therefore, they may be produced naturally or by fermentation processes in non-heated beverages, such as wine.

The most commonly used method for methoxypyrazine analysis is gas chromatography/mass spectrometry (GC/MS), of which LOD and LOQ in wines are generally  $> 0.03 \text{ ng L}^{-1}$  and  $1 \text{ ng L}^{-1}$  (Alberts et al., 2009; Hjelmeland et al., 2016). Also, solid phase extraction method is the most commonly used method for sample preparation in methoxypyrazine analysis in wine, and generally, nearly 100% recovery efficiencies were achieved (Ortega et al., 2001).

## In Wine

Fig. 5 shows the amounts of methoxypyrazines found in wines along with their structures. These pyrazines are not formed by heat but by biosynthesis (Helwi et al., 2015). They are present in various wines at levels ranging from  $1 \text{ ng L}^{-1}$  (2,5-



Methoxypyrazine	Structure	Level (ng L <sup>-1</sup> )	References
3-isobutyl-2-methoxypyrazine or 2-methoxy-3-isobutylpyrazine		2 – 14 5.6 – 11.0 12 – 27	Kotseridis et al., 1999 Chapman et al., 2004 Sala et al., 2002
“		2 – 4	Kotseridis et al., 2008
“		0.03 – 3.9	Alberts et al., 2009
“		1.1 – 7.8	López et al., 2011
“		4.5 – 17.9	Botezatu et al., 2014
“		5.4 – 6.4	Hjelmeland, et al., 2016
“		26.1 – 27.8	Ryan et al., 2005
“		14.0 ± 1.1	Kögel et al., 2015
“		1.9 – 5.0	Culleré et al., 2009
“		0.8 – 20.9	López et al., 2011
“		1.9 – 15.0	Ortega et al., 2001
“		1.6 – 9.6	Schmarr et al., 2010
3-sec-butyl-2-methoxypyrazine or 2-methoxy-3-(2-methylpropyl) pyrazine		5 – 10 7 0.03 – 3.2	Sala et al., 2002 Kotseridis et al., 2008 Alberts et al., 2009
“		1.1	López et al., 2011
“		<4 – 7.5	Botezatu et al., 2014
“		5.4 ± 0.3	Kögel et al., 2015
“		0.59 – 0.67	Hjelmeland, et al., 2016
“		26.1 – 27.8	Ryan et al., 2005
3-isopropyl-2-methoxypyrazine or 2-methoxy-3-isopropylpyrazine		<4 – 21.7 9.1 ± 1.3 0.2 – 1.0	Botezatu et al., 2014 Kögel et al., 2015 Schmarr et al., 2010
2,5-dimethyl-3-methoxypyrazine		42.3 – 96.5	Botezatu et al., 2014
“		51.0 ± 00.1	Kögel et al., 2015
“			
3-ethyl-2-methoxypyrazine or 2-methoxy-3-ethylpyrazine		2.5 ng/L	Hjelmeland, et al., 2016

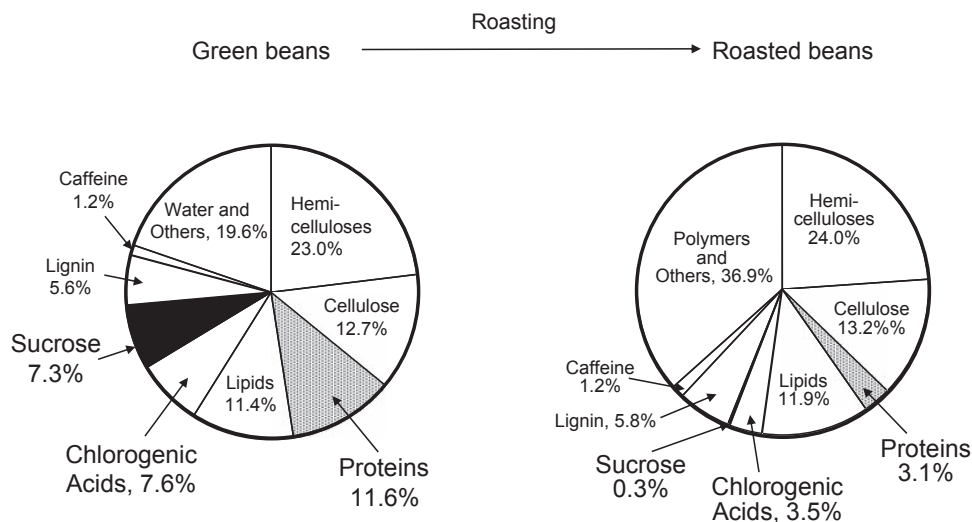
**Figure 5** Amounts of methoxypyrazines found in wines along with their structures.

dimethylmethoxypyrazine) to 27.8 ng L<sup>-1</sup> [2-methoxy-3-(2-methylpropyl) pyrazine]. Addition of 2,5-dimethylmethoxypyrazine (0, 50 and 120 ng L<sup>-1</sup>) to wines lower intensity ratings for cherry and red berry descriptors and higher ratings for earthy/musty and green/vegetal descriptors (Botezatu and Pickering, 2012). There are many reports on relationships between the presence of methoxypyrazines in wine and grapes with ladybeetles. 2-Isopropyl-3-methoxypyrazine increased beetle density in wine, whereas it decreased when grapes were heated prior to fermentation (Kögel et al., 2015). Botezatu et al. (2013) reported that ladybeetles caused formation of tainted wine associated with 2-isopropyl-3-methoxy pyrazine and consequently promoted a negative impact on wine quality. A strong correlation between the levels of 3-isobutyl-2-methoxypyrazine in wine-grapes and wines was observed (Ryona et al., 2009). Levels of 3-alkyl-2-methoxypyrazines remained the same during alcoholic and malolactic fermentation but they increased during the first day of maceration (Sala et al., 2002).

When 2-isobutyl-3-methoxypyrazine with a green pepper-like odor was found in Cabernet Sauvignon grapes in 1975, it was sensational because its odor threshold level was extremely low of 0.002–0.005 ng L<sup>-1</sup> (Sidhu et al., 2015). Consequently, these methoxypyrazines have received much attention as chemicals that play an important role in wine flavors (Lei et al., 2017).

### Heterocyclic Favor Chemicals Formed in Roasted Coffees

Components of green coffee beans are ideal to produce Maillard reaction products, mainly heterocyclic flavor chemicals, by heat-treatment (roasting). Fig. 6 shows the chemical compositions of green and roasted coffee beans. Some chemicals, such as hemicelluloses, cellulose, lipids, lignin, and caffeine, did not show any significant changes after roasting. On the other hand, sucrose,



**Figure 6** Average composition (percentage) of chemicals in coffee beans before and after roasting.

proteins and chlorogenic acids decreased considerably. It is obvious that the Maillard reaction occurred between sucrose and proteins and the resultant heterocyclic compounds were formed as described above (refer to Fig. 3).

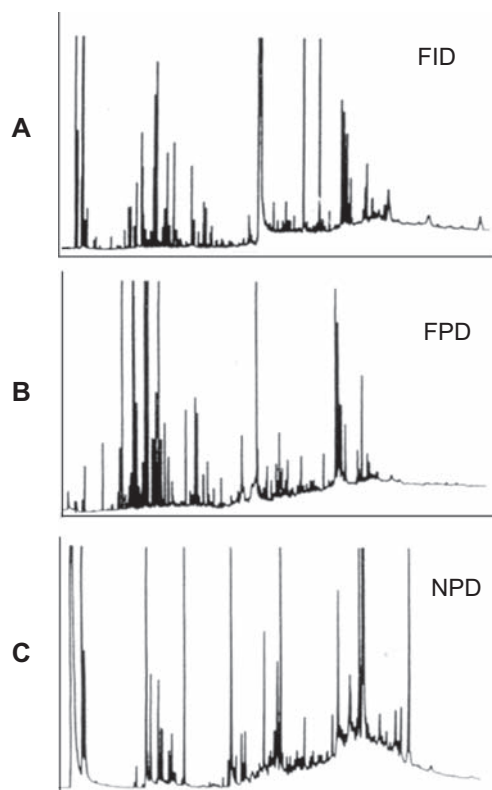
### Maillard Reaction Products

Fig. 7 shows typical gas chromatograms of coffee volatiles extracted from a brewed coffee taken by three different detectors. Chromatogram A was taken by a flame ionization detector (FID). Chromatogram B was taken by a flame photometric detector (FPD). Chromatogram C was taken by a nitrogen-phosphorous detector. FID is a comprehensive detector and sensitive to most organic compounds. FPD is a selective and highly sensitive detector for sulfur-containing compounds including thiophenes and thiazoles. NPD is a selective and highly sensitive detector for nitrogen containing chemicals including pyrroles, thiazoles, oxazoles, pyridines, pyrazines, and imidazoles.

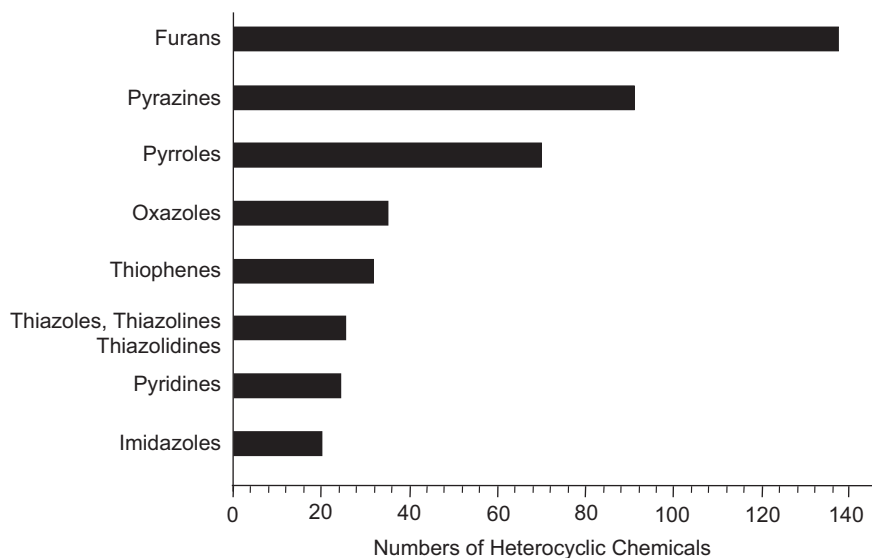
Tremendous numbers of heterocyclic flavor compounds have been found in brewed coffee (Fig. 8) and their role in characteristic brewed coffee flavors has been reported in many references (Goldman et al., 1967; Vitzthum and Werkhoff, 1976; Tressl and Silwar, 1981; Shibamoto et al., 1981; Shibamoto, 1991; Flament and Bessiere-Thomas, 2002). The numbers of such compounds found must keep increasing as more studies are conducted. To date, the most abundant compounds are furans followed by pyrazines, pyrroles and sulfur-containing compounds. This trend is exactly the same as that of the number of heterocyclic compounds found in the Maillard reaction as shown in Fig. 2. It is obvious from these reports that pyrazines and thiazoles are strongly associated with roasted or toasted flavors of heat-treated foods and beverages.

### Degradation Products From Chlorogenic Acids

As mentioned above, chlorogenic acids reduce significantly during roasting (refer to Fig. 6). Chlorogenic acids are naturally occurring flavonoids and more than 70 isomers have been found in various natural plants including coffee beans, fruits, and vegetables (Upadhyay and Mohan Rao, 2013). They are divided into three groups: caffeoylquinic acids (CQA), dicaffeoylquinic acids (diCQA) and feruloylquinic acids (FQA). They are esters of quinic acid and caffeic acid and each moiety possesses unique nature as shown in Fig. 9. The nine chlorogenic acids found in green coffee beans are shown in Fig. 10 (Fujioka and Shibamoto, 2007). Total amounts of chlorogenic acids in four commercial green beans ranged from  $86.42 \pm 2.04 \text{ mg g}^{-1}$  to  $61.15 \pm 1.40 \text{ mg g}^{-1}$ . When these green beans were roasted under light roast condition ( $230^\circ\text{C} \pm 12 \text{ min}$ ) and French roast condition ( $250^\circ\text{C} \pm 21 \text{ min}$ ), reduction rates ranged from 45.2% to 54.0% and >99%, respectively (Moon et al., 2009). These results indicate that obvious degradation of chlorogenic acids occurred in roasting. Among the 9 chlorogenic acids found in green coffee beans, the levels of 5-CQA were the greatest, ranging from  $50.7 \pm 1.61 \text{ mg g}^{-1}$  to  $40.15 \pm 0.71 \text{ mg g}^{-1}$ , which is nearly 70% of total chlorogenic acids in coffee beans. When 1 g of 5-CQA was heated at  $250^\circ\text{C}$  for 30 min, 2,5-dimethylfuran ( $59.8 \mu\text{g g}^{-1}$ ), pyridine ( $5.3 \mu\text{g g}^{-1}$ ) and 5-methylfurfural ( $1.8 \mu\text{g g}^{-1}$ ) were found. One study reported that thermal degradation of 5-CQA produced many heterocyclic compounds with strong antioxidative activity, such as phenols (Kamiyama et al., 2015). These reports indicate that many biologically active heterocyclic compounds are formed in brewed coffee.



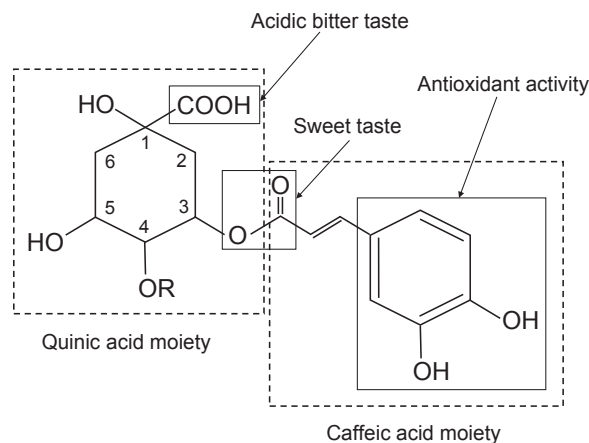
**Figure 7** Typical gas chromatograms of coffee volatiles extracted from a brewed coffee taken by three different detectors. A  $30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$  DBWAX fused silica capillary column was used. The oven temperature was held at  $50\text{ }^{\circ}\text{C}$  for 10 min and then programmed to  $200\text{ }^{\circ}\text{C}$  at  $3\text{ }^{\circ}\text{C}/\text{min}$ . The linear carrier gas flowrate was  $30\text{ cm/s}$  at split ratio of 1:40.



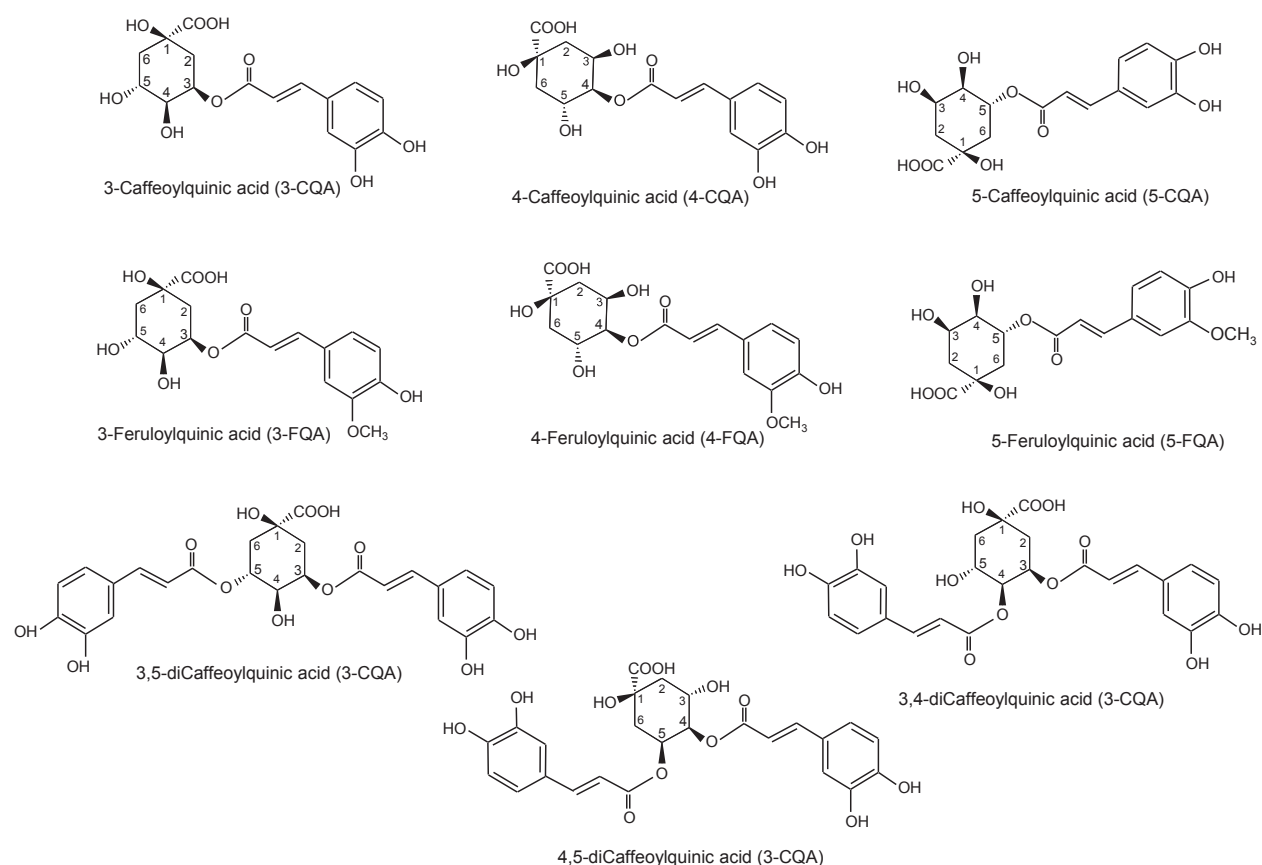
**Figure 8** Numbers of heterocyclic flavor compounds found in a brewed coffee.

#### 4(5)-Methylimidazole [4(5)-MI]

Even though formation of imidazoles was found in a Maillard reaction system consisting of a sugar and an amino acid more than 50 years ago (Tsuchida and Komoto, 1967), imidazoles have not received much attention among flavor chemists because of their lack of characteristic flavors. Fig. 11 shows proposed formation pathways of imidazoles formed in Maillard reaction model systems. In this proposal, dicarbonyls and aldehydes are formed from either sugars or lipids and ammonia is formed from amino acids



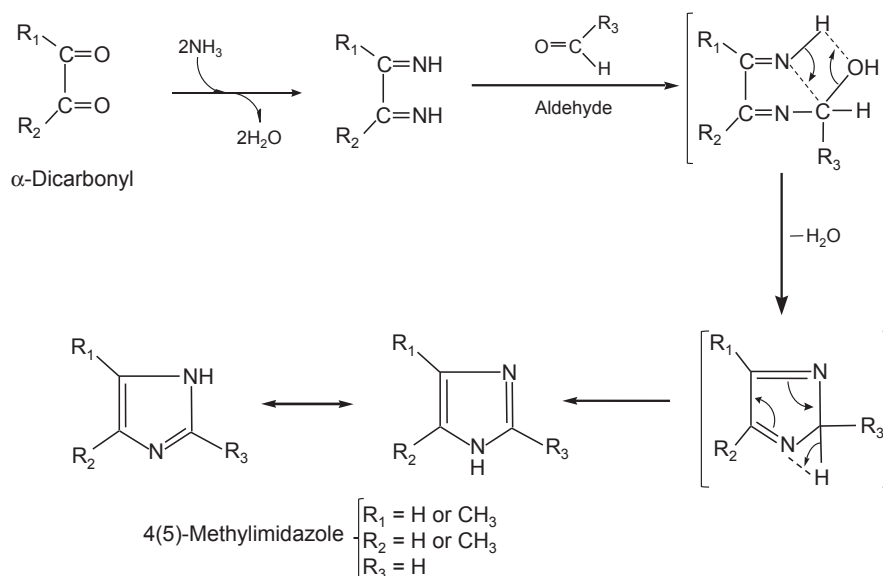
**Figure 9** Structure of chlorogenic acid and nature of each moiety.



**Figure 10** Chlorogenic acids found in green coffee beans.

produced by Strecker degradation (Strecker 1863; Shibamoto and Bernhard, 1978; Yaylayan, 2006; Shibamoto, 2014). Moreover, imidazoles were considered chemicals contributing off-flavors to cooked foods (Arctander, 1969). The time when imidazoles, in particular 4(5)-MI, had begun to receive much attention is rather recent. The National Toxicology Program (NTP) identified 4(5)-MI as a cancer-causing chemical in 2007. Subsequently, many analytical results on 4(5)-MI in foods and beverages, including coffee and colas, as well as in Maillard reaction systems have been reported (Hengel and Shibamoto, 2013).

Mainly, 4(5)-MI forms in caramel products, which are prepared with a sugar and ammonia. There are four types of caramel color. Types I and II are prepared from carbohydrates without ammonia, whereas Types III and IV are prepared from carbohydrates with ammonia. It is obvious that these products are produced by Maillard reaction systems. In particular, Type IV has been used commonly for soft drinks, such as colas, and beer. Levels of 4(5)-MI in beverages were summarized in one review article (Hengel



**Figure 11** Proposed Formation Pathways of Imidazoles.

and Shibamoto, 2013). The levels of 4(5)-MI in caramel color Types III and IV ranged from 0.025 ppm to 463 ppm and from 0 to 1276 ppm. Brand colas analyzed for 4(5)-MI in 2009 and 2013 contained from 188 ng mL<sup>-1</sup> to 613 ng mL<sup>-1</sup> and from 7 ng mL<sup>-1</sup> to 28 ng mL<sup>-1</sup>, suggesting that cola manufacturers have tried to reduce the levels of 4(5)-MI. On the other hand, the levels of 4(5)-MI in non-colored soft drinks ranged from 6.7 ng mL<sup>-1</sup> to 44 ng mL<sup>-1</sup> in 2009 and from 1.7 ng mL<sup>-1</sup> to 5.5 ng mL<sup>-1</sup> in 2013. Selected brand beers contained 4(5)-MI from 0 to 424 mg mL<sup>-1</sup> in dark beer (Cunha et al., 2011). Also, selected roasted-brand coffees had 4(5)-MI in the levels ranging from 0.307 ppm to 2.05 ppm (Casal et al., 2002; Klejdus et al., 2006).

After NTP reported that 4(5)-MI was a possible carcinogen in 2007, many studies on the toxicity, including carcinogenicity (Chan et al., 2008), of 4(5)-MI have been conducted using various experimental animals (Hengel and Shibamoto, 2013). According to these reports, California State Proposition 65 set its acceptable daily intake (ADI) as 16 µg day<sup>-1</sup>. However, it is extremely difficult to set ADI for chemicals naturally present or formed during processing in foods and beverages, such as 4(5)-MI and acrylamide. For example, by a simple calculation, one cup (150 mL) of coffee contains 4(5)-MI at levels ranging from 9.62 µg mL<sup>-1</sup> to 273.15 µg mL<sup>-1</sup> (Lee and Lee, 2016), most of which exceed the ADI set by Proposition 65.

## Conclusions

Heterocyclic compounds formed from the Maillard reaction possess characteristic cooked or roasted flavors. Among over 1200 heterocyclic compounds found in Maillard reaction systems, furans and pyrazines play an important role in the palatability of beverages. Many heterocyclic compounds, either synthetic or Maillard reaction products, have been used to prepare preferable beverages. Beverages which require freshness do not generally benefit from the formation of heterocyclic compounds but some naturally occurring heterocyclic compounds, such as furfurals and methoxypyrazines, contribute important flavors to beverages, such as fruit and vegetable juices. On the other hand, the most popular beverage, coffee, requires various heterocyclic compounds, the formation of which is strongly associated with the Maillard reaction. Recently, the biological activities of heterocyclic compounds, such as antioxidants, have begun to receive much attention in addition to their flavors. Therefore, there is still much research to be performed on the heterocyclic compounds found in foods and beverages.

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## Xanthine Oxidase in Dairy Foods

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### Nomenclature

XOR xanthine oxidoreductase

XDH xanthine dehydrogenase

FAD flavin adenine dinucleotide

H<sub>2</sub>O<sub>2</sub> hydrogen peroxide

RSNO S-Nitrosothiols

MFGM milk fat globule membrane

NAD<sup>+</sup> nicotinamide adenine dinucleotide

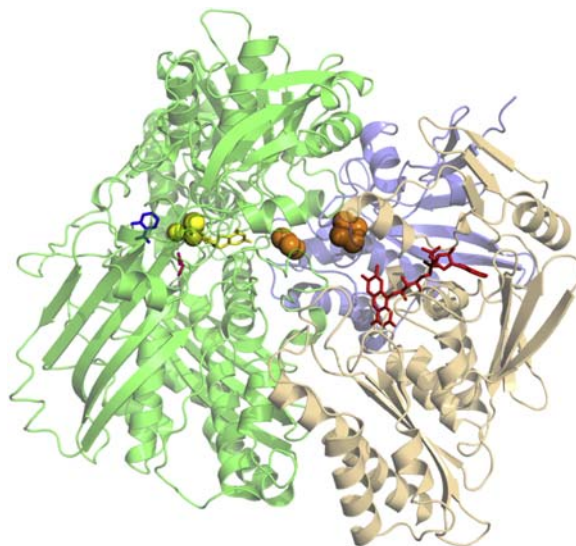
HPLC high-performance liquid chromatography

### Background

Xanthine oxidase, a complex iron-sulphur molybdenum flavoprotein enzyme and a form of xanthine oxidoreductase (XOR), is one of the most important enzymes found within the milk of mammals and is broadly disseminated amongst different species, from bacteria to humans (Ardan et al., 2004; Bray, 1975; Chang et al., 1992). Xanthine oxidase can also be obtained from xanthine dehydrogenase (XDH), through a reversible (oxidation of –SH groups to S–S bonds) or irreversible (trypsin or pancreatin proteolysis) reaction. It was more than a century ago when Schardinger (1903) identified this enzyme in the form of XOR for the first time in cow's milk. The newly discovered enzyme was later named 'xanthine oxidase' by Burian (1905).

### The Structure and Function of Xanthine Oxidase

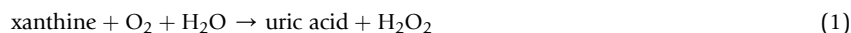
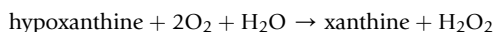
The molecule is a large protein, a dimer with a density of 290 kDa, while every 145 kDa monomer is composed of 20, 45, 85 kDa domains. As shown in Fig. 1, there are two flavin molecules (bound as flavin adenine dinucleotide; FAD) in each dimer, attached to two molybdenum atoms as well as eight iron atoms. These iron atoms, which are the important part of [2Fe–2S] ferredoxin iron-sulfur clusters, play an important role in the electron transfer reactions as part of the ferredoxin iron–sulfur clusters (Harrison, 2002). The molybdenum atoms, which are considered as the active sites of xanthine oxidase, are bounded as molybdopterin



**Figure 1** The chemical structure of xanthine oxidase. Bounded FAD (red), FeS-cluster (orange), molybdopterin cofactor with molybdenum (yellow), and salicylate (blue). Obtained from Wikipedia at [https://en.wikipedia.org/wiki/Xanthine\\_oxidase](https://en.wikipedia.org/wiki/Xanthine_oxidase).

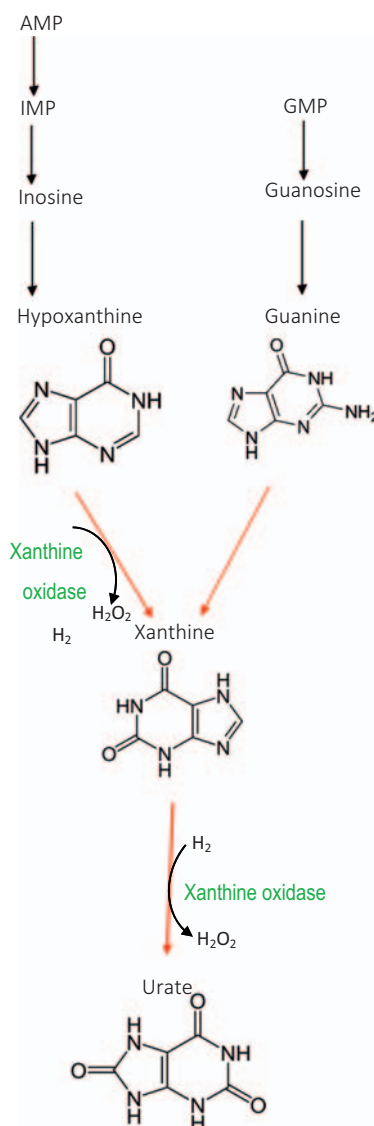
cofactors. In addition to the molybdopterin unit with the molybdenum atom in the active site of xanthine oxidase, there are sulfur atoms and a terminal OH group (Hille, 2006).

Xanthine oxidase plays a significant role in the catabolism of purines, pyrimidines, petrin, and a lot of aldehyde substrates in mammals including humans (Enroth et al., 2000; Harrison, 2002). Xanthine oxidase is the enzyme responsible for the oxidation of xanthine to urate, before which xanthine is formed from the oxidation of hypoxanthine. XOR and xanthine oxidase catalyze the oxidation of hypoxanthine to xanthine and then xanthine is catalyzed to uric acid by the same enzymes (Eq. 1).



The mechanism by which xanthine oxidase catalyzes the oxidation of hypoxanthine and xanthine along with the chemical structure of these purins and the end product (urate) is also represented in Fig. 2. To convert xanthine to uric acid (the second reaction in Eq. 1), an oxygen atom has to be transferred from molybdenum to xanthine, and so there are several intermediates involved in such a reaction. When water is present, the reformation of the active molybdenum center happens and the oxygen atom (introduced to the substrate by xanthine oxidase) originates from water not from dioxygen ( $\text{O}_2$ ).

Xanthine oxidase can also catalyze the decomposition of S-Nitrosothiols (RSNO), which are considered as reactive nitrogen species. Xanthine oxidase converts RSNO to nitric oxide, by which the formation of peroxynitrite under aerobic conditions happens (Trujillo et al., 1998). In addition, it has been reported (Bonini et al., 2004) that in the presence of catalase and bicarbonate, this



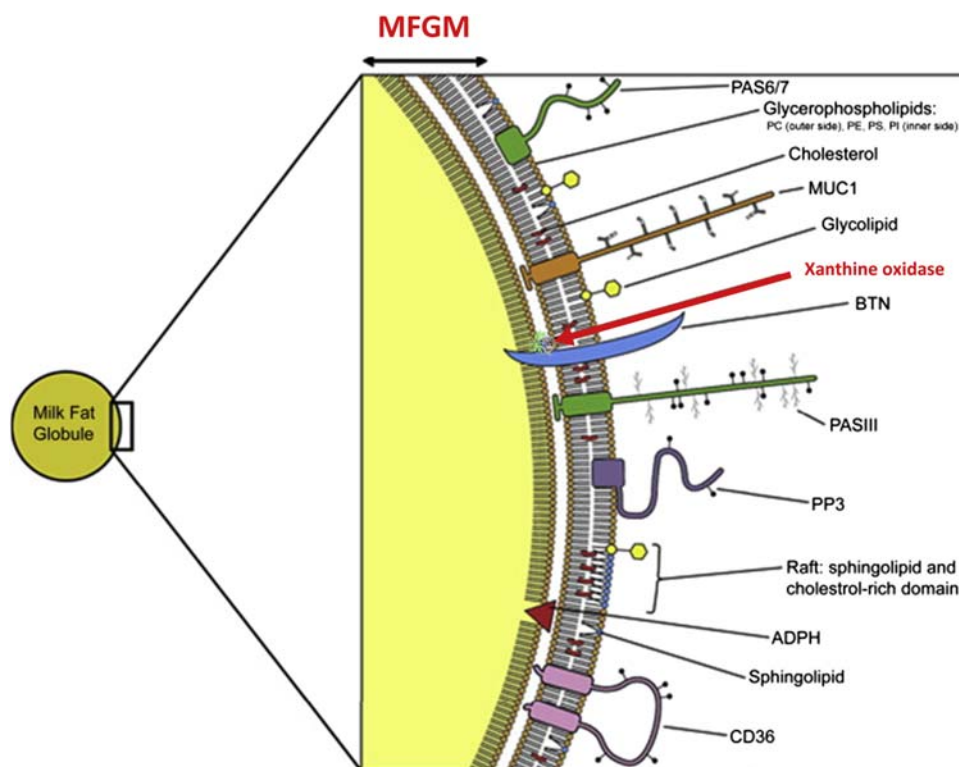
**Figure 2** The main catalization mechanism of purins by xanthine oxidase.

enzyme can contribute to the production of the strong one-electron oxidant carbonate radical anion (from oxidation with acetaldehyde). In regards with the effects of the processing conditions such as heat treatment, it has been reported that xanthine oxidase aggregates at heating temperatures about 60 °C, when heated for 10 min or more (Ye et al., 2002). In particular, pasteurization of raw milk can inactivate xanthine oxidase, and consequently affect the flavor quality of the product(s) (Walstra et al., 2005).

### Quantification of Xanthine Oxidase in Milk and Dairy Foods

Apart from the existence of xanthine oxidase in other tissues and organs, there has been an interest in the form of the enzyme found in cow's milk (within the fat globule membranes) for a long time (Battelli et al., 1973). It was found that this enzyme represents approximately 8% of the intrinsic proteins existing in the milk fat globule membrane (MFGM) (Briley and Eisenthal, 1975), while now it is believed that this enzyme accounts for about 20% of the total proteins in MFGM (Singh, 2006). The location of xanthine oxidase within the MFGM trilayer has not been fully understood yet, and since the enzyme is recovered after washing raw cream with  $MgCl_2$ , it is suggested that this enzyme is a peripheral protein (Mather and Keenan, 1975). The suggested location of xanthine oxidase within MFGM is shown in Fig. 3 (Gallier et al., 2014). This type of enzyme (oxidase type found in cow's milk) can be converted into a  $NAD^+$ -dependent dehydrogenase; however, this conversion happens to a small extent as XDH is inhibited by  $NADH$ , due to its competition with  $NAD^+$  (Enroth et al., 2000; Harrison, 2002).

When studying the enzymology of milk and dairy products, the quantification of xanthine oxidase present in different types of milk and dairy products is very important. There are some old published standard methods, which are based on measuring the factors such as oxygen uptake and coupling of xanthine oxidation to the reduction of a dye or cytochrome C (Gudnason and Shipe, 1962; Greenbank and Pallansch, 1962; Zikakis and Treece, 1971). However, such methods only estimate the apparent activity of the enzyme (xanthine oxidase) and are not an accurate quantification. Among all, the most appropriate method used for measuring the activity of xanthine oxidase in milk and dairy products is the old reference method of Cerbulis and Farrell Jr (1977). Until very recently, this method was still considered as a standard technique for measuring xanthine oxidase activity in dairy products (Wiking et al., 2003; Kathiriarachchi et al., 2014; Aboul-Enein et al., 2003; Sharma et al., 2014). Briefly, the sample is mixed into a phosphate buffer (pH 7.4) and water is added before adding a standard solution of xanthine as a xanthine oxidase substrate. The mixture is then incubated at 25 °C for 5–6 min followed by addition of an acid (trichloroacetic acid, 20%, w/v) in order to stop the reaction. The sample is then centrifuged and the solid phase is separated from the liquid phase. To determine the apparent concentration of xanthine oxidase, the absorbance of the accumulated uric acid in the liquid phase is measured at 290 nm and the activity is expressed as milliunits per mL of sample ( $mU\ mL^{-1}$ ) (Cerbulis and Farrell Jr, 1977), equal to  $nmole/min/mL$ .



**Figure 3** The location of xanthine oxidase within the milk fat globule membrane (MFGM). Modified from Gallier et al. (2014).

**Table 1** Xanthine oxidase activity of different milk and dairy foods as measured by the developed HPLC method

Sample	Decrease of initial xanthine/ $\text{mmol min}^{-1}$	Fat content (%)
Raw milk	$4.72 \pm 0.13$	5.2 <sup>1</sup>
Commercial full-fat milk	$2.77 \pm 0.09$	3.3
Commercial low-fat milk	$1.78 \pm 0.15$	0.5
Unwashed raw cream	$27.9 \pm 1.8$	ND
Washed raw cream	$34.1 \pm 1.1$	ND
Commercial cream	$23.9 \pm 1.2$	36.9
Jarlsberg cheese	$25.0 \pm 1.1$	27.1
Parmesan cheese	$20.6 \pm 1.1$	29.9
Mozzarella cheese	$12.0 \pm 1.0$	22.3
Blue vein cheese	$8.3 \pm 0.6$	29.8
Edam cheese	$13.7 \pm 1.1$	27.8

ND: not determined.

Modified from Rashidinejad et al. (2016).

Although this method can be a fast and convenient technique for measuring xanthine oxidase, recently, it was challenged and replaced by an accurate HPLC method (Rashidinejad et al., 2016) for the following reasons. First and foremost, as the method by Cerbulis and Farrell Jr (1977) relies on the absorbance of uric acid formed at 290 nm, we can say that this technique is only an apparent estimation of the activity of xanthine oxidase in the corresponding samples. The second reason is related to the assumption made regarding measuring the absorbance of urate at 290 nm, which does not consider other compounds in the sample which may be present in the liquid phase and contribute to the absorbance at 290 nm (e.g. water-soluble peptides, amino acids, and phenolic compounds). The next reason is that obtaining a clear supernatant containing urate, as reported by other researchers, using a conventional centrifugation process (e.g. 2000×g for 10 min) at temperatures ranging from 5–25°, is not possible since the supernatant contains a fat layer sitting on top of the sample, above an almost a clear phase in the middle of the centrifugation tube (i.e. the middle phase) (Rashidinejad et al., 2016). Possibly, the previous researchers have measured the absorbance of the middle phase, not the supernatant. The supernatant may be contaminated with fat globules, which will interfere with the spectrophotometric analysis. Further, if there has been any filtration or similar processes involved in order to separate the clear phase (i.e. the middle phase) from the supernatant (containing fat), this is not reported by the researchers who used the method. Last but not least, as xanthine itself is used as a substrate for this enzymatic assay established by Cerbulis and Farrell Jr (1977), some of the xanthine may remain as the unreacted substrate, which interestingly, shows a relatively strong absorbance at 290 nm (Rashidinejad et al., 2016). HPLC analysis showed that the maximum peak for urate could be obtained at 298 nm, not 290 nm (Rashidinejad et al., 2016). Therefore, measuring the absorbance of even a very clear phase after centrifugation could be giving a total absorbance from both the remaining substrate (xanthine) and the generated product (urate), thus confounding the analysis.

The improved HPLC method was able to quantify the amount of both substrate (xanthine) and the product (urate) in a variety of milk, cream, and cheese samples (Table 1). Validation, linearity, and reliability of this newly developed method were assessed by testing different concentrations of xanthine and uric acid in standard solutions and plotting the corresponding standard curves. The response of xanthine oxidase to xanthine was also investigated and the linearity tested. This is an isocratic HPLC method with short retention times for xanthine and urate (6.76 min and 4.92 min, respectively), meaning that the method is both fast and convenient for quantification of the activity of xanthine oxidase in milk and dairy products.

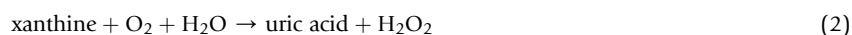
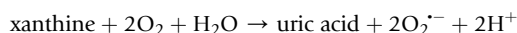
## The Effect of Xanthine Oxidase on Flavor Development in Dairy Products

Most of the aldehydes present in cow's milk, such as acetaldehyde, hexanal, propanal, *trans*-2-nonenal, *trans*-2-heptenal, and *trans*-2-hexenal are considered as substrates for xanthine oxidase. Accordingly, xanthine oxidase is known as a substantial factor contributing to oxidative reactions in milk (Morpeh, 1983; Bounds and Winston, 1991; Steffensen et al., 2002). The association between the activity of xanthine oxidase and oxidative deterioration of milk and dairy foods and the effect of such enzymatic activity on the development of the off-flavors in milk and dairy products has been investigated long ago (Aurand et al., 1967; Aurand and Woods, 1959). It was observed that a high level of xanthine oxidase was associated with the occurrence of a spontaneously-oxidized flavor in milk and the inhibitors of this enzyme could prevent the off-flavor development (Aurand et al., 1967; Aurand and Woods, 1959). As xanthine oxidase induces oxidation of the aforementioned aldehydes and produces different acids in milk and dairy foods (Harrison, 2006; Anderson and Brooker, 1975), it can lead to some considerable changes in the amounts and/or ratios of volatile acids and aldehydes in these products (Kathiriarachchi et al., 2014). This correspondingly, may affect the flavor development in dairy products, such as cheese (Rashidinejad et al., 2016). Xanthine oxidase has also been reported to interact with compounds such as minerals (e.g. copper), zinc superoxide dismutase, polyphenols, arsenate, and nanoparticles (Hille et al., 1983; Zhou et al., 2005; Al-Hakeim et al., 2014; Chang et al., 1992; Nagao et al., 1999; Lin et al., 2002).



## Health Concerns About Dairy Foods Containing Xanthine Oxidase

One of the substantial aspects for which xanthine oxidase in bovine milk has been under consideration is the speculation regarding its contribution to the growth of illnesses such as coronary artery and gout-related diseases, as well as its contribution in oxidative damage to living tissues (e.g. liver and kidney) in the human body (Bierman and Shank, 1975; Oster, 1971; Harrison, 2006; Lin et al., 2002). This may happen via the oxidation of hypoxanthine and xanthine to urate, and correspondingly, the formation of super oxide radicals which can promote the oxidation level in the body leading to xanthine oxidase being considered as a significant biological source of superoxide radicals (Fridovich, 1970; Cos et al., 1998) (Eq. 2). Super oxide radicals (e.g. peroxynitrite), formed by xanthine oxidase, can react with DNA, cells, and proteins and cause damage/toxicity to the cells. In addition, reactive oxygen species can be coupled with reactive nitrogen signalling and contribute to a central part of myocardial and vascular function, indicating the link of excess amount of xanthine oxidase with cardiovascular health (Zimmet and Hare, 2006). Regarding gout, which may be caused by xanthine oxidase, as this enzyme is a metabolic pathway in the formation of urate, allopurinol (as the inhibitor of xanthine oxidase) is used for the treatment of the disease (i.e. gout) (Heunks et al., 1999).



On the other hand, a rare genetic disorder called ‘xanthinuria’ can also happen, where a high concentration of xanthine in blood forms due to the lack of xanthine oxidase, which accordingly may result in some serious health problems such as renal failure. Obviously, to date, there is no definite cure for this problem; doctors advise the sufferers to take a diet low in purine and intake a high amount of fluids daily (Reiter et al., 1990).

In 1971, it was strongly believed that the consumption of homogenized milk could result in excessive mortality from coronary artery disease due to the release of xanthine oxidase through homogenization process (Oster, 1971); however, this claim was not supported by the later research (Bierman and Shank, 1975). McCarthy and Long (1976) reported that xanthine oxidase activity in blood serum of pigs receiving 7.6 L of full-fat milk daily during a 100-day period of study, was not significantly ( $p > 0.05$ ) different than the pigs that did not receive any milk in their diet. Also in the case of humans, there was not seen any casual or statistically-significant association between the activity of xanthine oxidase in blood and the average daily milk consumption, age, or sex (McCarthy and Long, 1976). Interestingly, there seems to be not-enough new scientific evidence on the association between milk/dairy consumption and the activity of xanthine oxidase in human blood, or any comprehensive study to confirm a link between milk/dairy food intake and gout and/or hyperuricemia caused by xanthine oxidase in milk and its products.

Whether the new findings in the future find a relationship between the aforementioned health problems and xanthine oxidase, and correspondingly, the consumption of dairy products or not, it is crucial to have enough knowledge about the concentration of xanthine oxidase in different types of milk and dairy products. According to the newly developed HPLC method for quantification of xanthine oxidase in different dairy products (Rashidinejad et al., 2016) introduced previously (section **Quantification of Xanthine Oxidase in Milk and Dairy Foods**), it was reported that raw milk possessed a higher xanthine oxidase activity than the commercial milk. And, the commercial full-fat milk contained a higher xanthine oxidase activity than the commercial low-fat milk (Table 1). The amount of xanthine oxidase in unwashed raw cream was lower than washed raw cream; whereas, both cream samples (unwashed and washed) presented greater values for xanthine oxidase activity than for the commercial cream. Testing several different varieties of cheeses (e.g. Parmesan, Jarlsberg, Colby, Mozzarella, Blue, and Edam), it was found that Jarlsberg and Blue had the highest and lowest activities of xanthine oxidase, respectively (Table 1). The differences in the activity of xanthine oxidase found between full-fat and low-fat milk and between full-fat and low-fat cheeses, can be related to the fact that xanthine oxidase is located in the MFGM (Fox and Kelly, 2006; Harrison, 2006).

As mentioned before (section **The Structure and Function of Xanthine Oxidase**), it is believed that processing conditions of milk such as homogenization can result in the reduction of the activity of the enzyme in this product (Cerbulis and Farrell Jr, 1977). This can be the reason for a higher degree of xanthine oxidase activity in raw milk compared with commercial pasteurized milk, regardless of the different fat contents in the two samples. It has been reported that washing cream products may selectively decrease (or even remove) MFGM components, which can accordingly decrease the enzymatic activity (Kathiriarachchi et al., 2014; Zittle et al., 1956). Rashidinejad et al. (2016) stated that xanthine oxidase activity in cheese samples depended upon the cheese preparation method; however, they did not confirm if a mature cheese with a longer ripening period could contain a higher or lower activity of xanthine oxidase. It appears that the cheeses manufactured from raw milk (e.g. Jarlsberg) can show the highest xanthine oxidase activity (Cerbulis and Farrell Jr, 1977; Rashidinejad et al., 2016).

It is notable that the effect of milk processing on the activity of xanthine oxidase has been of interest for a long time. For instance, about half a century ago, Greenbank and Pallansch (1962) studied the effect of different processing methods (e.g. pasteurization, homogenization, condensation, and drying) on the activity of xanthine oxidase in milk and dairy products. These researchers (Greenbank and Pallansch, 1962) found that the heat-treatment of full-fat milk to 195 °F for 15 seconds could inactivate the xanthine oxidase activity meaningfully. However, when they condensed the same milk to 50% total solids followed by homogenization at 4500 psi, xanthine oxidase was reactivated. It was also found that when the same milk was exposed to sonic vibrations (frequency of 10,000 vibrations/s for 7.5 h), about 94% of the enzyme was inactivated.



## Conclusion

In contrast to the possible health concerns attributed to the xanthine oxidase activity in milk and dairy products, this enzyme is influential in flavour development of many of these products, both due to the negative effects from inactivation during the processing and flavorful oxidative changes during storage (e.g. during cheese ripening). The old methods for analyzing xanthine oxidase in dairy products do not provide the appropriate quantification of the enzyme, but a new HPLC method has been developed for the accurate quantification of both xanthine (substrate) and urate (product). Such quantification would be very beneficial for understanding the contribution of xanthine oxidase to flavor development in dairy products, as well as for understanding the effect of the enzyme on the occurrence of the proclaimed corresponding diseases. It appears that the processing conditions such as homogenisation and heat treatment have a substantial effect on the amount of xanthine oxidase and its activity in milk and dairy products.

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## Bioactive Peptides

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### Introduction

Bioactive peptides are derived from food proteins and exert a positive effect in humans due to its health-promoting properties. Bioactive peptides can exert several beneficial effects like preventing diseases or modulating the physiological systems once they are absorbed in the human body. There is a broad range of functions, depending on the sequence of the bioactive peptides, so that they can be involved in the gastrointestinal system such as the anti-obesity and satiety peptides, the cardiovascular system such as antihypertensive, antithrombotic, antioxidant and hypocholesterolemic peptides, the immune system such as antimicrobial, cytomodulatory and immune-modulatory peptides, and the nervous system such as opioid peptides.

Bioactive peptides usually contain between 3 and 20 amino acid residues and remain inactive while the sequences are kept within the parent protein. They are active once released by enzymatic hydrolysis by peptidases during food processing and/or during gastrointestinal digestion. In order to exert a positive health effect, bioactive peptides must cross the gastrointestinal (GI) barrier and survive enzyme degradation.

Numerous bioactive peptides have been reported in recent years as naturally present or generated from food proteins of different origins like milk, eggs, soya, fish, and meat. In this sense, the most extensively studied bioactivity during the last decade has been the antihypertensive activity through the measurement of angiotensin I-converting enzyme (ACE) inhibitory activity. The reason for this interest is mainly because high blood pressure is one of the major, independent risk factors for cardiovascular diseases, and the main reason of death in developed countries. Peptides with this type of activity are generically termed as bioactive peptides even though other activities such as antioxidant, antimicrobial, opioid, antithrombotic, antidiabetic, etc ... also fit into the general bioactive peptide term.

### Bioactive Peptides From Food Proteins

There are sequences of bioactive peptides that remain inactive while forming part of the parent proteins but, once released, they exert its bioactivity. Bioactive peptides can be released during food processing either by spontaneous endogenous enzymatic hydrolysis of food proteins, or by controlled hydrolysis with commercial peptidases. Bioactive peptides can also be released during the gastrointestinal digestion of the ingested foods. However, the released bioactive peptides must remain intact through gastrointestinal digestion and must be absorbed intact when crossing the intestinal barrier and reach the blood stream to exert their physiological action. All these pathways of generation are schematized in [Fig. 1](#) and briefly summarized below.

#### Bioactive Peptides Naturally Generated From Food Proteins

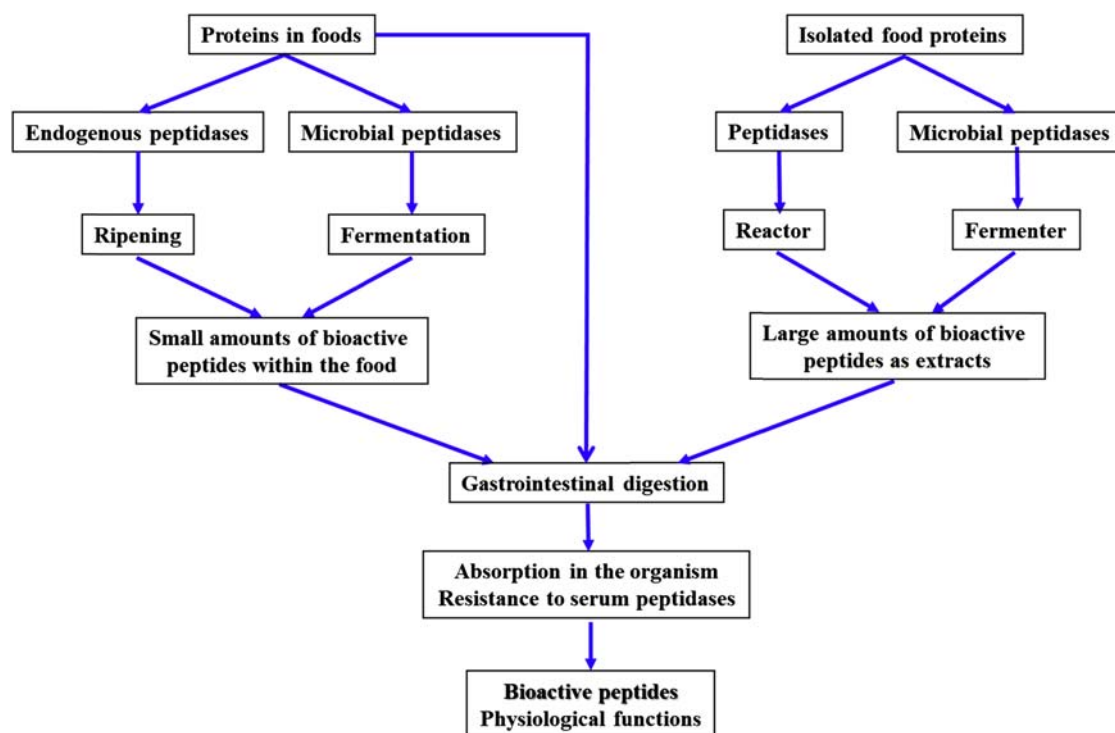
There are several pathways for the release of bioactive peptides from food proteins during processing. All of them involve the presence of endogenous endo- and exo-peptidases and adequate processing conditions for such enzymes to be active. Under these circumstances, bioactive peptides are released from meat and fish proteins which are hydrolyzed during ageing or ripening at mild temperatures. In other cases, bioactive peptides can be released by a combined action of endogenous and microbial peptidases, like in fermentation or curing processes of meat, fish and dairy products (i.e., dry fermented sausages, dry-cured ham, cured cheese, yogurt, etc.). Some lactic acid bacteria contribute with active endo- and exo-peptidases.

#### Bioactive Peptides Generated Through Hydrolysis of Food Proteins With Commercial Proteolytic Enzymes

The most usual way to produce bioactive peptides consists of the controlled hydrolysis of specific food proteins by commercial peptidases from animal, plant or microbial sources, or combinations of these. Main peptidases used for protein hydrolyzates are papain, thermolysin, Alcalase, Neutrase, Flavourzyme, or Actinase E, and its characteristics are compiled in [Table 1](#). In some cases, fermentations with Generally Recognised As Safe (GRAS) bacteria such as *Lactobacilli* also contribute to large generation of bioactive peptides. In most cases, proteins from food by-products constitute the substrate for obtaining enriched bioactive peptides fractions, so that an added-value is obtained while reducing the environmental impact. The resulting bioactive peptides must be extracted, partially purified, characterized or identified, and the *in vitro* and *in vivo* bioactivity determined. A scheme for the full process is shown in [Fig. 2](#).

#### Bioactive Peptides Generated Through Gastrointestinal Digestion of Ingested Food Proteins

Bioactive peptides that are encrypted into the protein structure may be released during gastrointestinal digestion by peptidases such as pepsin, trypsin and chymotrypsin. The routes of generation are usually confirmed in laboratory by using specific proteins and



**Figure 1** Scheme of the generation of bioactive peptides from protein hydrolysis in foods and/or the hydrolysis of isolated food proteins. Reproduced with permission from Toldrá, F., Reig, M., Aristoy, M.C. & Mora, L. Generation of bioactive peptides during food processing. Food Chemistry, 2018, in press. <https://doi.org/10.1016/j.foodchem.2017.06.119>.

simulated ingestion and gastrointestinal digestion with trypsin and/or chymotrypsin reproducing the conditions of all steps in real digestion. The bioactivity of the generated peptides is usually assayed *in vitro* but it is really necessary to perform additional *in vivo* assays with animals to verify such physiological effect.

### Functional Activity of Bioactive Peptides

Bioactive peptides exert its physiological effect when they are consumed. Once ingested, peptides are susceptible to be hydrolyzed by enzymes involved in the gastrointestinal digestion. Smaller peptides are released either by brush border membrane peptidases, intracellular peptidases or peptidases from the intestinal microbial flora. A requirement for peptides to be bioactive is that they must remain intact during their transport in the intestine, and be able to cross the intestinal membrane and be transported intact into bloodstream for reaching their target sites, and exert their physiological benefit. Different types of activity have been reported in the literature (see Fig. 3), and the most relevant are summarized below.

#### ACE Inhibitory Activity Peptides

These peptides are inhibitors of the angiotensin-1 converting enzyme (ACE or ACE-1) and can thus prevent hypertension. ACE has a relevant role in the regulation of blood pressure in the renin-angiotensin system because this enzyme converts the inactive decapeptide angiotensin I into the potent vasoconstricting octapeptide angiotensin II and this results in an increase in blood pressure (see Fig. 4). Such enzyme is also able to inactivate bradykinin. So, ACE inhibitory peptides can reduce the blood pressure a few hours after oral administration through the inhibition of ACE that is a dipeptidyl carboxypeptidase usually membrane-bound in vascular endothelial cells.

The intensity of ACE inhibition by specific peptides is measured through respective  $IC_{50}$  values but they do not always correlate with their real antihypertensive physiological effect in the body. Sometimes, promising peptides with low  $IC_{50}$  values are inactive by oral administration because they may be hydrolyzed during gastrointestinal digestion into smaller peptides with poorer inhibitory activity on ACE enzyme.

There are hundreds of ACE inhibitory peptides reported in the literature from many types of hydrolyzed foods like milk, fish, meat, eggs, soybean, corn, wheat, seaweed, etc. Some of them have been assayed for *in vivo* antihypertensive activity (see Table 2). Such assays use spontaneously hypertensive rats (SHR) who are orally administered the peptides and its antihypertensive effects are followed after several hours of ingestion. Fig. 5 shows an example of such *in vivo* assay with 3 different peptides. The antihypertensive effect of such peptides was clearly established with spontaneous hypertensive rats, that showed a decrease of systolic blood pressure within the range 25–35 mm Hg after 6 h of oral administration (Fig. 5).

**Table 1** Examples of commercial peptidase preparations for proteins hydrolysis

Enzyme preparation	Origin	Manufacturer	Activity
Flavourzyme 1000M	<i>Aspergillus oryzae</i>	Novozymes	3 endopeptidases, 2 aminopeptidases, 2 dipeptidylpeptidases, 1 $\alpha$ -amylase
Valkerase	<i>Bacillus licheniformis</i>	Bri Enzymes	Keratinase, Serin Endopeptidase
Prolidase	<i>L-lactis cremoris</i> Other many sources		Dipeptidase
Biopraser SP-20FG	<i>Bacillus sp</i>		Subtilisin, Endo metalloprotease, Aminopeptidase
Neutrase	<i>Bacillus subtilis</i> <i>B. amyloliquefaciens</i>	Novozymes	Metalloprotease
Alcalase 2.4 L	<i>Bacillus licheniformis</i>	Novozymes	Subtilisin, Alkaline serin endopeptidase, Extracellular neutral metallo protease, Aminopeptidase
Esperase	<i>Bacillus lentus</i>	Novozymes	Subtilisin, Alkaline serin endopeptidase
Protamex	<i>Bacillus licheniformis</i> <i>Bacillus amyloliquefaciens</i>	Novozymes	Subtilisin, Serin endopeptidase, Metallo endopeptidase Neutral protease
Protex 6L	<i>Bacillus licheniformis</i>	Genencor	Alcaline serine endopeptidase
Protease N-01	<i>Bacillus subtilis</i>	ASA Spezialenzyme GmbH	Endoprotease
Protease M			Endo & Exo
Promod 439L	<i>Bacillus licheniformis</i>	Biocatalysts	Subtilisin
Pronase	<i>Streptomyces griseus</i>	Sigma-Aldrich	Endo & Exo
Corolase 7089	<i>Bacillus Subtilis</i>	AB Enzymes GmbH	Neutral Endopeptidase
Corolase PP	<i>Porcine pancreatic gland</i>	AB Enzymes GmbH	Endopeptidase, Amino- & carboxypeptidase
Corolase 2TS	<i>Bacillus thermoproteolyticus</i> <i>Bacillus stearothermophilus</i>	AB Enzymes	Endopeptidase
GC710		Genencor Biotech	Neutral proteinase
GC106	<i>Aspergillus niger</i>	Genencor Biotech	Acid proteinase Aspartic-type peptidase
Pancreatic trypsin Novo	<i>Porcine pancreatic glands</i>	Novozymes	Endopeptidase
Trypsin	<i>Bovine pancreas</i>	Sigma-Aldrich	Endopeptidase
Chymotrypsin A	<i>Bovine pancreas</i>	Sigma-Aldrich	Endopeptidase
Chymotrypsin C	<i>Bovine pancreas</i>	Sigma-Aldrich	Endopeptidase
Pepsin	<i>Porcine Gastric mucosa</i>	Sigma-Aldrich	
Alkaline protease	<i>Bacillus licheniformis</i>	Genencor	Serine-type peptidase
Seabzyme L 200	<i>Carica papaya</i>	Speciality Enzymes & Biotechnologies	Endoprotease
Bromelain	<i>Pineapple stem</i>	Great food (Biochem)	Cysteine-type peptidase
Collupulin	<i>Carica papaya</i>	Gist-brocades	Cysteine-type peptidase
Papain	<i>Carica papaya</i>	Sigma-Aldrich	Cysteine-type peptidase
Ficin (ficin)	<i>Figs latex</i>	Sigma-Aldrich	Cysteine-type peptidase

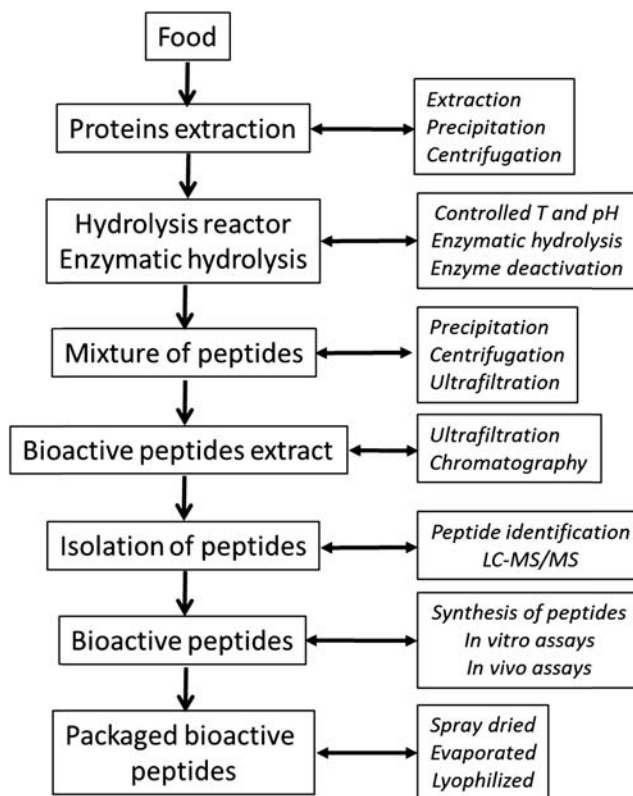
## Opioid Peptides

Opioid peptides have a good affinity for an opioid receptor and are thus able to exert opiate-like effects by affecting the nerve system and gastrointestinal functions. Peptides from exogenous sources are denominated exorphins. The stability and lower side effects are the main advantages of food derived opioid peptides when compared to endogenous and synthetic opioid peptides. Such peptides are usually characterized by a tyrosine residue at the amino terminal end and an aromatic residue located in the third or fourth position. Exorphins have been reported in hydrolyzates from milk casein, wheat gluten and blood hemoglobin.

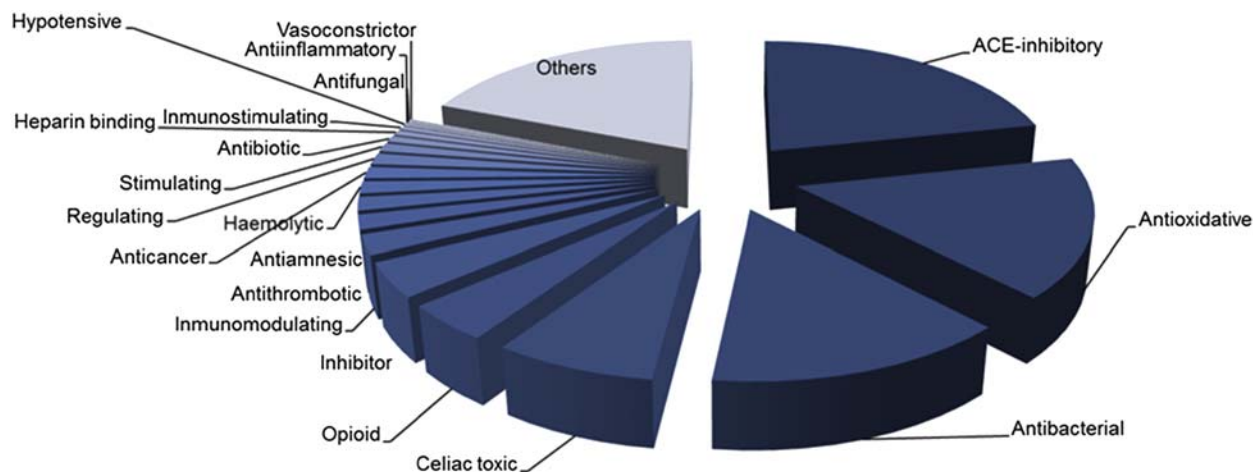
## Antioxidant Peptides

Depending on the chemical reactions involved, antioxidant activity of peptides can be classified into two groups: i) those peptides able to reduce free radicals by hydrogen donation in a competitive reaction, and ii) those peptides able to transfer one electron to reduce an oxidant. The methods used in the first case are oxygen radical absorbance capacity assay (ORAC), total radical trapping antioxidant parameter (TRAP) and  $\beta$ -carotene bleaching assay, while in the second case are 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging assay, ferric-reducing antioxidant power, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.

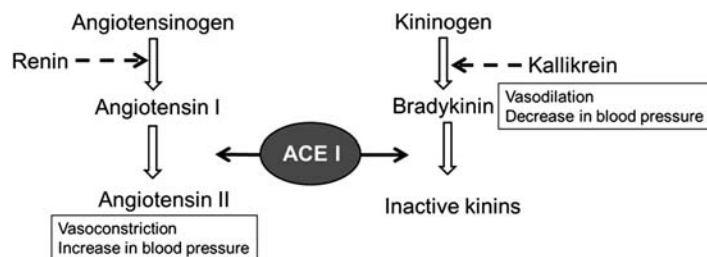
Antioxidant peptides may contribute to decrease the risk of cardiovascular disease as well as certain types of cancer. Such peptides prevent the formation of free radicals or introduce substances that compete for the existing radicals. Most of them have



**Figure 2** Flow diagram for the generation of bioactive peptides through the enzymatic hydrolysis of food proteins.



**Figure 3** Activity profile of main bioactive peptides included in the free access BIOPEP database.

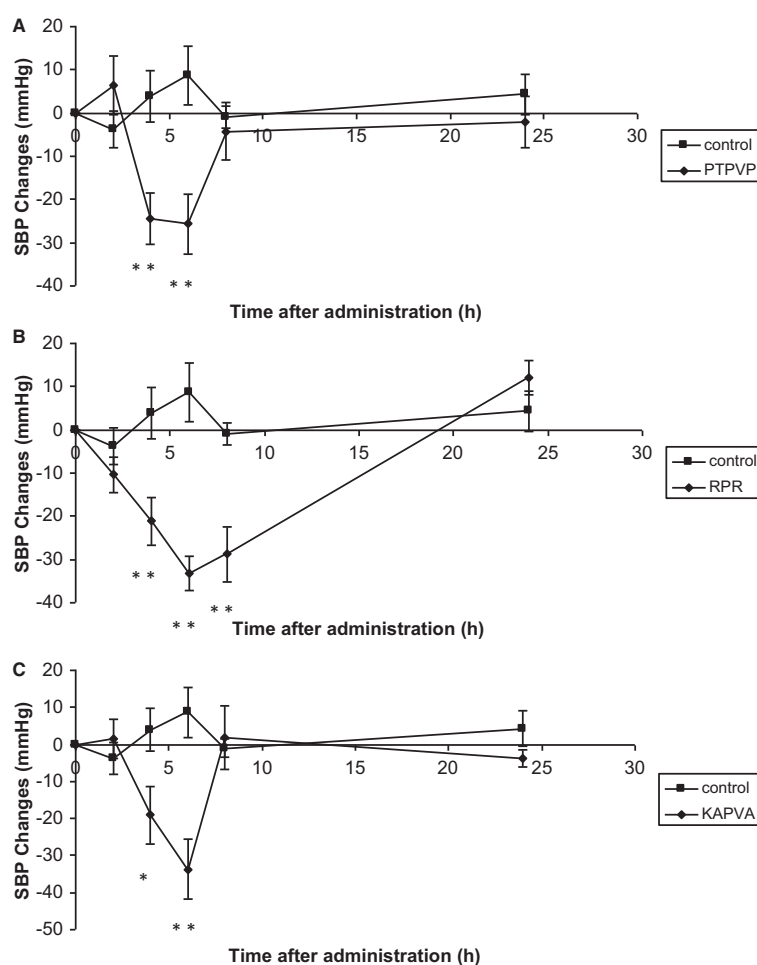


**Figure 4** Brief description of the Renin-Angiotensin system, the main blood pressure and water balance system in the human body. Reproduced with permission from Mora, L., Aristoy, M.C. & Toldrá, F. (2016) Bioactive peptides in foods. In: Encyclopedia of Food and Health (B. Caballero, P.M. Finglas and F. Toldrá, Eds.), Academic Press/Elsevier Science Ltd., London, UK, vol. 1, 395–400.



**Table 2** Examples of ACE inhibitory peptides with *in vivo* antihypertensive activity assayed on spontaneously hypertensive rats

Food	Enzymatic generation	Peptide sequence	IC <sub>50</sub> (μM)	Dose (mg/kg)	SBP decrease (mmHg)	References
Dry cured Ham	Muscle endogenous enzymes	AAATP	100	1	−25.6	Escudero et al., 2013
Goat meat	Protamex + Flavourzyme	FQPS	27	2.4	−10.6	Mirdhayati et al., 2016
Salmon skin	Trypsin	GLP	9.08	20	−31	Lee et al., 2014
Egg	Thermolysine + pepsin	IQW	2.9	15	−19	Majumder et al., 2015
		LKP	1.4	15	−30	
Whey protein	Alcalase + trypsin	IW	0.7	19	−7	Martin et al., 2015
Bovine lactoferrin	Pepsin	LIWKWL	0.47	10	−25.3 (24H)	Ruiz-Giménez et al., 2012
		RPYL	56.5	10	−18.9 (1H)	
		LNNSRAP	105.3	10	−15.3 (1H)	
Cattlefish	Bacillus mojavensis A21 & cuttlefish hepatopancreas enzymes	VELTP	5.22	10	−20	Balti et al., 2015
Rice	Alcalase + Corolare PP	TQVY	18.2	30	−40	Li et al., 2007



**Figure 5** Antihypertensive effect of single oral administration of ACE inhibitory peptides PTPVP, RPR and KAPVA. Each point indicates the mean of systolic blood pressure of 8 SHRs, and the vertical bars represent the standard error. Treatment in each case was control (distilled water) and (a) peptide PTPVP; (b) peptide RPR; (c) peptide KAPVA; dose 1 mg peptide/kg B.W, respectively. Significant difference from control at each time: \* $P < 0.05$  and \*\* $P < 0.01$ . Reproduced with permission from Escudero, E., Toldrá, F., Sentandreu, M.A., Nishimura, H., and Arihara, K. (2012) Antihypertensive activity of peptides identified in the *in vitro* gastrointestinal digest of pork meat. Meat Science, 91, 382–384.

between 4 and 16 amino acids, and the molecular mass ranges from 400 to 2000 Da. Radical-scavenging properties have been reported in peptides containing tyrosine, tryptophan and phenylalanine. If aromatic amino acids are present in peptides at the water–lipid interface, such peptides can get access to scavenge free radicals from the lipid phase.

**Table 3** Examples of antioxidant peptides generated in foods or food hydrolyzates

Food	Enzymatic generation	Assay used	Peptide sequence	References
Tomato seeds	<i>Bacillus subtilis</i>	DPPH	GQVPP	Moayedi et al., 2017, 2018
Rice	Alcalase, Flavourzyme Protamex, Pepsin, Papain, Trypsin	DPPH, ABTS, FRAP	RPNYTDA TSQLLSDQ TRTGDPPF NFHPQ	Yan et al., 2015
Pine nut	Alcalase	DPPH, Ferric reducing power. ABTS radical, cellular antioxidant activity	KWFCT QWFCT	Yang et al., 2017
Chicken egg white	Protease P	ORAC	AEERYP DEDTQAMP	Nimalaratne et al., 2015
Chicken egg white	Alcalase	ORAC/DPPH	DHTKE FFGFN MPDAHL	Liu et al., 2015
Fish (sardinelle)	<i>Bacillus subtilis</i> & <i>Bacillus amyloliquefaciens</i>	DPPH, Ferric reducing power	NVPVYEGY SLEAQAEKY GTELDLKY	Jemil et al., 2016
Fish (carp head)	Pepsin	DPPH	MKAVCFSL	Zhang et al., 2017
Oyster	<i>Bacillus cereus</i> protease	DPPH, Ferric reducing power	LANAK PLSVGRPPVGKLT VKVLEHPVL	Umayaparvathi et al., 2014
Dry-cured ham	Endogenous enzymes	DPPH, Ferric reducing power	SNAAC	Mora et al., 2014
Dry-cured ham	Endogenous enzymes	DPPH, Ferric reducing power	SAGNPN, GLAGA	Escudero et al., 2013, Gallego et al., 2018
Dry-fermented sausage	Microbial fermentation	DPPH and Hydroxyl radical-scavenging activity	GYP	Ohata et al., 2016

Several antioxidant peptides have been reported in hydrolyzates of soybean, milk, eggs, fish and meat proteins. An example of some of the identified antioxidant peptides in a variety of foods or food hydrolyzates are shown in **Table 3**. They can be also generated by endogenous enzymes during the processing of some foods such as cured and fermented foods.

### Peptides Against Type-2 Diabetes

Type-2 diabetes affects millions of adults worldwide constituting a major health issue. Overnutrition and obesity have a clear incidence on the development of diabetes type-2. Certain bioactive peptides have been reported to enhance insulin sensitivity and activate AMP-activated protein kinase via multiple signaling pathways.

The enzymes  $\alpha$ -glucosidase and dipeptidyl dipeptidase IV (DPPIV) are somehow regulating the glucose levels in postprandial plasma. So, an effective strategy to manage diabetes type-2 would be based on the inhibition of both enzymes. Dipeptidyl peptidase IV is a serine protease able to cleave preferentially X-proline or X-alanine dipeptides from the N-terminus of different substrates and its inhibition would reduce its action on glucagon-like peptide (GLP-1) and glucose-dependent insulintropic peptide (GIP) keeping a lower degradation of GLP-1. In fact, some drugs for controlling diabetes are based on the inhibition of DPPIV although they may exert secondary effects. Encrypted peptides in canary seeds, traditionally consumed for treating diabetes and hypertension, have shown inhibitory activity against DPPIV and ACE, which are targets for diabetes and hypertension treatments.

Peptides against type-2 diabetes have been reported in hydrolyzates of fish, seafood, milk or meat, and also naturally generated by endogenous enzymes in ripened foods like dry-cured ham. An example of some of the identified peptides against type-2 diabetes in foods or food hydrolyzates are shown in **Table 4**.

### Immunomodulating Peptides

Peptides exerting its action on immune system affect both the immune system and cell proliferation responses and have good applications in clinical medicine. So, immunomodulatory peptides can regulate lymphocytes proliferation in humans, modulate certain cytokines production, as well as stimulate the macrophages activity. Most of the reported immunomodulatory peptides are released from milk proteins either by enzymatic hydrolysis or by gastrointestinal digestion after consumption even though they may be scarce in the last case for any significant effects on human body. Enzymatic hydrolysis is necessary in order to get therapeutic amounts of immunomodulatory peptides. For instance, some milk caseins hydrolyzates stimulate the immune system while other peptides generated by pancreatin or trypsin may inhibit the proliferative responses of murine splenic lymphocytes and Peyer's patch cells. Other immunomodulating peptides were reported after trypsin hydrolysis of proteins from Alaska Pollock. The amino acid sequences were Asn-Gly-Met-Thr-Tyr, Asn-Gly-Leu-Ala-Pro, and Trp-Thr, and 20  $\mu$ g/mL of the purified peptides were reported to exert lymphocyte proliferation rates of 35.9%, 32.9%, and 31.3%, respectively.

**Table 4** Examples of mineral-binding and anti type-2 diabetes peptides in foods or food hydrolyzates

Activity	Food	Enzymatic generation	Assay used	Sequence	References
Mineral binding: Calcium	wheat germ	Alcalase	Lab assay	FVDVT	Wang et al., 2018
Mineral binding: Iron	Whey proteins	Flavourzyme/Protamex	CaCo-2 cells for iron absorption	EG and GT	Ou et al., 2010
Mineral binding: Calcium, Zinc and Iron	Alaska pollock skin	Pepsin + Trypsin	CaCo-2 cells for metal absorption	GPAGPHGPPG	Chen et al., 2017
Mineral binding: Calcium	Cod Skin gelatin	Trypsin	Lab assay	GDKGESGEAGER GEKGEGGHR	Wu et al., 2017
Anti type-2 diabetes	Fish (carp)	pepsin	DPP IV inhibitory activity	IADHFL	Zhang et al., 2017
Anti type-2 diabetes	Milk proteins	Pepsin + Pancreatin	DPP IV inhibitory activity and Cellular (CaCo-2 cells) glucose transporter inhibition	LKPTPEGDL, LPYPY, IPIQY, IPI and WR	Lacroix et al., 2017
Anti type-2 diabetes	Dry-cured ham	Muscle endogenous enzymes	DPP IV inhibitory activity	KA and AAATP	Gallego et al., 2014

### Antimicrobial Peptides

Antimicrobial peptides have been mainly isolated from milk and egg and constitute a natural defense of the organism against pathogens. Antimicrobial peptides are effective against different bacteria such as *Staphylococcus aureus* and *Escherichia coli* among others, as well as against yeast. The mode of action depends on the type of peptide but its effects are by forming pores on bacterial membranes, causing thinner membranes interacting with other components in the cell or acting in a detergent-like manner.

The most studied is the fragment 17–41 of lactoferrin, known as lactoferricin. Other proteins that are good sources of antimicrobial peptides are ovotransferrin, lysozyme, alpha-lactalbumin and beta-lactoglobulin.

### Mineral-Binding Peptides

Several food protein-derived peptides generated by enzymatic hydrolysis have shown good ability to trap mineral and trace elements constituting a strategy for their improved absorption. For example, they facilitate calcium absorption being very useful for the prevention of dental caries, osteoporosis, hypertension, and anemia. Further, some peptides can also help the remineralization and the increased absorption of calcium and other minerals in the intestine. Peptides from milk and whey protein hydrolysates show good mineral-binding properties (see Table 4). For instance, casein-derived phosphopeptides can form salts with calcium through their phosphorylated serine residues. Fish protein hydrolyzates were reported to be less effective in general than those from casein hydrolyzates even though some peptides derived from hoki, cod and Alaska Pollack frame proteins exerted similar calcium-binding properties. Iron-binding peptides have been reported in whey, walnut, egg and cod skin gelatin hydrolyzates.

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## Relevant Websites

<http://mbpdb.nws.oregonstate.edu/> Milk bioactive peptide database.

<http://crdd.osdd.net/raghava/ahtpdb/cond.php> AHTPDB database of antihypertensive peptides.

[http://bioware.ucd.ie/~compass/biowareweb/Server\\_pages/biopred.php](http://bioware.ucd.ie/~compass/biowareweb/Server_pages/biopred.php) Peptide locator for identification of bioactive peptides within a protein sequence.

[http://bioware.ucd.ie/~compass/biowareweb/Server\\_pages/peptideranker.php](http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php) Peptide Ranker for prediction whether peptides are bioactive.

<http://bioware.ucd.ie/~enzpred/Enzpred.php> Enzyme Predictor for identification of cleavage sites in a protein.

<http://www.uwm.edu.pl/biochemia> BIOPEP database for identification of potentially biologically active peptide sequences.

<http://bactibase.pfba-lab-tun.org> Database of antimicrobial peptides (bacteriocins).

<http://www.uwm.edu.pl/biochemia/index.php/pl/biopep/32-bioactive-peptide-databases> Compilation of bioactive peptides databases.

# Resistant Starch Preparation Methods

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## Glossary

**Prebiotic** A carbohydrate-based substance which acts as a metabolite for the growth of probiotics.

**Probiotic** Microorganisms living in the large intestine with capability of fermentation and producing short chain acids that exert health benefits from the gastrointestinal system.

**Resistant starch** A part of starch that is resistant to digestion in the small intestine and can be fermented by colon microbiota.

## Introduction

Due to the importance of prebiotics in prevention of certain diseases, they have increasingly attracted the attention of food technologists (Verma and Banerjee, 2010). Prebiotics are non-digestible oligosaccharides that offer many beneficial effects on the gastrointestinal system. Typical prebiotics are dietary fibers (DF) where the most well-known are inulin, oligosaccharides and resistant starch (RS) (Buttriss and Stokes, 2008). The functional features of RS, together with its potential physiological benefits, provide an opportunity to increase the level of DF in the diet through common foods. However, processing procedures, especially thermal processing, may decrease the RS content of food products, whether naturally containing RS or RS-enriched products (Sullivan et al., 2017). In this respect, researchers started to look for ways to either increase the amount of RS or making it more resistant to processing conditions. In this chapter, in addition to review the preparation methods of RS, health benefits of this unique prebiotic will also be discussed.

## Resistant Starch

Resistant starch is mainly composed of the linear part of the starch molecule (amylose) which is fermented by probiotics, including *Bifidobacterium* species in the colon. This gives rise to the production of short chain fatty acids, mainly butyric acid, which plays a significant role in prevention of colorectal cancer. The effective dosage of RS which exerts beneficial health effects was reported as 6–12 g/d, whereas the recommendation for daily intake of another DF was 38 g/d (Fuentes-Zaragoza et al., 2011; Behall et al., 2006). RS naturally occurs in starch-based seeds, cereal grains and cooled heat-treated starchy foods. The highest amount of RS can be found in raw potato with 75 g/100 g (dry basis). Also, green banana is remarkable due to its high RS percentage of about 70 g/100 g of peeled fruit (Wang et al., 2017).

There are five types of RS introduced to date. In table 1, types, sources and description of each type are summarized.

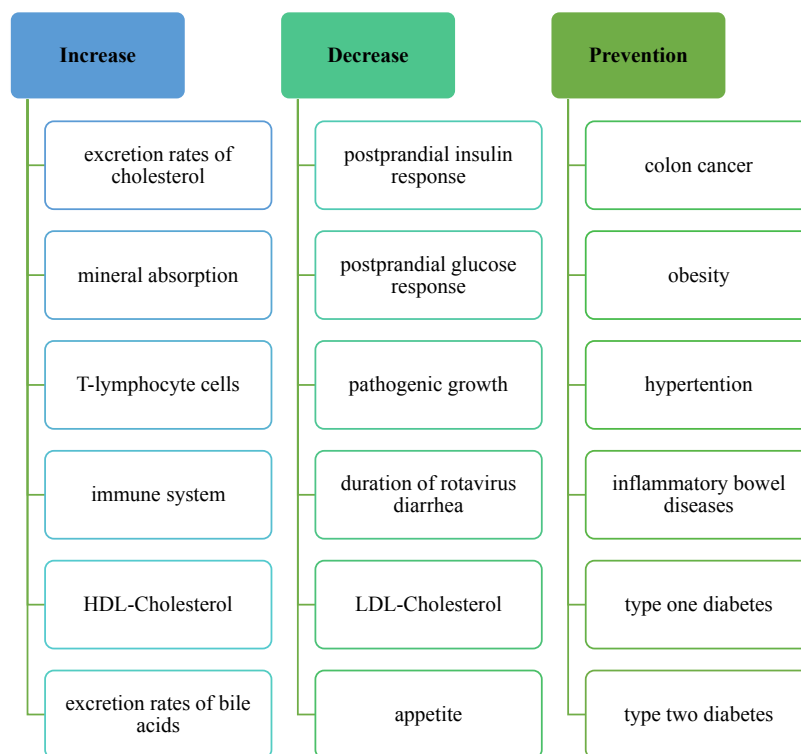
## Resistant Starch Health Benefits

Considering that several digestive diseases are triggered by inadequate or inappropriate diet, increased consumption of indigestible carbohydrates is important. Among numerous DF, RS has approximately half of the calorific value (8 kJ/g) compared with digestible starch (15 kJ/g) (Chen et al., 2017). Numerous health benefits have been shown for RS. In addition to its direct impact on the reduction of the glycemic index, most health effects have been ascribed to the prebiotic nature of RS (Boll et al., 2016).

**Table 1** Resistant starch types, description and sources (Milasinovic-Seremesic et al., 2012; Birt et al., 2013; Khalili and Amini, 2015)

Type	Description	Source
RS1	Physically inaccessible in cell walls	Grain, legumes, seeds
RS2	Granular native starch with high crystalline structure	Raw starchy foods (potato, pasta, high-amylose corn, unripe banana)
RS3	Retrograded starch	Retrograded starchy foods
RS4	Chemically modified starch	Esterified, Etherified or phosphorylated cross-linked starch
RS5	Amylose-lipid complex	Fatty acid treatment of debranched starch





**Figure 1** Health effects of resistant starch (Homayouni et al., 2014; Amini et al., 2015).

Colonic microbiota, specifically *Bifidobacterium bifidum* sp., starts to ferment RS into short chain fatty acids. Therefore, with reducing pH level of the environment by the presence of acetic, propionic and butyric acid, the proliferation of carcinomatous cells begins to be prohibited. Some studies have shown a positive effect of RS intake on prevention of colorectal cancer (Homayouni et al., 2014; Malcomson et al., 2015; Amini et al., 2015; Singh et al., 2016; Hung et al., 2016; Yuan et al., 2017; Yin and Zhao, 2017; Panebianco et al., 2017; Cray et al., 2017). As a result, the indirect effects of RS consumption are mostly due to the activity of probiotic microbiota which is illustrated in Fig. 1.

## Preparation Methods

There are three main strategies to produce resistant starch-rich powdered ingredients: physical, chemical and enzymatic processes. In the physical method, the main procedure is repeated heating–cooling cycles which lead to reorganization of linear chains of starch into a new structure which is resistant to hydrolysis by digestive enzymes (Sarawong et al., 2014). When heating is applied to a high-amylose starch dispersion, disaggregation and breaking of both amylose and amylopectin occurs and leads to formation of shorter linear chains. In the next process, cooling down of these chains brings about the formation of double helical aggregates which are denser and more resistant to be hydrolyzed. Consequently, RS produced by this method is more resistant to heat treatment and can be used as a functional ingredient for fortification of food products (Abioye et al., 2017).

Chemical methods promote modification of starch granules by lintnerization, acetylation, phosphorylation, oxidation, hydroxypropylation, esterification and combinations of these treatments (Nagahata et al., 2013). In each of these treatments, a chemical reagent prevents enzymes from binding properly to the starch. For instance, acetyl groups can be esterified to starch using free hydroxyl groups present on the glucose units with acetic anhydride commonly being used (Sha et al., 2012). In oxidation, by the utilization of reagents such as oxygen, ozone, sodium hypochlorite, periodate and hydrogen peroxide, carbonyl and carboxylic groups are produced that impedes the starch digestive enzymes (Chung et al., 2008). For hydroxypropylation treatment, propylene oxide is commonly used. Under high alkaline conditions, the esterified propylene is utilized to free hydroxy groups from the starch structure resulting in a bulky structure which cannot be digested by carbohydrase enzymes (Juansang et al., 2012). Lintnerization is the term applied to mineral acid-treated starch. By disruption of amorphous sections of starch, acid treatment (mainly by hydrochloric acid) leaves behind a higher ratio of crystalline parts which are more difficult for enzymes to access. In table 2 some of the main chemical modifications are shown.

Enzymatic treatments focus on debranching the  $\alpha$ -1-6 amylopectin bonds by pullulanase and isoamylase which results in rearranging the structure later in the retrogradation process (Reddy et al., 2013). Other enzymes such as  $\alpha$  and  $\beta$ -amylase are used to hydrolyze the starch amorphous regions and leave a tightly packed crystalline structure behind. Consequently, the aim of using debranching enzymes is to hydrolyze amylopectin branch chains and provide more linear parts (Cai and Shi, 2010). In

**Table 2** Chemical treatments for the production of resistant starch

<i>Chemical method</i>	<i>Starch type</i>	<i>Main chemical process</i>	<i>Remarks</i>	<i>Reference</i>
Lintnerization	Banana Corn Waxy corn	HCL (heating for 75 °C and storage up to 78 h)	<ul style="list-style-type: none"> <li>• Time consuming and less productive</li> </ul>	<a href="#">Aparicio-Saguilán et al., 2005</a> , <a href="#">Brumovsky and Thompson, 2001</a> , <a href="#">Ozturk et al., 2011</a> , <a href="#">Nagahata et al., 2013</a>
Phosphorylation	Pea Normal corn Canna Rice Wheat	Using sodium trimetaphosphate and sodium tripolyphosphate in alkaline conditions (around 3 h), following gelatinization at 100 °C	<ul style="list-style-type: none"> <li>• Legal limitations by FDA at excess amounts (0.4%)</li> <li>• Destructive effects on bread's rheology at high level of substitution</li> </ul>	<a href="#">Dupuis et al., 2014</a> , <a href="#">Thompson, 2001</a> , <a href="#">Yeo and Seib, 2009</a>
Carboxymethylation	Potato Rice	Addition of monochloroacetic acid in alkaline conditions with subsequent microwave treatment	<ul style="list-style-type: none"> <li>• Reducing process time by microwave heating</li> <li>• Low RS yield</li> </ul>	<a href="#">Liu et al., 2012</a> , <a href="#">Kittipongpatana and Kittipongpatana, 2013</a>
Oxidation	Normal corn Rice Pinto bean	Injection of O <sub>3</sub> , H <sub>2</sub> O <sub>2</sub> , O <sub>2</sub> in alkaline conditions for 30 min	<ul style="list-style-type: none"> <li>• Enzymatic hydrolysis by <math>\alpha</math>-amylase</li> <li>• Slightly increase in bean starch</li> </ul>	<a href="#">Chung et al., 2008</a> , <a href="#">Simsek et al., 2012</a>
Acetylation	Rice Canna Normal corn	Treatment with vinyl acetate (30 °C, 5 h)	<ul style="list-style-type: none"> <li>• Around 16% vinyl acetate led to higher amount of RS</li> </ul>	<a href="#">Sha et al., 2012</a> , <a href="#">Simsek et al., 2012</a> ; <a href="#">Juansang et al., 2012</a>
Hydroxypropylation	Canna Normal corn Waxy corn	Addition of propylene oxide in alkaline conditions Followed by gelatinization (40min)	<ul style="list-style-type: none"> <li>• Long treatment (40 h)</li> <li>• Decrease of RS compared to initial amount</li> </ul>	<a href="#">Juansang et al., 2012</a> , <a href="#">Chung et al., 2008</a> , <a href="#">Han and Bemiller, 2007</a>
Citric Acid treatment	Normal Waxy Hylon VII	12 h reaction with 0.1 or 0.01 M citric acid followed by oven heating OR Non-autoclaved method: 16 h reaction + 9 h heating at 120 °C	<ul style="list-style-type: none"> <li>• Decrease of RS2 amount</li> <li>• Long procedure time</li> <li>• Only suitable for high amylose cereals</li> </ul>	<a href="#">Sun et al., 2012</a> , <a href="#">Shin et al., 2007</a> , <a href="#">Xie and Liu, 2004</a>

**Table 3** Enzymatic treatments for the production of resistant

<i>Enzymatic method</i>	<i>Starch type</i>	<i>Main enzyme process</i>	<i>Remarks</i>	<i>Reference</i>
$\beta$ -amylase	corn Wheat	20 h of $\beta$ -amylase reaction followed by 3 autoclave cycles	<ul style="list-style-type: none"> <li>• Long preparation procedure</li> <li>• Two-fold increase in RS</li> </ul>	<a href="#">Hickman et al., 2009</a>
$\beta$ -Amylase and Pullulanase	corn starch	$\beta$ -Amylase in conjunction with Pullulanase	<ul style="list-style-type: none"> <li>• Optimum amount of enzyme concentration is of importance</li> </ul>	<a href="#">Zhang and Jin, 2011</a>
Isoamylase	Waxy corn Waxy wheat Waxy potato	1g/100 g starch dry basis Isoamylase (50 °C, 24 h) followed by retrogradation	<ul style="list-style-type: none"> <li>• Pre-cooking is needed</li> <li>• Long post-treatment (48 h)</li> <li>• Significantly increased RS3</li> </ul>	<a href="#">Cai and Shi, 2010</a>
Pullulanase	Gelatinized red kidney bean Cassava potato	Pullulanase, 40U/g, 10 h followed by heat treatment at 50 °C	<ul style="list-style-type: none"> <li>• Thermal treatment is needed</li> <li>• Long post-treatment (24h/25 °C)</li> </ul>	<a href="#">Reddy et al., 2013</a> , <a href="#">van Hung et al., 2012</a>

this regard, subsequent retrogradation will produce higher levels of RS3 with double helical structures stabilized by hydrogen bonds. However, in order to achieve maximum enzyme performance, optimal pH, temperature and time of reaction should be precisely controlled. **Table 3** shows the main enzyme processes in enzymatic treatment of starchy crops.

Regarding that a combination of mentioned methods could also increase the RS amount, most of them ended with retrogradation processes resulting in RS3 increments. The conditions and percentage of produced RS3 in the finished product are summarized in **table 4**.

**Table 4** Combination treatments for increasing resistant starch

Source	Procedure	%RS (g/100 g)	Remarks	Reference
Taro corm	autoclaving, enzymatic debranching, retrogradation and oven drying processes for two times (60 °C)	35.1 ± 1.9% (dry basis)	• A combination of methods in two cycles resulted in 16-fold increase in RS amount	Simsek and El, 2012
Maize starch	twin-screw extrusion, cooking, mild acid hydrolysis, hydrothermal treatments (110 °C)	increased from 11% to 20%	• Acid modified normal-maize starch (AMMS) produced a greater RS	Hasjim and Jane, 2009
Cassava starch	enzymatic debranching, steam cooking, drying (45 °C) and freezing (−28 °C)	19–20	• Refrigeration of cassava starch gave rise to higher RS compared to freezing storage	Abioye et al., 2017
High amylose corn starch (Hylon V and VII)	storage in 23 °C then drying at 50 °C after each extrusion cooking with high moisture feed for 3 cycles	Hylon V: from 43 to 40 Hylon VII: from 53 to 45.1	• RS3 made from Hylon VII had high emulsion stability • Repeated autoclaving-storage cycles are necessary to increase the RS3 content to the desired levels	Masatcioglu et al., 2017
High-amylose corn starch	Hydrothermal Pressure (15 MPa for 2 h) followed by retrogradation (120 °C/24h, 4 °C)	At 100 °C reached to 27%	• The hydrothermal pressure resulted in a significant increase in the total RS content if the samples were gelatinized at temperatures below 120 °C	Pu et al., 2013

## Conclusion

Resistant starch is an exceptional prebiotic with numerous additional health benefits. It also brings technological positive effects for food products as a functional ingredient. However, processing conditions, especially heat treatment, can attenuate its content. Consequently, there are ways in order to isolate and increase the amount of resistant starch with the aim of increasing its capability to be resistant during food processes.

Chemical reagents could increase the amount of resistant starch type four in starchy foods, however, long reaction times and some health concerns about the dosage of chemicals and the residue are limiting factors. Enzymatic debranching is shown to be more effective due to the high concentration of produced resistant starch type three. It also seems to be an essential step when used with other methods, such as heat and cool cycles. Though, it needs a controlled environment, effective enzyme activity and subsequent heat treatments. Although the heating-cooling cycles could increase the resistant starch type three in produced products, there are several factors that needed to be investigated including the rate of cooling/heating process, number of cycles and various temperatures. Additionally, the combination effect of fatty acid addition and other methods for producing resistant starch type five has not been considered yet.

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## Interactions of Milk Proteins With Minerals

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### Glossary

**Colloid** A mixture in which one substance of microscopically dispersed insoluble particles is suspended throughout another substance.

**Micelle** Aggregates of amphipathic molecules (e.g., surfactants, detergents, soaps) that are in dynamic equilibrium with surfactant monomers.

**Pasteurization** A partial sterilization process that kills harmful bacteria by heating milk to a specific temperature for a set period of time.

**Peptide** Chain of amino acid monomers of between 2 and 50 residues in length

**Inductively coupled plasma mass spectrometry** A type of *mass spectrometry* which is capable of detecting multiple metal and (some) non-metal elements in the same sample at concentrations as low as one part in 10<sup>15</sup>.

**Ultrafiltration** Filtration using a medium fine enough to retain colloidal particles, viruses, or large molecules

### Nomenclature

Ca Calcium

Fe Iron

Ca<sup>2+</sup> Calcium ions

P Phosphorus

P<sub>i</sub> Inorganic phosphate

Mg Magnesium

K Potassium

Na Sodium

Cl Chlorine

S Sulphur

Cu Copper

Mn Manganese

Zn Zinc

ms<sup>-2</sup> Meter per second squared

°C Degrees Celsius

ICP-MS Inductively coupled plasma mass spectrometry

AAS Atomic absorption spectroscopy

AES Atomic emission spectroscopy

CaCl<sub>2</sub> Calcium chloride

CPPs Caseinophosphopeptides

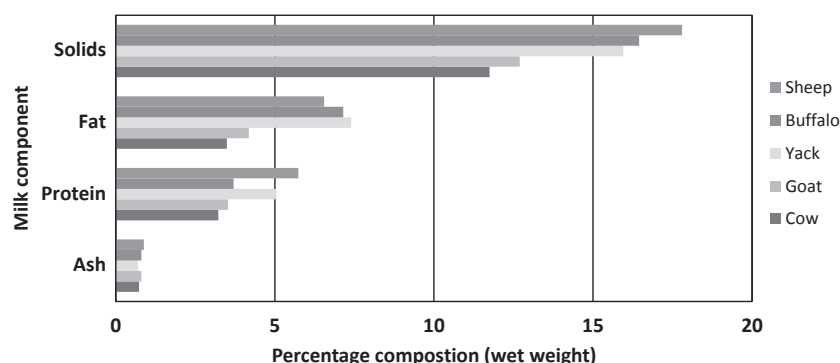
DMT-1 Divalent metal transporter 1

HT-29 cells Human colon adenocarcinoma cells

Caco-2 cells Human epithelial colorectal adenocarcinoma cells

### Overview

Mineral-protein interactions are important in milk since minerals can influence milk protein configuration and structure, and thus can play an important role in the biological and structural functions of milk (Gaucheron, 2011). Milk functionality including stability, nutritional properties, and technological/processing characteristics are all affected by the way in which proteins and minerals interact within the milk matrix (de la Fuente, 1998).



**Figure 1** Composition of milk fractions. Data sourced: Boyazoglu and Morand-Fehr (2001), Hadjipanayiotou (1995), Hernandez et al. (2014), Moreno-Montoro et al. (2015), Payandeh et al. (2015), Alves et al. (2015), Mayer and Fiechter (2012), Pavić et al. (2002), Leitner et al. (2016), Abdel-Salam and El-Sayed (2014).

Mineral and protein interactions can take a number of forms including electrostatic, ionic, and covalent (coordinate) bonding. The type of bonding that occurs is reliant on the specific protein(s) involved (Harris, 2014). Many of the interactions present are similar in nature to those present in other biological fluids such as blood (Lenton et al., 2015).

Milk proteins make up between 3% and 6% of the wet weight of milk (Fig. 1) and are involved in a wide range of functions including nutrient transport and lipid metabolism. The major proteins present in milk include the casein family (comprising  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$  and  $\kappa$  caseins), the whey protein fraction (including  $\alpha$ -lactoglobulin,  $\beta$ -lactoglobulin, and others), and various growth factors, such as epidermal growth factor (Claeys et al., 2014). In addition there is a large number of low abundance proteins (Ha et al., 2015) involved with cell surface interactions, peptidase activity, antioxidant function, cell surface binding, and lipoprotein receptor binding (McSweeney and Fox, 2003; Ha et al., 2014).

The mineral composition of milk varies depending on species, lactation time, lactation stage and diet and is typically measured holistically as ash content (Fig. 1) (Králičková et al., 2012; Bilal et al., 2014). Although required in low concentrations, minerals play key roles in metabolic pathways and are key to maintaining cell homeostasis (Fraga, 2005; Gaucheron, 2005). Milk contains high levels of key nutritional minerals, including calcium (Ca), magnesium (Mg), iron (Fe), and zinc (Zn) (Table 1). The minerals in milk can be present in a range of forms, with the majority of minerals being present either in a soluble (ionic) form or as colloidal salts (Fox et al., 2015a). Selenium (Se) is generally found incorporated into amino acids, specifically as selenocysteine and selenomethionine (Cobo-Angel et al., 2014; Zheng et al., 2016). Likewise, phosphorus (P) within milk is present in two forms, inorganic (Pi) and organic (P). Similar to Se, the organic P is incorporated in proteins by covalent bonding (Fox et al., 2015c).

**Table 1** Range of mineral concentration of milk from different species <sup>c</sup>

Source				
Minerals (mg/100 mL)	Cow	Goat	Sheep	Human
Calcium (Ca)	107–133	106–192	136–218.44	22–41
Phosphate (P)	63–119	91–140	80–159.67	12–43
Magnesium (Mg)	9–16	10–21	8–19.7	12–43
Potassium (K)	113.7–178	123.85–237	97.43–225.6	46–55
Sodium (Na)	39.56–58	31.7–50	29–74.01	12–15
Chloride (Cl)	90–106	100–198	0–160	32–60
Sulphur (S)	32 <sup>a</sup>	28 <sup>a</sup>	0–29 <sup>b</sup>	14 <sup>a</sup>
Iron (Fe)	0.02–0.08	0.03–0.08	0.04–0.1	0.03–0.2
Copper (Cu)	0–0.06 <sup>b</sup>	0.01–0.05	0.01–0.09	0.02–0.08
Manganese (Mn)	0–0.02 <sup>b</sup>	0.01–0.03	0.01 <sup>a</sup>	0.01–0.07
Zinc (Zn)	0.07–0.53	0.24–0.56	0.38–0.77	0–0.38 <sup>b</sup>

<sup>a</sup>Data were obtained from only one source.

<sup>b</sup>Minimum value below detection limit.

<sup>c</sup>Data obtained from: Park et al. (2007), Al-Wabel (2008), Hanuš et al. (2008), Bornaz et al. (2009), Ivanova (2011), Mayer and Fiechter (2012), Zamberlin et al. (2012), Payandeh et al. (2015).



## Biological Functions Resulting From Mineral and Protein Interactions

### Facilitating Protein Function

The correct function and/or activity of a number of enzymatic proteins and non-enzymatic proteins present in milk depends on interaction with a mineral (Vegarud et al., 2000). This can occur in a number of ways. Mineral binding through chelation often stabilizes protein conformation, resulting in improved activity. Minerals can also act as part of the catalytic sites within enzymes (Vegarud et al., 2000). For example, lactoperoxidase, an enzyme present in milk with a known antibacterial function has a coordinately bound heme group containing Fe at its active site that facilitates the catalytic conversion of hydrogen peroxide to water, and thiocyanate to hypothiocyanite (Kussendrager and Van Hooijdonk, 2000). The activity of lactoperoxidase is significantly higher in the presence of  $\text{Ca}^{2+}$  ions (Kussendrager and Van Hooijdonk, 2000; Koksai et al., 2016). Lactoferrin is another protein present in milk that has been shown to have a range of functions including tumor inhibition, antibacterial activity, bacteriostatic activity, immunomodulation activity, and hemolytic function (Giansanti et al., 2016). With respect to lactoferrin bacteriostatic activity, a number of mechanisms have been suggested including one that is considered critical in the milk matrix - the sequestering of Fe ions. In this case, the binding of lactoferrin to free Fe ions restricts the access of microbes to an essential nutrient thus restricting growth (Giansanti et al., 2016; Sedykh et al., 2016).

### The Casein Micelle and Calcium Phosphate

One of the key interactions between minerals and proteins that occur in milk is the binding between colloidal calcium phosphate and the casein micelle. The casein micelle is a complex protein aggregate made up of proteins from the casein family, including  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$  and  $\kappa$  caseins. The internal structure of the casein micelle is still an area of active scientific investigation (Ingham et al., 2015) even though the characteristics of the individual casein proteins have been extensively studied (De Kruif and Holt, 2003). The structure of the colloidal calcium phosphate casein micelle complex plays an important role in the functional properties of milk, including gel formation, emulsification, and foam formation (Fox et al., 2015a). Casein proteins contain a relatively high number of phosphates bound to serine that enable interaction with ionic Zn and Ca (Horne, 2014).

Besides ionic interactions, the casein micelle has been shown to interact with colloidal calcium phosphate present in milk. Colloidal calcium phosphate is present in equilibrium with  $\text{Ca}^{2+}$  and hydrogen phosphate ( $\text{HPO}_4^{2-}$ ) in milk. It is believed that colloidal calcium phosphate interacts with the casein micelle through associations with the phosphoserine residues present in casein proteins (Horne, 2014; Xu et al., 2016) with up to four phosphoserine residues interacting with each calcium phosphate group (Choi et al., 2011).

It is understood that colloidal calcium phosphate stabilizes the structure of the casein micelle as well as helping maintain the suspension of colloidal calcium phosphate (Fox et al., 2015a). The decalcification of the casein micelle has been shown to cause the disassociation of the micelle structure. Xu et al. (2016) decalcified milk protein concentrates by ultrafiltration and ion exchange treatment and found that the average casein micelle diameter, determined by particle size distribution, was reduced with increasing decalcification (with a z-average diameter of 179.4 and 98.6 nm at 0% and 83.6% decalcification, respectively). Inversely, when  $\text{Ca}^{2+}$  or calcium plus phosphate was added to milk an increase in the stability of the casein micelle was observed with increased resistance to gel formation by both rennet coagulation and heat (Kamal et al., 2017; Balakrishnan et al., 2018).

### Stabilization of Solubility and Ionization States of Minerals

Protein and mineral interactions in milk are able to aid maximal mineral absorption, because for minerals to be available for absorption, they must be present in the intestine in a specific form (Benito and Miller, 1998; Stahl et al., 2002). For example, inorganic iron must be present in the ferrous ( $\text{Fe}^{2+}$ ) rather than the ferric ( $\text{Fe}^{3+}$ ) form so that it can be transported through intestinal membranes by active transport proteins (Ganz, 2013; Abbaspour et al., 2014). The interactions between proteins and minerals are able to minimize changes in oxidation states and solubility during storage, processing, and digestion. This occurs through the same chelation and ionic interaction effects that help in stabilizing the structure and function of proteins (Benito and Miller, 1998; Zhao et al., 2014). Milk is considered a key source for nutritional Ca, due to high Ca concentration and the high bioavailability of the milk Ca (Heaney, 2000; Adolphi et al., 2009; Pereira and Vicente, 2017). One aspect of this increased absorption can be linked to the way in which Ca interacts with proteins in the milk matrix. The formation of amorphous calcium phosphate complexes can occur in the intestine due to the effects of pH, in addition to the native colloidal calcium phosphate discussed above (Lenton et al., 2015). These complexes are considered undesirable as they reduce the bioavailability of both Ca and P. The presence of phosphopeptide sequences within caseins in milk increases the solubility of the amorphous calcium phosphate complexes, which in turn, increases the availability of both Ca and P for absorption (Kitts et al., 1992; Cross et al., 2005; Sun et al., 2016). The absorption of Ca and P are considered critical for the correct development and maintenance of bone structures (Weaver, 2017).

### Mineral Co-dependent Interactions

One aspect of mineral metabolism that needs to be highlighted is the co-dependent and at times antagonistic nature of mineral absorption. An example is the relationship between Fe and Cu. Copper is able to support Fe absorption by facilitating oxidation and subsequently allowing Fe to bind to ferroxidase hephaestin enabling absorption from the gut into the circulatory system (Vulpe

et al., 1999; McKie et al., 2000; Datz et al., 2013). Inversely, competitive interactions can occur among Ca, Mg, Fe, and Zn in a dose-dependent manner (Bressler et al., 2007). It is believed that the competitive interactions are regulated by divalent metal transporter 1 (DMT-1) (Thompson et al., 2010). The competitive interactions between these minerals can be reduced due to the effects of milk protein and peptides. The most well studied of these are the interactions between caseinophosphopeptides (CPPs), derived from milk and Ca (Fiorilli et al., 2012). Cosentino et al. (2010) showed that the presence of CPPs resulted in an increase in the absorption of Ca in human colon adenocarcinoma cells and human epithelial colorectal adenocarcinoma cells (HT-29 and Caco-2 cells). Cell signaling, bio-modulatory effects and alternative absorption pathways were some of a range of potential mechanisms that were proposed by the authors. Furthermore, CPPs have been shown to bind other divalent cations including Fe and Zn but there is some doubt in the literature as to whether there is any beneficial effect. Garcia-Nebot et al. (2010) found that milk-derived CPPs aided in the bioavailability of Fe in Caco-2 cells when used to supplement fruit juice beverages. Subsequently, in more recent work by the same lab, also using Caco-2 cells, no effect of individual CPP fractions was demonstrated (Garcia-Nebot et al., 2015). These results indicate that further investigation is required in relation to the role of CPPs and mineral absorption.

### Milk Peptides

The proteins present in milk can be enzymatically hydrolyzed into peptide fragments. This can occur during digestion, cheese making, or due to addition of specific enzymes (Korhonen and Pihlanto, 2006). These fragments have properties that are considered independent of the proteins they are derived from (Meisel, 1998). The most commonly identified example of this is CPPs as discussed above. However, CPPs are one of a vast range of peptides formed during the digestion of milk (Mohanty et al., 2016).

The natural (gastrointestinal) digestion of milk produces a specific set of peptides. However, a wide range of peptides can be formed from milk when different hydrolysis methods (e.g. microbial fermentation or use of pure enzymes) are applied (Saavedra et al., 2013). Bacterial fermentation (using *Lactococcus lactis*, and *Lactobacillus helveticus*), and isolated proteases from bacteria and or fungi (including protease from *Aspergillus sojae*, *Aspergillus oryzae* and *Bacillus licheniformis*) are two examples of methods shown to produce novel peptides from milk proteins (Saavedra et al., 2013; Morais et al., 2014). Peptides produced using these systems have been shown to have a vast range of functions including antibacterial activity, antioxidant activity, angiotensin converting enzyme inhibition, haemolytic activity and to direct mineral binding functions (López-Expósito et al., 2006; Corrêa et al., 2011, 2014; Meira et al., 2012). Peptides derived from whey protein concentrate hydrolyzed with alcalase have been shown to bind Fe resulting in a significant increase in Fe solubility (Kim et al., 2007). Likewise, whey proteins hydrolyzed with flavorzyme and protamex has been shown to produce Ca binding peptides with binding characteristics independent of those of CPPs (Zhao et al., 2014). Other milk proteins including  $\beta$ -casein,  $\alpha$ -Lactalbumin, and  $\beta$ -lactoglobulin have also been identified as targets for the production of peptides (Saavedra et al., 2013).

*In silico* methods have been used for the identification of bioactive peptides from a range of protein sources including milk (Saavedra et al., 2013). Even so, the application of *in silico* methods allows for rapid identification of peptide targets, the application of these methods is limited because for a natural food product the technology just provides potential peptide sequences. Once a potential peptide sequence is identified the process of hydrolysis, isolation, characterization and the measurement of activity is still required (Carrasco-Castilla et al., 2012).

### Factors Affecting Mineral and Milk Protein Interactions

Milk is typically processed using one or more steps before it is sold for consumption, including pasteurization (thermal processing), cheese making (concentration, fermentation), drying (thermal processing), and freezing (cold processing) (de la Fuente et al., 1997; Huppertz, 2016). The mineral composition of milk can alter the processing parameters of the milk and the processing technology can affect the interactions between the mineral fraction of the milk and milk proteins. These interactions are collectively confounded by the differences between milks of different species (Fig. 1) (Park and Haenlein, 2013). As an example of the impact of minerals on the protein functionality, sheep milk has a larger native micelle structure and lower colloidal calcium level compared to cow milk. This means that processing (including both thermal and cold processing) may result in smaller changes of physical and functional properties of sheep milk than observed with cow milk. This is particularly true with respect to common cheese-making properties of rennet coagulation time and curd firmness (Raynal and Remeuf, 1998; Raynal-Ljutovac et al., 2007).

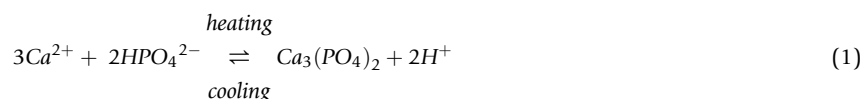
### Heat Processing

The safety of any food product is extremely important to ensure the wellbeing of consumers and to maintain consumer trust. The most common food preservation method used in dairy processing is pasteurization by heat treatment. Pasteurization not only minimizes potential detrimental bacterial effects (both pathogenic and spoilage) on milk products but also inactivates enzymes that can cause quality changes and shorten shelf life (Raynal-Ljutovac et al., 2007; Huppertz, 2016). The main mechanism of pasteurization is protein denaturation, which is carried out to inactivate the biological function of bacteria. Because the interactions between minerals and proteins are closely associated with protein functionality and structure, it is only logical that the interactions between minerals and proteins will also be affected (Fox et al., 2015b). The inactivation of enzymes such as lactoperoxidase, for

example, reduces the sequestering of Fe within milk and consequently this will affect the bactericidal capability of lactoperoxidase (Giansanti et al., 2016; Sedykh et al., 2016).

The solubility of minerals can also be altered by temperature changes. While an increase in temperature is normally expected to increase the solubility of a given salt, calcium phosphate, however, has reduced solubility in milk with increasing temperature (Kamal et al., 2017). Although this process is mostly reversible, exposure to high temperatures or long heating times can result in an insoluble deposition (or colloidal form) of calcium phosphate (Fox et al., 2015b). Extended heat treatments can also affect the structure of the casein micelle, due to the close relationship between calcium phosphate and the casein micelle (O'Connell and Fox, 2000). As a secondary effect of changes in the solubility of calcium phosphates, the pH of milk changes during heating. The formation of insoluble calcium phosphates occurs within an equilibrium as summarized by Eq. (1). As the pH change is related to the calcium phosphate solubility this change in pH is reversed upon cooling (Chandrapala et al., 2010; Fox et al., 2015a).

Calcium phosphate equilibrium in relation to heating (Fox et al., 2015b)



## Freezing

It is not typical for cow milk to be frozen prior to further processing and the use of any sub-zero processing is typically the final processing step, such as in the production of ice-cream (Huppertz, 2016). However, goat and sheep milk are commonly frozen (de la Fuente, 1998), as the result of the highly seasonal lactation periods that are associated with small ruminates (de la Fuente, 1998). Similar to pasteurization, freezing of milk alters the mineral distribution within its matrix. Pazzola et al. (2013) identified that freezing fresh sheep milk at  $-20^\circ\text{C}$  for up to five months resulted in negative changes in its renneting characteristics that may be due to an alteration in the ratio of soluble Ca to soluble P (de la Fuente et al., 1997). With respect to goat milk, Kljajevic et al. (2016) found no significant changes in milk coagulation properties after a frozen storage period of 60 days at  $-27^\circ\text{C}$ . It must be noted that neither of these works assessed the direct interactions between proteins and minerals.

With respect to cow milk ice cream, the interactions between minerals and proteins have not been extensively investigated. Jonkman et al. (1999) identified that the conversion of cow milk into ice cream did not result in the alteration of the casein micelle structure, although an increase in the association between minerals and the casein micelle was observed when assessed by ultrafiltration. It is understood that the addition of calcium chloride ( $\text{CaCl}_2$ ) reduces the emulsification capacity of milk proteins during the ice cream making process. This, in turn, results in an increase in the size of ice crystals and an increase in fat partial coalescence, both of which are considered undesirable characteristics for ice cream (Costa et al., 2008).

## Cheese Making

The way in which calcium in milk interacts and associates with micellar structures is a key factor in determining the renneting properties of milk and therefore the initial efficiency of the cheese making process (Park et al., 2007). In cow milk, the large association of calcium with the casein micelle results in a protective effect that slows the coagulation process (Hickey et al., 2015). Variation in the cheese making characteristics between different milk types has been associated with differences in casein micelle mineralization (calcium phosphate associations). This is exemplified by comparing sheep milk and cow milk. Even though there is a higher total calcium concentration in sheep milk, this is predominantly in the ionic form rather than being associated with the colloidal structure of the casein micelles, which results in a faster coagulation time during the renneting step (Storry et al., 1983; Ramos and Juarez, 2002). In addition, the high curd strength found in sheep milk cheeses increases the whey drainage time and reduces the cheese yield (Riddell-Lawrence and Hicks, 1989; Catarino et al., 2013; Hickey et al., 2015).

## Quantification of Mineral and Protein Interactions

As protein and mineral interactions within milk are an important characteristic, consideration of the methods available for the measurement of the interactions is of interest. The quantification of total mineral content within milk is standard practice, using methods such as inductively coupled plasma mass spectrometry (ICP-MS), atomic absorption spectroscopy (AAS) or atomic emission spectroscopy (AES), which accurately and constantly determine the elemental composition and concentration of minerals in milk (Ataro et al., 2008; Pereira et al., 2016). When more expedient measurements are required, a range of methods including mid-infrared spectroscopy have been applied (Toffanin et al., 2015). Likewise, the determination of milk protein content by total nitrogen determination is commonplace within both industry and scientific investigations (Chang and Zhang, 2017). However, the determination of mineral and protein interactions in milk is complex. This is because the interactions between proteins and minerals are in many cases extremely sensitive to pH, temperature, and ionic strength. Most of the common methods for the isolation of proteins use the manipulation of at least one of these factors (Gaucheron, 2005).

One method of analysis proposed by [de La Fuente et al. \(1996\)](#) to determine protein and mineral interactions is to use high-speed centrifugation ( $980665 \text{ m s}^{-2}$  for 1 hour). The main criticism of this method is the potential for low molecular weight peptide fractions to remain in the supernatant resulting in an underestimation of the bound mineral content ([de la Fuente et al., 2002](#)). Therefore, this method should be referred to as the determination of non-sedimentable minerals ([Gaucheron, 2005](#)). Ultrafiltration is another method that can be applied to aid in the separation of free and bound minerals. The use of low molecular weight cut off membranes (cut off less than 10–15 kDa) is able to separate out the majority of the protein phase allowing for the analysis of bound minerals ([Malacarne et al., 2015](#)). However, similar criticisms can be applied to this method as with high-speed centrifugation ([Gaucheron, 2005](#)). It must be noted that one of the benefits of ultrafiltration is the ability to carry out testing the separation of the various phases at elevated temperatures. For example, [On-Nom et al. \(2010\)](#) carried out separation at temperatures up to  $80^\circ\text{C}$ .

The determination of interactions is possible with individual proteins and peptides derived from milk. This can be carried out using isotopic measurements, protein characterization, and mineral specific assays ([Kitts and Yuan, 1992](#); [Guo et al., 2014](#); [Sun et al., 2016](#)) and has been carried out on a number of milk-derived proteins and peptides ([Zhao et al., 2014](#)). The examination of mineral binding on an individual protein or peptide is useful to understand the functionality of novel proteins and peptides derived from milk ([Korhonen, 2009](#)). However, as milk is a complex biological fluid the isolation of novel proteins and peptides can be a challenging exercise ([Guerrier et al., 2008](#); [Pa'Ée et al., 2015](#)).

## Conclusions

The interactions between the proteins and minerals present in milk are critical to the functions of milk, and the nutritional benefits that milk provides to the consumer. Minerals act to stabilize protein structure and act to facilitate enzymatic catalysis. However, the role that proteins play in increasing mineral bioavailability is just as important. This is because milk is seen as a key source of nutrients, especially during growth and development. Bioactive peptides derived from milk have also been an area of intensive research due to their applications as nutraceutical and medicinal agents. The development of *in silico* methods has resulted in the identification of a vast range of novel peptides in milk. The activity of many of these peptides is dependent on or associated with ionic minerals, particularly Fe and Ca. However, further work on the isolation and characterization of these peptides is still required. During processing, a number of changes occur within the milk matrix. Differences between the composition of milk from different species, specifically related to the casein micelle composition (and structure) can result in differences in the processing of milk, as is particularly notable in the cheese making process. Finally, the quantification of mineral and protein associations is difficult because of the complexities of separating out the bound and non-bound phases without inducing changes. The use of centrifugation and ultrafiltration are common within literature but these methods are limited and do not provide a true snapshot of milk mineral and protein interactions in their native state. Likewise, though the analysis of individual protein and or peptide fractions is well established, these methods provided a limited insight into the nature of the milk matrix at a system level. An understanding of the native protein and mineral interactions within milk, as well as how these interactions change due to processing is important to maximize the nutritional benefits of both unprocessed and processed dairy products.

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## Protein-Stabilised Emulsions

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### Glossary

**Creaming** is the upward movement of droplets in an emulsion due to lower density droplets moving upwards in the continuous phase.

**Emulsion** is a thermodynamically unstable mixture of two or more immiscible liquids.

**Emulsion stability** is the ability of the emulsion to resist changes in its physicochemical properties over time.

**Interface** is the point where immiscible phases such as oil and water are in contact.

### Introduction

Many proteins, in particular milk proteins, are surface active molecules that consist of both hydrophilic and hydrophobic functional groups (Dickinson, 1997; Wilde et al., 2004). Proteins have been used extensively as emulsifiers in foods (Rodríguez Niño et al., 2001; Sliwinski et al., 2003; Sünder et al., 2001) to form stable oil-in-water (o/w) emulsions such as milk, cream, ice-cream, mayonnaise, salad dressing and gravies (Tornberg and Hermansson, 1977). An oil-in-water emulsion consists of dispersed oil droplets in a continuous water phase. Milk proteins such as casein and whey proteins adsorb to the surface of oil droplets, reduce the surface tension between oil and water, and form a protective film surrounding the oil droplet to stabilise it (Walstra, 2002). Low molecular weight emulsifiers such as mono- and diglycerides are commonly added to improve emulsion stability (Krog, 2011; Novales et al., 2005; Sagalowicz et al., 2006). The biphasic nature of an emulsion means that it can only keep droplets dispersed for a limited time, thus affecting shelf life. Emulsions undergo destabilisation over time according to a number of different mechanisms. In this chapter, destabilisation mechanisms, extrinsic and intrinsic factors affecting emulsion stability, and the effect of mono- and diglycerides on emulsion properties are discussed.

### Protein-Stabilised Emulsions

Proteins such as casein and whey proteins from milk have a globular structure and adsorb onto most surfaces (Walstra, 2002). During the formation of emulsions, globular proteins rearrange their configuration to expose the hydrophobic amino acid groups and adsorb to the oil-water interface. The adsorption of protein to the interface will lower the surface tension and upon disruption will lead to the formation of smaller oil droplets. The adsorbed proteins form a protective layer around the oil droplets. These proteins at the interface provide repulsive forces such as steric and electrostatic forces, which stabilise the droplets for long term stability (Bos and van Vliet, 2001; Tcholakova et al., 2006; Wilde et al., 2004). Extrinsic factors (homogenisation, thermal treatment) and intrinsic factors (pH, ionic strength, biopolymers, emulsifiers) affecting emulsion stability will be discussed later in Sections [Extrinsic Factors Affecting Emulsion Stability](#) and [Intrinsic Factors Affecting Emulsion Stability](#) of this chapter.

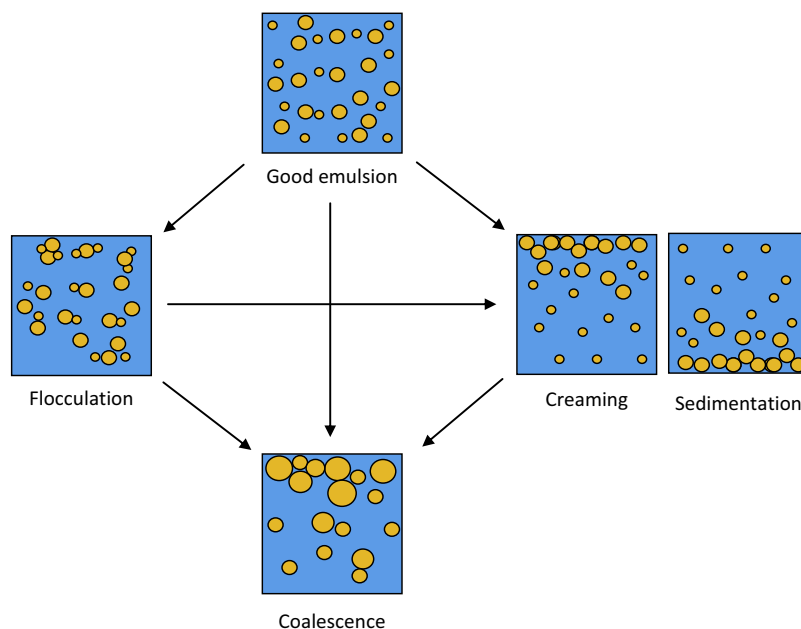
### Emulsion Destabilisation Mechanisms

Emulsion stability is the ability of the emulsion to remain unchanged in its physical properties over time (McClements, 2007). A protein-stabilised emulsion is a thermodynamically unstable mixture that can undergo destabilisation mechanisms such as flocculation, creaming, sedimentation and coalescence. [Fig. 1](#) shows the common destabilisation mechanisms that occur in food emulsions.

#### Flocculation

Flocculation happens when neighbouring droplets come closer to each other and form colonies in the continuous phase. The aggregation is often not clearly visible and may serve as a precursor to creaming and coalescence. Flocculation could also take place during or after creaming. It may be reversible or not depending on the strength of the intermolecular bond between droplets. However, flocculation may be a desired physical property in some food products such as whipping cream. The occurrence of flocculation could derive from the chemical nature of the emulsifier, phase volume ratio and concentration of electrolytes. The following steps could prevent flocculation from happening.

- 1) To make a uniform droplet size using an appropriate size reduction process.



**Figure 1** Physical changes to the oil droplets in a protein-stabilised emulsion.

- 2) To modify the charge surrounding the droplet so that it will exert repulsive forces with neighbouring droplets.
- 3) To increase the viscosity of the continuous phase and make the globules relatively immobile.

Flocculation is common with protein-stabilised emulsions. One classic example is the feathering of cream in hot coffee. The protein-stabilised oil globules, when exposed to the hot and acidic pH in the coffee, will undergo conformation changes at the interface, thus causing the oil globules to become more attracted to each other (Krog, 2011).

### Creaming

Creaming is an upward movement of dispersed globules in a continuous phase due to the density difference between the two phases. Creaming usually happens in an o/w emulsion due to the upward movement of the relatively lower density oil globules in an aqueous phase. Sometimes the movement of different globule sizes may result in a different colour shade of the emulsion layers. Creaming is a reversible process as the globule does not merge with another globule and the micelle structure created by the emulsifier remains intact. In some instances, a little agitation such as shaking may revert the mixture to a good emulsion. Creaming is different from flocculation as there are no attractive and repulsive forces among the globules.

The creaming rate of an emulsion globule in a dilute o/w emulsion system follows Stoke's law. It describes the movement of a small spherical globule during the creaming process that freely moves without collision and has no physical or chemical attraction. According to Stoke's law, the creaming rate is given by the equation below.

$$v = \frac{d^2(\rho_o - \rho_c) g}{18 \eta}$$

where  $v$  is the creaming rate,  $d$  is the diameter of droplet,  $\rho_o$  is the density of droplet,  $\rho_c$  is the density of continuous phase,  $g$  is the acceleration of gravity, and  $\eta$  is the viscosity of the continuous phase.

From the equation, the three main factors that affect creaming are droplet size, viscosity of continuous phase and the difference in the densities between emulsion droplets and continuous phase. Theoretically, creaming can be reduced by the following actions:

- 1) Reduce the particle size by homogenisation. For example, reducing the diameter of the globule by half will increase creaming stability by a factor of four.
- 2) Increase the viscosity of the continuous phase by adding thickening agents such as xanthan gum or starch.
- 3) Reduce the difference in the densities between the dispersed phase and continuous phase.

### Sedimentation

Sedimentation is the opposite phenomenon of creaming and normally observed in water-in-oil emulsions. Sedimentation happens when the dispersed phase is denser than the continuous phase and the gravitational forces pull the denser globules towards the bottom of the emulsion. Similar to creaming, sedimentation follows Stoke's law.

## Coalescence

Coalescence is a process where the globules merge with each other to form a larger globule. In this irreversible process, the globules come close to each other and cause breakage of the emulsifier film in order to merge. This will normally result in an increased droplet size and reduce the number of globules. Eventually, coalescence will lead to 'breaking' where all of the emulsion film is destroyed and a complete separation of oil and water phases occurs. A similar phenomenon is Ostwald ripening, where large droplets grow at the expense of the small droplets due to mass transport of dispersed phase through the continuous phase. However, it is considered to be negligible in o/w emulsions due to the low solubility of triglycerides in the continuous phase (McClements, 2007). Some of the common factors for coalescence to occur include insufficient amount of emulsifying agent, large globules, and weak repulsion between globules. One preventative action to reinforce the mechanical strength of the interfacial film involves the selection of the right emulsifying agent and the right choice of mixing process, such as high-shear dispersion and high-pressure homogenisation.

## Extrinsic Factors Affecting Emulsion Stability

### Homogenisation

Homogenisation is a process to mix two immiscible liquids so that they can be in the same phase. This will turn one of the liquids into small globules that disperse uniformly in the other liquid. In the milk processing industry, homogenisation is used to reduce oil globules to about 1  $\mu\text{m}$  in diameter and to lower the tendency of creaming and coalescence (Bylund, 2015). Small oil globules are created when milk is forced through a narrow gap at high velocity. Two events happened at that time where oil globule membranes are partially disrupted and milk proteins adsorb at the interface (Lopez, 2005). Atarés et al. (2012) reported that homogenisation reduces the droplet size and size range distribution of o/w emulsions containing sunflower oil and whey proteins. High-pressure homogenisation is more effective in producing smaller droplets compared to mechanical homogenisation (Perrier-Cornet et al., 2005). The type of homogenisation process will determine the droplet size and emulsion stability.

### Heat Treatment

Various heat treatments have been utilized in the dairy industry to destroy any pathogenic microorganisms while retaining the nutritional benefits of protein-stabilised emulsions. The types of milk proteins (casein and whey proteins) in the emulsion experience different effects during heat treatment. Casein is very stable at high temperature (Fox et al., 2015), where there is relatively little aggregation upon heating. Casein-stabilised emulsions do not aggregate at 120–130 °C (Guo et al., 1989), and it has been demonstrated that heated casein has better adsorption at the interface (Liang et al., 2017). In contrast, whey proteins undergo conformational changes such as unfolding, denaturation and aggregation when heated above 65 °C (Croguennec et al., 2004; Jang and Swaisgood, 1990). These conformational changes affect whey protein functionality as emulsifiers. Heat treatment of whey protein-stabilised emulsions resulted in aggregation of oil droplets (Euston et al., 2000), increased viscosity and increased the droplet size (Sliwinski et al., 2003). Whey protein-stabilised emulsions are reported to be highly sensitive to heat treatment (Liang et al., 2013).

## Intrinsic Factors Affecting Emulsion Stability

### pH

In a protein-stabilised emulsion, the proteins adsorb to the interface and form a thin protective membrane around the oil. The emulsions are stable against flocculation due to the strong electrostatic repulsion between the charged droplets (Dickinson, 1997; McClements, 2004; Tangsuphoom and Coupland, 2008). The protein-stabilised emulsions are sensitive to the pH of the emulsion. For example, casein in milk aggregates and flocculates as a non-soluble component near to its isoelectric point around pH 4.6. Flocculation has been observed in emulsions near to their isoelectric point when there is no net interface charge (Rampon et al., 2004; Tangsuphoom and Coupland, 2008). The change in pH leads to the folding and unfolding of proteins, which occurs via an intermediate stage called the molten globule (Matsumura et al., 1994). The molten globule state is the intermediate stage between the native and the completely unfolded state, and characterised by the retained secondary structure and unfolded tertiary structure (Nylander et al., 2008). Some proteins at the molten globule state adsorb at a greater degree to the interface than the native state. For example, the adsorption of  $\alpha$ -lactalbumin to the interface increased with the lowering of pH, where the protein is in the molten globule state (Nylander et al., 2008).

### Ionic Strength

The presence of ions such as  $\text{Na}^+$ ,  $\text{Fe}^{2+}$  and  $\text{Ca}^{2+}$  in the protein-stabilised emulsion can interact with the charged droplets and affect emulsion stability. These ions can bind to the oppositely charged droplets and subsequently reduce the electrostatic repulsion between charged droplets. The ionic strength of the emulsion has a significant effect on flocculation (Das and Kinsella, 1990;

Dickinson, 1997; Tangsuphoom and Coupland, 2008; Tcholakova et al., 2006). Flocculation becomes rapid when ionic strength exceeds a certain limit.

### Biopolymers

Protein-stabilised emulsions usually contain one or more biopolymers (McClements, 2004). Biopolymers are naturally occurring polymers found in living organisms, such as proteins and polysaccharides. Both proteins and polysaccharides are usually used together in the food industry. Polysaccharides interact with protein-stabilised emulsions and influence functional properties such as thickening, gelling and emulsifying (McClements, 2004; Ye, 2008). Biopolymers may interact with the adsorbed protein to behave differently at the same emulsion conditions or during manufacturing processes (Dickinson, 2003). For example, polysaccharides have been shown to reduce droplet aggregation in emulsions with high salt concentrations (Harnsilawat et al., 2006).

### Emulsifiers

Low molecular weight emulsifiers or surfactants interact with proteins at the interface that can alter emulsion stability. Emulsifiers can be categorised according to their ability to dissolve in water or oil. Examples of common oil-soluble emulsifiers are lecithin and mono- and diglycerides, while polysorbates are water-soluble emulsifiers. Alternatively, emulsifiers can be grouped according to their head group, either non-ionic (no charge) or ionic (anionic or cationic). For examples, mono- and diglycerides have no charge while sodium stearyl lactylate has a charged head.

Emulsifiers can quickly adsorb to the interface and lower the interfacial tension to a greater extent than proteins (Bos and van Vliet, 2001). It is well known that emulsifiers compete with proteins at the interface and can displace protein from the interface, which may cause emulsion destabilisation (Dickinson, 1997). One aspect of protein displacement is the ability of ionic emulsifiers to bind with protein, which alter heat stability of proteins and adsorption strength to the interface (Nylander et al., 2008). Dodecyl trimethyl ammonium bromide, being a cationic emulsifier, binds to the proteins to promote protein unfolding and aggregation, which results in emulsion destabilisation (Kelley and McClements, 2003b). Anionic emulsifiers such as sodium dodecyl sulfate improve thermal stability of emulsions by enhancing the electrostatic repulsion between droplets and increasing the denaturation temperature of the adsorbed protein (Kelley and McClements, 2003a). Anionic emulsifiers can act synergistically with protein to improve emulsion stability, particularly creaming (Dickinson and Ritzoulis, 2000).

Another factor affecting protein displacement is the solubility of emulsifiers in water or oil and their concentrations in the emulsion (Nylander et al., 2008). Water-soluble polysorbates displace protein to a greater extent than oil-soluble mono- and diglycerides (Euston et al., 1995). Oil-soluble emulsifiers such as mono- and diglycerides and sorbitan esters at low concentration do not displace protein (Dickinson and Hong, 1994), thus enhance the robustness of emulsion droplets against destabilisation. The knowledge on protein displacement is important to food technologists in order to select the right types of emulsifiers when formulating emulsions.

### Effects of Mono- and Diglycerides on Physicochemical Properties and Emulsion Stability

Mono- and diglycerides are a mixture of mono-, di- and triglycerides together with a small amount of glycerol and fatty acids. Mono- and diglycerides are oil-soluble emulsifiers derived from the hydrolysis of oils and fats and found in many food products. Mono- and diglycerides can alter the physicochemical properties and emulsion stability of a protein-stabilised emulsion (McClements, 2004).

Mono- and diglycerides are more surface active than proteins, and thus spread rapidly at the interface and lower the interfacial tension (Fredrick et al., 2013). At low concentrations of mono- and diglycerides, protein adsorbed at the interface is high (Dickinson and Hong, 1994; Euston and Hirst, 2000). At this stage, mono- and diglycerides fill between the small gaps at the interface without displacing protein (Euston and Hirst, 2000; Munk et al., 2014). However, a higher protein displacement is seen when increasing the shear or lowering the temperature of the emulsions containing mono- and diglycerides (Carrera Sánchez and Rodríguez Patino, 2004; Davies et al., 2001; Dickinson and Tanai, 1992).

Creaming is the most common emulsion destabilisation mechanism amongst dairy-based emulsions. According to Stoke's Law, large droplets have a faster rate of creaming compared to smaller ones and are more likely to separate due to gravity (McClements, 2007). Mono- and diglycerides can effectively reduce droplet size and size range distribution of protein-stabilised emulsions (Dickinson and Hong, 1994; Krog, 2011; Liang et al., 2016; Matsumiya et al., 2010). Thus, reducing the number of large globules should delay the occurrence of creaming. Recent studies of protein-stabilised emulsions by our group found that unsaturated mono- and diglycerides reduce droplet size range distribution and enhance creaming stability to a greater extent than saturated mono- and diglycerides (Loi et al., 2018).

## Conclusions

The separation of oil and water phases in the emulsions over time have been observed in many foods and the common mechanisms of emulsion destabilisation are flocculation, creaming, sedimentation and coalescence. Simple model emulsions have been used to understand destabilisation mechanisms and the factors affecting destabilisation. Intrinsic and extrinsic factors can influence the physicochemical and emulsion stability of model emulsions. However, the complex interactions in food emulsions often makes it difficult to predict physicochemical properties and stability of emulsions. Studies on the complex interaction between biopolymers and emulsifiers will advance our understanding on stability and shelf life of food emulsions.

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# Application of HPLC in characterisation of Triacylglycerols and Detection of Adulteration in Cold Pressed Seed Oils

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## List of Abbreviations

TAG Triacylglycerol

LnLnLn Trilinolenoyl-glycerol

LLL Trilinoleoyl-glycerol

SOO Stearoyl-dioleoyl-glycerol

OOLn Dioleoyl-linolenoyl-glycerol

OLL Oleoyl-dilinoleoyl-glycerol

LnLP linolenoyl-linoleoyl-palmitoylglycerol.

## Introduction

Due to the economic importance of edible oils, it is imperative to develop and improve methods to verify authenticity, detect adulteration and define the composition and purity of blends (Ashurst and Dennis, 1996). Edible seed oils are predominantly made up of triacylglycerols (TAG) that usually follow a typical pattern in the glycerol molecule but have unique characteristics in each of the different oilseeds. The advantage of analysing the fatty acid positional profile in TAGs, compared to just the fatty acid profiles alone, is that the distribution of fatty acids on the glycerol backbone is stereo-specific. This fatty acid distribution is genetically modulated and consequently determination of the fatty acid positional profile in TAGs is of value (Aparicio and Aparicio-Ruiz, 2000).

The TAG compositional data is a valuable indicator that provides the quantitative assessment of the quality and purity of seed oils. The high specificity and precision of the TAG compositional information that can be obtained for different kinds of oils have enabled the food industry and researchers to more precisely confirm authenticity and adulteration of oils. Several techniques have been developed to analyse the TAG compositions in seed oils. This chapter briefly reviews the use of HPLC in the determination of intact TAGs and quality assessment in edible seed oil, with particular emphasis on oil products extracted by means of cold pressing.

## Cold Pressed Seed Oils

Cold pressed oils are obtained from fruit or seeds either by mechanical processes such as traditional grinding using granite mill-stones or by pressing using modern stainless-steel presses commonly found in large commercial operations. Pressing and grinding processes produce heat through friction and for oil extraction the temperature must not rise above 120°F (49 °C) for any oil to be considered cold pressed. The main advantage of cold pressed oils is that the low processing temperature during oil extraction retains the bioactivity of phenolics and essential fatty acids naturally present in the extracted oil (Teh and Birch, 2013). In addition, cold pressing retains more of the flavour, aroma and nutritional value in the extracted oil, producing a product that has minimal chemical contamination and modification (Parker et al., 2003) since no solvents are used in the extraction process. Whilst such an oil extraction process has desirable features, the main disadvantage is that the oil extraction yield from the seed is lower compared to other extraction processes that use organic solvents. Studies were carried out on cold pressing lemon seed resulted in a lower oil yield (36.84%) compared to 71.29% for solvent extraction (Yilmaz and Güneşer, 2017).

## Importance of Analysing and Characterising Intact Triacylglycerols in Seed Oils

Edible cold pressed seed oils have attracted a lot of interest due to the health benefits associated with retained bioactive compounds in the oil. The retention of essential fatty acids such as  $\alpha$ -linolenic acid (18:3n-3), vitamin E and other important oil characteristics including improved oxidative stability, has created an awareness in the industry of the value of characterising and profiling the positional fatty acid profile in the TAG components of the cold pressed seed oils (Parry et al., 2005). Most of the recent research has focused on determination of the lipid composition of cold pressed seed oils with an emphasis on quality assurance to address purported adulteration of cold pressed oils. As consumers are willing to pay a premium for quality assured cold pressed seed

oil, validation is imperative. Many of the reported studies have focused on examining the triacylglycerol profiles of closely related oils to obtain detailed compositional information. This can provide information on the raw materials used, the processing conditions, and can provide a means of quality control of purity and whether the oils have been blended (Jakab et al., 2002). The characterization of several seed oils has been reported, including oil extracts from grape (Bail et al., 2008), canola, hemp and flax seeds (Mungure and Birch, 2014).

As the distribution of TAG composition affects the physicochemical and oxidative stability of oils, this information is useful to enable development of appropriate handling and processing methods to ensure oil quality is retained. Nederal et al. (2012) showed that TAGs comprised mainly of linoleic acid at all three glycerol esterification positions (LLL) in pumpkin seed oil had less oxidative stability compared to the TAGs comprised of a mixture of trioleic acid (OOO), and those with some TGAs substituted with other fatty acids (SOO and LOO) are easier to abstract a hydrogen atom from and to initiate oxidative processes due to the linoleic acid being more unsaturated.

Validation of the origin of seed oil is increasingly a priority due to the possibility of substitution of oils obtained from alternative sources. Amaranth seed has been reported to be an ethical and healthy alternative source of edible seed oil that also contains squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene), an expensive terpenoid compound. Amaranth seed oil is now increasingly used as an ethical source instead of oils containing squalene that have been more traditionally sourced from whale (*Physeter microcephalus*), shark liver (*Cantrophorus squamosus*) and fish skin (Jahaniaval et al., 2000). The composition of TAGs has also been analysed to determine the differences in similar species grown in different geographic and climatic conditions. A study by Jahaniaval et al. (2000) showed that various Amaranth accessions displayed genetic variation and showed sizeable differences in the fatty acid profiles of TAGs.

A variety of techniques have been applied to analyse TAGs in oil samples, including MALDI-TOF MS/MS (Zollner et al., 1997; Bail et al., 2008), LC-MS/MS, thin layer chromatography (TLC), high temperature gas-liquid chromatography (HT-GLC) and silver ion-reversed phase HPLC (Ag-RP-HPLC) (Carelli and Cert, 1993). The following section reviews different HPLC techniques that have been used to characterise triacylglycerol fatty acid compositions in selected seed oils.

## Methods of Analysing Triacylglycerols Using Reversed Phase-HPLC

Many of the methods have been developed as a result of the need to achieve quality assurance in the virgin olive oil processing industry. Typically, TAGs have been analysed by HPLC using a reversed phase C-18 column, with acetone/acetonitrile as the mobile phase. HPLC triacylglycerol determination methods have been applied in analysing seed oils. The European commission has adopted an official method for the determination of TAG compositions (Butinar et al., 2010; de la Mata-Espinosa et al., 2011). This method involves a stereospecific analysis of TAGs and is commonly known as  $\Delta$ ECN42. The ECN number is the number of carbon atoms (excluding the carbons on the glycerol) less the doubled sum of the number of double bonds in a TAG (Jabeur et al., 2014). This is summarised in Eq. (1) below:

$$\text{ECN} = \text{CN} - 2n \quad (1)$$

where CN is the carbon number and n is the number of double bonds per TAG present in the attached 3 fatty acids, but not including the carbons in the glycerol (Piravi-Vanak et al., 2009). Several different detection systems have been used in HPLC separation of TAGs. Although ultra-violet/visible spectroscopy and refractive index (RID) have been the most commonly used detection systems, evaporative light scattering display (ELSD) and more recently charged aerosol detector (CAD) systems have been used (de la Mata-Espinosa et al., 2011). The different analytical techniques are discussed in detail below.

### HPLC-ELSD

The principle of ELSD is to nebulize or spray the mobile phase effluent in a stream of nitrogen gas forming a dispersion of droplets. The mobile phase is evaporated leaving a fine mist of dried particles, and the light scattered by the particles is recorded and an electrical signal is generated. Nederal et al. (2012) used HPLC-ELSD to analyse/compare the TAG composition and the oxidative stability of pumpkin seed oils extracted following roasting or by cold pressing the seed. The TAGs in these oil extracts were identified using known TAG standards and unknowns were determined using the ECN number system explained above, and up to 13 TAGs were identified. The results obtained showed that cold pressing de-husked pumpkin seed produced higher yields of TAG with OLL fatty acid profile compared with cold pressing the complete seeds. The saturated fatty acids namely stearic and palmitic were shown to have a preference of *sn*-1, -3 positions compared to the unsaturated fatty acids taking *sn*-2 position (Nederal et al., 2012).

### HPLC-CAD

HPLC-CAD has been reported to provide significant advantages of increased sensitivity and shorter analysis time compared with current protocols officially adopted by the European Commission for TAG identification in virgin olive oil (de la Mata-Espinosa et al., 2011).

With HPLC-CAD, TAG identification is commonly done using known standards. [de la Mata-Espinosa et al. \(2011\)](#) applied two different approaches with the first one adding the TAG standards into a test sample solution and secondly by comparing the retention times for the TAG from a standard solution with the sample solution. After the identification, the chromatograms were verified by comparison to official results published by the International Olive Council (IOC). HPLC-CAD provided the same results with 13 major TAGs identified as reported with the IOC protocol. HPLC-CAD offered better sensitivity and detection of lower abundance TAGs.

### HPLC-ESI-MS

[Mungure and Birch \(2014\)](#) used HPLC coupled with ESI-MS to characterise cold pressed canola, flax, and hemp seed oil. TAGs were separated by HPLC and the detection was carried out using a diode array detector at wavelengths of 215 nm (flax and canola) and 205 nm (hemp). Collected intact TAGs were introduced by direct infusion onto the electrospray ionisation (ESI) source of a mass spectrometer set to positive ion mode with a high collision energy of up to 10 eV. Eight TAGs were detected for cold pressed hemp at wavelength  $\lambda = 215$  nm. Seven TAGs were detected at  $\lambda = 205$  nm in cold pressed flax and canola oils. The TAG fractions obtained from HPLC were subjected to ESI-MS. The results obtained are summarised in [Table 1](#), in terms of elution order characterised according to partition number/ECN number, and TAG abundances. It is interesting to note that all three cold pressed seed oils had the highest TAG abundance with highest fatty acid content. For instance, flax was reported to have linolenic acid as the most abundant fatty acid (Ln = 58.8%) and the TAG with the highest abundance was LnLnLn. The results reported for flax by [Mungure and Birch \(2014\)](#) were in agreement with that reported by [Choo et al. \(2009\)](#). A representative chromatogram of cold pressed hemp seed oil is presented in [Fig. 1](#), for which HPLC fractions of the TAGs were collected prior to ESI-MS.

Cold pressed canola oils presented a different pattern compared to the other two oils, with the most abundant TAG being OOLn, with LLL and LnLP co-eluting and the two constituted up to a fifth in total abundance.

### HPLC-RID

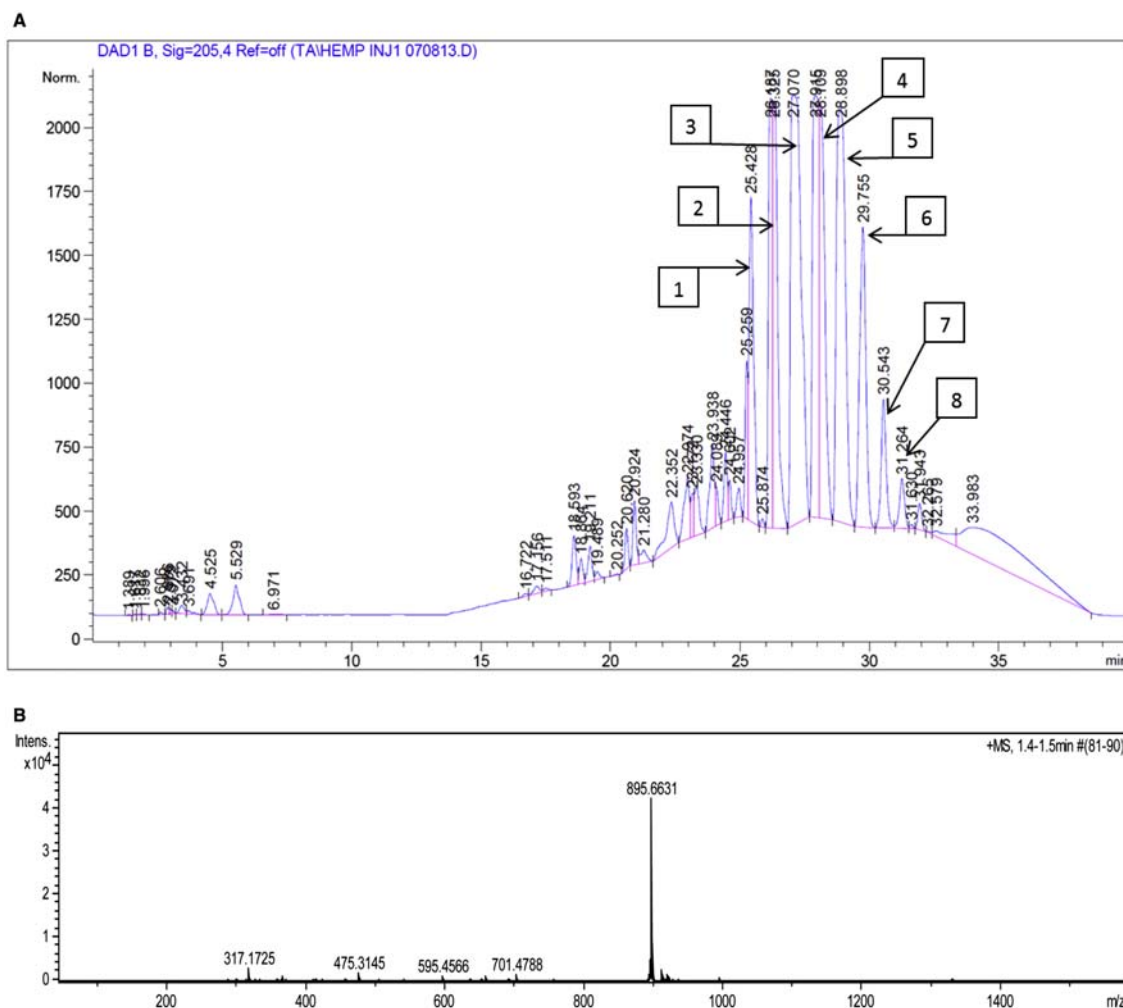
This method involves detection by refractive index and is one of the most widely used detection methods. It has been used for assessing genuineness of selected Slovenian cold pressed pumpkin seed oils ([Butinar et al., 2010](#)). The pumpkin seed oil was subjected to RP-HPLC using propionitrile as the mobile phase eluant and fractions containing TAGs were collected and then identified using TAG standards derived from olive oil reference TAGs. The use of propionitrile and column temperature control were reported to provide more accurate and repeatable data with improved baseline stability ([Butinar et al., 2010](#)). A total of 29 TAGs were identified in this study which is considerably greater than that reported in previous investigations by [Jakab et al. \(2002\)](#) and ([Andrikopoulos et al., 2001](#)) who identified only 12 TAGs. This method demonstrated that coupling stereospecific analyses with other chemical determinations (i.e. fatty acid profiles using GC) could be useful in evaluating the authenticity of the cold pressed seed oils.

## Conclusion

Most of the current work on the evaluation of edible cold pressed oil adulteration is based on high-performance liquid chromatography analysis. Improvements in the detection of TAGs eluted from HPLC such as using CAD, have provided increased precision and have achieved excellent recoveries of compounds compared to earlier HPLC configurations. In some cases, a combination of HPLC and chemometrics has enhanced the correlation of obtained compositional data with accredited reference quality parameters in the cold pressed oil product. By comparing the relative proportions of TAGs, in addition to the positional identification of fatty acids on individual TAGs, is collectively becoming an important measure of characterisation and quality assessment.

**Table 1** Summary of the most abundant TAGs present in various cold pressed seed oils

Cold pressed seed oil	Detection system	Number of TAGs identified	Most abundant TAG	References
Canola	ESI-MS	7	OOLn	<a href="#">Mungure and Birch, 2014</a>
Flax	ESI-MS	7	LnLnLn	<a href="#">Mungure and Birch, 2014</a>
Hemp	ESI-MS	8	LLL	<a href="#">Mungure and Birch, 2014</a>
Pumpkin seed oil Northern Slovenia (Stajersko prekmursho bučno olje)	RID*	29	OLL	<a href="#">Butinar et al., 2010</a>
De-husked pumpkin seed oil	RID	13	OLL	<a href="#">Nederal et al., 2012</a>
Husked pumpkin seed oil	RID	13	OLL	<a href="#">Nederal et al., 2012</a>



**Figure 1** (A) Representative RP-HPLC chromatogram of hemp seed oil extract run by the author. The 8 major fractions containing TAGs were collected and subsequently analysed by ESI-MS. RP-HPLC was conducted on an Agilent1260 infinity Bio-inert Quaternary HPLC system with a C18 Phenosphere-Next column and using a diode array detector. Detection was achieved at wavelength of 205 nm). (B) A representative mass spectrum for fraction 1 from ESI-MS ( $m/z = 895$ , peak 895-sodated trilinolenoyl-glycerol).

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# Enzyme Immobilization for Oligosaccharide Production

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## Glossary

OSs Oligosaccharides

XOSs Xylooligosaccharides

GOSs Galactooligosaccharides

IMOSs Isomaltooligosaccharides

## Introduction

Oligosaccharides (OSs) are carbohydrates with low molecular weight showing considerable variety in terms of sugar composition and degree of polymerization (DP). These compounds are naturally found in foods, in mixtures containing the parental polysaccharides, as well as monosaccharides. Due to the biological activities presented by the different OSs, there is a growing demand for the industrial production of these compounds. Some non-digestible OSs, such as fructooligosaccharides, galactooligosaccharides and xylooligosaccharides, are considered efficient functional foods because they can selectively stimulate the growth and/or activity of benefic bacteria. Such effects are involved with many positive aspects in relation to human health, including lower risks of intestinal infections and prevention of some types of cancer (Rastall, 2010; Rajendran et al., 2017).

The contemporary demand of green technologies has increased the use of enzymatic process at industrial level. The use of enzyme as biocatalysts offers benefits such as mild reaction conditions, biodegradability and specificity. In this context, OSs production, specially through the enzymatic route, has received major attention; and examples of target enzymes are fructosyltransferases, xylanases and  $\beta$ -galactosidases, among others, as presented in Chapter XX. The severe conditions of industrial processes however can result in enzyme destabilization, shortening their industrial lifespan. Immobilization, in turn, is an effective alternative to circumvent these concerns by enhancing enzyme properties, simplifying downstream processing and improving operational stability of biocatalysts (Mohamad et al., 2015). Due to the advantages of using immobilized enzymes, we included this chapter reviewing recent data dealing with different techniques used for the immobilization of different microbial enzymes involved in OSs production.

## Immobilization of Xylanolytic Enzymes for Xylooligosaccharides Production

Xylooligosaccharides (XOSs) are the only nutraceutical that can be obtained from lignocellulosic biomass which can be generally considered a low-cost and abundantly available raw material. Immobilization of enzymes acting on the components of plant cell walls, remarkably xylanases, which are responsible for hemicellulose degradation has been previously reviewed (Terrasan et al., 2015). From 2015, new studies dealing with immobilization of microbial xylanases for XOSs production have arisen and their respective data are summarized in Table 1. Most of them deals with xylanolytic enzymes of bacterial origin, such as those from mesophilic *Bacillus subtilis* (Milessi et al., 2016), *Paenibacillus* sp (Young Kyu Hwang, 2015) or from extremophilic strains such as *Selenomonas ruminantium* (Terrasan et al., 2016), and *Geobacillus thermodenitrificans* JK1 (Kieraitė et al., 2015). Yet, some other studies deal with fungal xylanases or microbial enzymes present in commercial mixtures/cocktails (Bagewadi et al., 2016; de Oliveira et al., 2018a; de Oliveira et al., 2018b; Zhang et al., 2016).

Among these several studies evaluating different techniques for xylanase immobilization on diverse supports, a high stabilization degree was achieved by covalently immobilizing the xylanase (XynA) produced by *Bacillus subtilis*. This enzyme was 75- and 8600-fold thermo-stabilized after immobilization on glyoxal-chitosan and glyoxal-agarose supports, respectively. Furthermore, it was observed that when applied for substrate hydrolysis, the agarose-glyoxal derivative released xylobiose, xylotriose and xylotetraose from both soluble and conventional birchwood xylan, in a process that reached 20% of substrate conversion up to ten cycles of substrate hydrolysis (Milessi et al., 2016). In another study, a bifunctional enzyme with xylanase and cellulase activity was constructed and successfully immobilized by covalent bonds on carbon-coated chitosan nanoparticles. Immobilization improved thermal and storage stability, and the derivative was able to release XOSs from purified oat spelt, birchwood and beechwood xylans, as well as from wheat bran insoluble xylan and wheat bran. Xylooligosaccharides with chain-length in the range 2–6 were the main products released from wheat hydrolysis (Liu et al., 2015). Purohit et al. (2017) developed a magnetic cross-linked enzyme aggregate (CLEA) using a xylanase isolated from the bacterium *Acinetobacter pittii* MASK 25. After optimizing conditions for enzyme-glutaraldehyde crosslinking with magnetic nanoparticles, the aggregate was applied for hydrolysis of physically pretreated rice straw and corn cobs. Similar to the

**Table 1** Immobilization of microbial xylanases for xylooligosaccharides production

Enzyme/origin	Methodology/support	Results	Substrate	Main product	References
<b>Bacteria</b>					
<i>Paenibacillus</i> sp. HPL-001	Adsorption; silica-based mesoporous cellular foam	Stability for 5 reactional cycles (100%)	Birchwood xylan	–	<a href="#">Lim et al., 2015</a>
<i>Acinetobacter pittii</i> MASK 25	Magnetic CLEA	–	Rice straw and corn cobs	X5 and X6	<a href="#">Purohit et al., 2017</a>
<i>Geobacillus thermodenitrificans</i> JK1	Adsorption; Sepabeads Entrapment; sodium alginate	–	Xylans from beechwood, distiller's grains and wheat bran	X2	<a href="#">Kieraitė et al., 2015</a>
<b>Fungi</b>					
<i>Penicillium citrinum</i> HZN13	Covalent; glutaraldehyde activated alginate beads	Stability for 7 operational cycles (90%)	Sweet sorghum bagasse	Xylose and XOSs with higher DP	<a href="#">Bagewadi et al., 2016</a>
<b>Commercial enzyme/cocktails</b>					
Bioxilanase L Plus (Biocon)	Covalent; glyoxal agarose with polyethylenimine coating	Improved activity and stability; high substrate conversion degree	Beechwood xylan	X2	<a href="#">de Oliveira et al., 2018b</a>
Depol™ 333 MDP (Biocatalysts)	Covalent; glyoxal agarose	Improved activity and stability	Beechwood xylan	X2	<a href="#">de Oliveira et al., 2018a</a>
<i>Thermomyces lanuginosus</i> (Sigma)	Entrapment; sodium alginate followed by covalent cross-linking with glutaraldehyde	Release of XOSs with lower DP; 70% activity after 2 operational cycles	Xylan from <i>Meranti</i> wood sawdust	X2 and X3	<a href="#">Sukri and Mimi Sakinah, 2018</a>
<i>Thermomyces lanuginosus</i> (Novozymes)	Entrapment; polyethylene glycol net-cloth grafted on polypropylene nonwoven fabrics	80% and 60% activity after 25 and 50 operational cycles, respectively	–	–	<a href="#">Zhang et al., 2016</a>
<b>Other</b>					
Chimeric bifunctional enzyme	Covalent; glutaraldehyde on carbon-coated chitosan nanoparticles	80% activity after 7 operational cycles	Wheat bran and insoluble xylan from wheat bran	–	<a href="#">Liu et al., 2015</a>

DP: degree of polymerization, CLEA: crosslinked enzyme aggregate; X2: xylobiose; X3: xylotriase.

verified with the crude xylanase, the magnetic aggregate predominantly produced xylopentaose and xylohexaose from both biomasses, nevertheless, the aggregate-mediated hydrolysis presented 15%-higher xylan conversion of both biomass into XOSs.

In addition,  $\beta$ -xylosidases such as that from *Talaromyces thermophilus* covalently immobilized on chitosan, when catalyzing reverse hydrolysis reaction, synthesized xylooligosaccharides with DP from 2 to 4 in the presence of high xylose concentration (40%, w/v), a reaction which was not performed by the free enzyme. This characteristic was attributed to the immobilization process wherein a hydrophobic microenvironment was created at the active site, favoring synthesis activity (Guerfali et al., 2009).

### Immobilization of $\beta$ -galactosidases for Galacto-Oligosaccharides Production

Most of immobilized  $\beta$ -galactosidase for galactooligosaccharides (GOSs) synthesis were from fungal origin specially from filamentous *Aspergillus* spp. or yeasts *Kluyveromyces* spp (Table 2). Among many different approaches evaluated for  $\beta$ -galactosidase immobilization, the  $\beta$ -galactosidase gene (bgaL3) from *Lactobacillus bulgaricus* L3 was fused with a cellulose binding domain (CBD) and expressed in *Escherichia coli*. The fusion protein could be directly adsorbed onto microcrystalline cellulose with 61% immobilization efficiency. The enzymatic and transglycosylation characteristics of the immobilized enzyme were similar to those from the free form. By using the immobilized catalyst, the yield of galactooligosaccharides (GOSs) reached 49% (w/w) from 400 g/L initial lactose at pH 7.6, 45 °C for 75 min, with productivity of 156.8 g/L/h. A reusability assay demonstrated the immobilized derivative retained over 85% of activity after 20 batches with GOSs yield above 40% (Lu et al., 2012).

In addition, the use of lactulose (4-O- $\beta$ -D-galactopyranosyl-D-fructose) instead of lactose as precursor of novel GOSs was evaluated (Cardelle-Cobas et al., 2016) due to the well-recognized prebiotic properties of lactulose (Olano and Corzo, 2009), and therefore GOSs produced from transglycosylation of lactulose might have higher added value when compared to conventional GOSs. On this regard, the  $\beta$ -galactosidase from *Aspergillus oryzae* was successfully immobilized on glutaraldehyde-agarose. After process optimization, it was verified that immobilization increased thermal stability, allowed the enzyme reuse by many operational cycles and, hence, the yield of OsLu (oligosaccharides from lactulose). Furthermore, it was observed that some reaction conditions, specially in relation to pH conditions, affected the selectivity of the enzyme (Cardelle-Cobas et al., 2016).

### Isomaltooligosaccharides (IMOSs) Production

IMOSs consist of  $\alpha$ -D-glucose residues that are linked by  $\alpha$ -(1–6) glycosidic bonds with a DP from 2 to 6 and can be obtained through three main enzymatic processes: a) transfer of glucosyl residue from sucrose to acceptors such as glucose, fructose, maltose and isomaltose catalyzed by dextranucrase, b) digestion of highly linear dextran with dextranase, or c) conversion of sucrose involving both dextranucrase and dextranase (Kubik et al., 2004; Wu et al., 2017). Individual and/or concomitant immobilization/application of these enzymes present some peculiarities, as presented below and in Table 3.

#### Immobilization of dextran(glucan)sucrase

Dextranucleases (EC 2.4.1.5) traditionally obtained from Gram-positive bacteria *Leuconostoc* spp. are enzymes that perform the transfer of the glucosyl moiety of sucrose forming dextran and that can also catalyze acceptor reactions by transferring glucosyl residues from sucrose to other carbohydrate acceptors, producing oligosaccharides with  $\alpha$ -(1–6)-glucosyl linkages. Studies dealing with dextranucrase immobilization, however, have not been successful with respect to yield and stability. The reasons are that the dextran associated with the enzyme can mask the reactive groups of the enzyme or the presence of residues such as lysine, cysteine, aspartic and glutamic acids at the active site, which can react with aldehyde and epoxy groups leading to enzyme inactivation, in the case of covalent immobilization (Ölçer and Tanrıseven, 2010; Parlak et al., 2013).

Despite its disadvantages such as physical instability and undesired influence of several anions, encapsulation of dextranucleases in alginate is the most common immobilization method since it usually renders high process yields. In this respect, the *Leuconostoc* sp dextranucrase in the native form and after crosslinking with glutaraldehyde was entrapped in alginate beads and then coated with a film of chitosan. The immobilization process presented similar yield when different concentrations of glutaraldehyde were evaluated and in presence or absence of a chitosan coating. The preparations of native dextranucrase entrapped in alginate beads were less stable and rendered a lower degree of sucrose conversion what was attributed to enzyme leakage from the support. High operational stability was observed for all preparations which contained the crosslinked enzyme and the best results were achieved with 10% glutaraldehyde crosslinking. In the first cycle of sucrose conversion it was obtained 90% yield, and it dropped to 60%–70% from the sixth batch. The final products mixtures contained 55%–60% isomaltose, 20% glucose, as well as isomaltotriose and tetraose at 10%–15% (Kubik et al., 2004). By using a bio-engineering approach, Parlak et al. (2013) designed a novel dextranucrase that could be successfully immobilized by covalent linkages on Eupergit C 250L, with 100% immobilization and 83.3% activity yields. The problems commonly associated to covalent immobilization of dextranucleases were solved by eliminating of the enzyme and by fusing a glutathione S-transferase to the dextranucrase, what allowed reaction with epoxy groups from the support. Optimal activity conditions were not affected and thermal stability was increased by immobilization. Immobilized enzyme was quite active performing dextran biosynthesis and acceptor reactions effectively. In fact, the novel enzyme formed more acceptor products than the native enzyme. Furthermore, the

**Table 2** Immobilization of microbial  $\beta$ -galactosidases for galactooligosaccharides production

Enzyme origin	Methodology/support	Results	Substrate (reaction)	Product	References
<b>Bacteria</b>					
<i>Bifidobacterium</i> sp; expressed in <i>E. coli</i>	Ionic; DEAE-Cellulose and Q-Sepharose Covalent; amino-ethyl- and glyoxal-agarose	Increased operational stability; GOSs synthesis up to 55 °C and during six cycles (Q-Sepharose derivative) with similar yields	Lactose	GOSs	Osman et al., 2014
<i>Thermotoga maritima</i> expressed in <i>E. coli</i>	Adsorption; epoxy-coated magnetic beads	Higher stability			Marín-Navarro et al., 2014
<i>Lactobacillus bulgaricus</i> L3 gene fused with a cellulose binding domain; expressed in <i>Escherichia coli</i>	Adsorption; cellulose	Activity retained after twenty batches; GOSs yield maintained above 40%			Lu et al., 2012
<b>Fungi</b>					
<i>Cluyveromyces fragilis</i>	Adsorption; magnetic nanobeads with epoxy groups	Higher catalytic activity and operational stability	Lactose	GOSs	Liu et al., 2015
<i>Aspergillus</i> sp.	Entrapment; calcium alginate	Improved mechanical strength			Feng et al., 2010
<i>Cluyveromyces lactis</i>	Adsorption; polystyrene nanofiber	Improved thermal stability and in alkaline pH; 30% of activity retained after nine operational cycles; enhanced product yield			Misson et al., 2015
<i>Aspergillus oryzae</i>	Covalent; glutaraldehyde agarose	Increased thermal and operational stability	Lactulose (transgalactosylation)	6' galactosyl-lactulose and other oligosaccharides with DP $\geq$ 3	Cardelle-Cobas et al., 2016
<b>Commercial enzyme/cocktails</b>					
<i>B. circulans</i> Biolactasa-NTL CONC X2	Covalent; chitosan derivatized with glutaraldehyde or epichlorohydrin	Increased thermal stability	Lactose	GOSs	Urrutia et al., 2013
<i>Cluyveromyces lactis</i> Maxilact LX 5000	Covalent; glutaraldehyde-activated chitosan	Increased optima pH and temperature; increased thermal stability			Klein et al., 2013
<i>A. oryzae</i> (Enzeco)	Amino-epoxy Sepabeads, glyoxyl-agarose and chitosan	Glyoxal agarose was the best derivative for lactose conversion and operational stability.			Huerta et al., 2011
<i>A. oryzae</i> (Sigma)	Covalent; glutaraldehyde crosslinked on magnetic hydrazide-Dacron particles	High operational stability			Neri et al., 2011a
<i>A. oryzae</i> (Sigma)	Covalent; glutaraldehyde crosslinking; magnetic particles coated with polyaniline	High operational stability			Neri et al., 2011b
<i>A. oryzae</i> (Sigma)	Entrapment; sol-gel matrix and polyvinyl alcohol hydrogel capsules	High operational and storage stability; continuous production in a packed-bed reactor	Lactose Whey	GOSs	Jovanovic-Malinovska et al., 2012
<i>Cluyveromyces lactis</i> (Sigma)	Covalent; silica-gel	Optimization for product synthesis; increased operational stability	Lactose and fructose	Lactulose	Song et al., 2013
<i>Bacillus circulans</i> (Biolacta FN5)	Covalent; cross-linking with glutaraldehyde; microporous polyvinylidene fluoride membrane	Optimization for operation in a reaction cell, with three immobilized membranes in series; increased operational and storage stability	Lactose	GOSs	Palai et al., 2014
–	Covalent; chitosan-treated Fe <sub>3</sub> O <sub>4</sub> nanoparticles	Increased optima pH and temperature; increased thermal stability			Chen and Duan, 2015

GOSs - galactooligosaccharides; DEAE - diethylaminoethyl.

**Table 3** Application of individually and co-immobilized dextransucrase and dextranases for isomaltooligosaccharides (IMOSs) production

Enzyme origin	Methodology/support	Substrate	Products	Results	References
<b>Dextransucrase</b>					
<i>Leuconostoc mesenteroides</i> B-512F	Covalent; glutaraldehyde activated-chitosan	Maltose/Sucrose	IMOSs	Immobilized enzyme on chitosan particles was more feasible for industrial applications than calcium-alginate beads	<a href="#">Graebin et al., 2016</a>
<i>Leuconostoc mesenteroides</i>	Entrapment; sodium alginate beads	Maltose/Sucrose	IMOSs	High operational stability, increased storage, thermal, and pH stabilities and produced gluco-oligosaccharide with DP 3–5.	<a href="#">Kothari et al., 2012</a>
<b>Dextranase</b>					
<i>Penicillium</i> sp. D8144	Convection; epoxy-activated monolithic Convective Interaction Media (CIM) disk	Dextran T40	IMOSs	The capacity of IMOSs to serve as carbon source was at least as good as commercialized fructo-oligosaccharides Fibrulose F97 and better than Orafit P95.	<a href="#">Chalane et al., 2017</a>
<i>Penicillium lilacinum</i>	Covalent; Eupergit C	Dextran	IMOSs	Immobilized enzyme with high industrial potential for production of IMOSs from dextran.	<a href="#">Aslan and Tanriseven, 2007</a>
<i>Penicillium</i> sp. D8144	Convection; epoxy-activated monolithic Convective Interaction Media (CIM) disk	Dextran T40	IMOSs DP 8–10	IMOSs with DP 11–20 generated during the first 120 min, which were then degraded to shorter IMOSs. Ratio of IMOSs with DP 8–10 increased all over the hydrolysis showing the possibility to produce IMOs with different chain length	<a href="#">Bertrand et al., 2014</a>
<b>Dextransucrase and dextranase</b>					
Dextransucrase from <i>Leuconostoc mesenteroides</i> /dextranase from <i>Chaetomium erraticum</i>	Entrapment, alginate	Sucrose	IMOSs	A mechanistic model was presented, which takes account the formation and hydrolysis of the main constituents of isomaltose, IMOSs and dextran	<a href="#">Erhardt et al., 2008</a>
Dextransucrase from <i>Leuconostoc mesenteroides</i> /dextranase from <i>Penicillium funiculosum</i>	Entrapment, alginate	Sucrose	IMOSs	Co-immobilization of dextransucrase and dextranase was simple, effective presenting potential for IMOSs production at industrial-scale	<a href="#">Ölçer and Tanriseven, 2010</a>
Dextransucrase from <i>Leuconostoc mesenteroides</i> NRRL B-512F/dextranase from <i>Chaetomium erraticum</i>	Entrapment, alginate	Sucrose	IMOSs	Enhanced thermal, operational and storage stability of dextransucrase, application to obtain enriched orange juice	<a href="#">Tingirikari et al., 2017</a>

DP - degree of polymerization; IMOSs - isomaltooligosaccharides.

immobilized enzyme showed no decrease in activity over 15 batch reactions and retained its initial activity for 35 days at 4 °C storage.

### Immobilization of Dextranases

Dextranases (EC 3.2.1.11), usually obtained from filamentous *Penicillium* spp. belong to a group of hydrolytic enzymes that specifically hydrolyze (1–6) linkages in dextrans producing IMOSs. These enzymes have been immobilized using different methods including physical adsorption, covalent binding, ionic binding and entrapment (Table 3). Recently, the dextranase from *Penicillium lilacinum* was covalently immobilized on Eupergit C, and the derivative presented much higher stability both at low and high pH, and also under high temperatures. Furthermore, no decrease in activity was verified even after 20 batch reactions for IMOSs production (Aslan and Tanriseven, 2007). Immobilization of the dextranase from *Aspergillus penicillioides* NRC 39 was evaluated by using different carriers and various techniques rendering an immobilization process with high yield when using covalent binding using 2% glutaraldehyde and prawn shell as support (El-Shamy and Atalla, 2014). In another case, a commercial dextranase from *Penicillium* sp (Sigma) was covalently immobilized on epoxy-activated macroporous monolithic support and applied to continuous operation reactor producing IMOSs with DP 8–10 from dextran. The IMOSs were generated all over the hydrolysis process and a residual enzymatic activity about 78% was verified after 5400 vol column (Bertrand et al., 2014).

### Co-immobilization of Dextranase and Dextran(glucan)sucrase

Considering dextran is a product of sucrose conversion, the hydrolysis of this polysaccharide by dextranases generates about 20% of unwanted branched oligomers. Therefore, for enzymatic synthesis, in addition to dextranase, the presence of a soluble or immobilized dextranase for the conversion of sucrose to dextran would be ideal (Table 3). In this case, dextranase and dextranase can be easily combined to produce these oligosaccharides from sucrose, mainly because of their similar optimum pH (Erhardt et al., 2008). Furthermore, from kinetic and economic points of view, it is more advantageous the use co-immobilized derivatives allowing simultaneous activity of dextranase and dextranase. In addition, co-immobilized derivatives can be applied not only to reduce reaction time, but also as an efficient approach to achieve higher product yields. In this sense, some studies have evaluated the co-immobilization of these enzymes and the application for IMOSs production.

In order to obtain a more sustainable process, sucrose instead of dextran has been used in a process which combines the use of dextranase for the conversion of sucrose into dextran and dextranases for the dextran decomposition. In this regard (Erhardt et al., 2008), created a biocatalyst from the co-immobilization of commercial dextranase pre-adsorbed onto bentonite and soluble dextranase which rendered high yields during IMOSs production. The dextranase from *Leuconostoc mesenteroides* and the dextranase from *P. lilacinum* were co-immobilized by encapsulating the dextranase and covalently attaching the dextranase to Eupergit C in alginate in presence of soluble starch, resulting in high immobilization yield and activity retention during 20 batch reactions producing isomaltooligosaccharides from sucrose and one month storage at 4 °C. In addition, the amount of produced oligosaccharides was increased after sucrose addition due to the co-immobilized dextranase (Ölçer and Tanriseven, 2010).

### Immobilization of $\beta$ -glucosidases for Glucooligosaccharides (GLOs) Production

GLOs can be produced by vegetable  $\beta$ -glucosidases which are usually extracted from almond (*Prunus dulcis* var. *amara*) and perform reverse hydrolysis and transglycosylation reactions. In this respect, a  $\beta$ -glucosidase from almond was immobilized on polyamine microspheres by cross-linking with glutaraldehyde for synthesis of octyl glucoside (OG) from glucose and octanol through reverse hydrolysis. Immobilization increased enzyme activity at pH 6.0–7.0 and optimal temperature was identical for both enzyme forms; however, both thermal stability and solvent tolerance were increased after immobilization. In a co-solvent system using *t*-butyl alcohol and water, the OG yield was increased by 1.7-fold compared to that from a system without co-solvent. Besides, initial reaction velocity was increased by three-fold after immobilization and OG synthesis was inhibited by surplus glucose. When OG synthesis was performed for 336 h in a fed-batch reaction to minimize inhibition by substrate, OG yield and glucose conversion rate reached 134 mM and 59.6%, respectively. Finally, when compared to the batch operation, fed-bath increased OG yield and glucose conversion rate by 340% and 381%, respectively (Wang et al., 2017).

### Other Oligosaccharides

#### Fructooligosaccharides (FOSs)

FOSs can be produced by the action of enzymes with transfructosylating activity either fructosyltransferase (EC 2.4.1.9) or  $\beta$ -fructofuranosidase (EC 3.2.1.26) derived from plants or microorganisms, mostly filamentous fungi such as *Aspergillus* sp. *Aureobasidium* sp. *Penicillium* sp. and *Fusarium* sp (Ganaie et al., 2014a). FOS production can be evaluated by using commercial fructofuranosidase and fructosyltransferase covalently immobilized on chitosan using glutaraldehyde (Lorenzoni et al., 2015), but it is more usually



performed by using immobilized cell systems which avoids laborious enzyme extraction and purification techniques. To accomplish cell immobilization system, entrapment of cells within porous matrices such as calcium alginate or chitosan gel beads are commonly evaluated approaches (Huang et al., 2016; Ganaie et al., 2014b). (Lorenzoni et al., 2015) operating two packed bed reactors (PBR) and two fluidized bed reactors (FBR) found FOS production achieved a 59% yield in the PBR and 54% in the FBR. They also observed that varying the flow rate it was possible to modulate the FOS composition and the operational stability in the PBR was evaluated for 40 days with no reductions in yields.

### Inulo-Oligosaccharides (IOSs)

The commercial *endo*-inulinase (EC 3.2.1.7) from *Aspergillus niger* (Megazyme) was covalently immobilized on glutaraldehyde-activated chitosan and the immobilized preparation was more stable at high temperature than the free enzyme, allowing continuous operation at 60 °C for prolonged periods. The biocatalyst presented prolonged storage stability at 4 °C and successfully produced IOSs from inulin and Jerusalem artichoke juice with DP 7–4 and the system produced higher IOSs content (from 35% to 65%) when operating continuously (Nguyen et al., 2011).

### Chitosan-Oligosaccharides

The chitosanase (EC 3.2.1.132) from *Bacillus pumilus* BN-262 an *endo*-type enzyme which produces dimeric and trimeric oligosaccharides from chitosan was immobilized by entrapment on agar-coated disks giving rise to a reactor with agar gel-coated multidisc impeller. The reactor was applied for repeated hydrolysis of chitosan using the immobilized enzyme, and the target products were obtained at high concentrations without activity loss of the immobilized biocatalyst (Ming et al., 2006).

### Mano-Oligosaccharides

The mannanase (EC 3.2.1.78) from *Penicillium occitanis* Pol6 was immobilized on chitin by cross-linking with glutaraldehyde. The immobilized enzyme exhibited improved thermal and pH stability in relation to its free counterpart. The immobilized biocatalyst also exhibited long-term storage stability retaining 70% of activity after 120 days. The main hydrolysis products yielded from locust bean gum were mannotriose and mannotetraose, which could be used as nutrient for lactic bacteria (Blibech et al., 2011).

### Future Perspectives

In this chapter, we reviewed recent data regarding immobilization of enzymes associated to the production of oligosaccharides with special focus on xylo-, galacto-, isomalto- and gluco-oligosaccharides production. Immobilization not only turn soluble enzymes into heterogeneous catalysts, instead it can also improve enzyme properties such as activity, specificity and stability under different conditions. Advances in immobilization science include developments of new matrices and the establishment of new and practical protocols, that can also take in consideration the absence of a support, the case of crosslinked aggregates (CLEAs). However, there is a gap in relation to studies evaluating the application of immobilized biocatalysts at larger scale. In addition, molecular biology tools to modify or create enzyme properties can improve or even allow the immobilization of a protein in certain support, as demonstrated in this chapter for the  $\beta$ -galactosidase harboring a cellulose binding domain for immobilization on cellulose; or the dextranucrase truncated at glucan binding regions to remove associated dextran and also containing amino acids that interact with reactive groups from the support, thus allowing immobilization of these enzymes; yet, even new catalytic activities can be added to an enzyme, as demonstrated with chimeric bifunctional enzyme with xylanase and cellulase activities.

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## Interactions of Macromolecules: $\beta$ -Lactoglobulin Interaction With Pectins

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### Glossary

**Complex coacervation** when oppositely charged macromolecules in solution are mixed, liquid–liquid phase separation can occur. The macromolecular complexes are concentrated in one liquid phase while the other liquid phase is depleted of the macromolecular complexes

**$\beta$ Lg**  $\beta$ -lactoglobulin

**DM** degree of methyl esterification (of pectins). The percentage of carboxylic acids that are esterified

**DP** degree of polymerisation

**ITC** isothermal titration calorimetry (the stepwise measurement of the heat of reaction produced by the interaction of molecules)

**SANS** small angle neutron scattering (used to study the size and shape of macromolecular complexes). Labelling a macromolecule with deuterium (e.g. protein) allows you to “see” it in a complex with unlabelled macromolecule (e.g. a polysaccharide)

**SAXS** small angle x-ray scattering (used to determine the size and shape of small particles)

**Self-assembly** a process by which individual molecules form defined molecular assemblies such as aggregates or clusters

**Self-organisation** a process by which the self-assembled aggregates or clusters create higher-ordered structures or networks

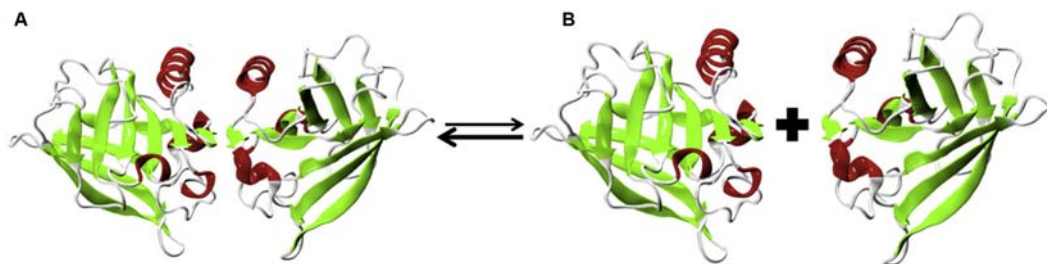
### Introduction

Why are people interested in the interaction of  $\beta$ -lactoglobulin and pectins? Because it is a good model for studying the protein-polysaccharide interactions found in a soft matter such as food (Schmitt and Turgeon, 2011; Aryee and Nickerson, 2014). Protein-polysaccharide interactions have been widely investigated in food emulsions (Augustin and Hemar, 2009; Damianou and Kiosseoglou, 2006), encapsulation of bioactives (Ron et al., 2010; Hosseini et al., 2015; Chen et al., 2016), gels (Laneville et al., 2006), foams (Ganzevles et al., 2006; Ganzevles et al., 2007) and as scaffolds for delivery of nutraceuticals and drugs (Xu et al., 2017b). An understanding of how such interactions occur can lead to better control of structure and texture of manufactured foods and more effective delivery of bioactive compounds to the target region of the human gut.

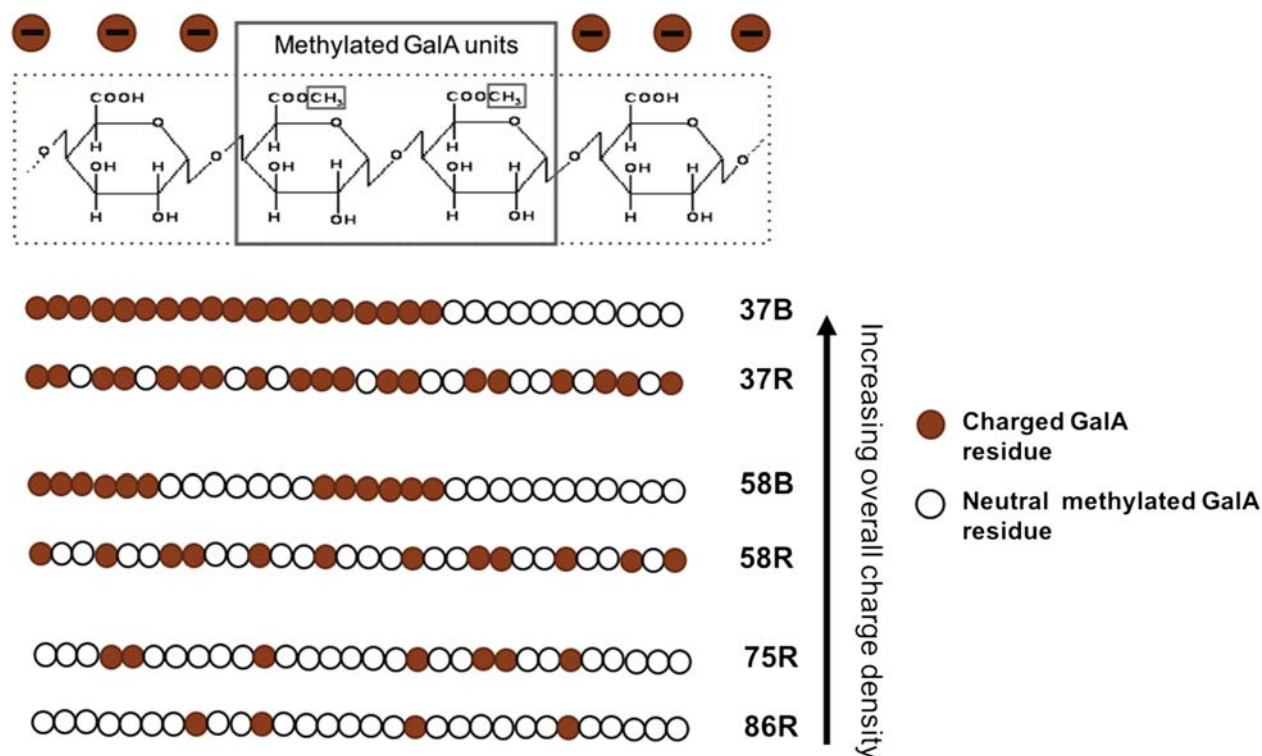
$\beta$ -Lactoglobulin ( $\beta$ Lg) is found in the milk of almost all mammals; humans being an exception. It is the most abundant protein in the whey from cow's milk from which it is isolated. It is a small globe-shaped protein comprising 162 amino acids with a mass of 18.4 kDa and an isoelectric point of pH 5.4 (Edwards et al., 2009). At the isoelectric point the overall charge is zero, while at pH less than 5.4 it will be positively charged and at pH greater than 5.4 it will be negatively charged. It is very robust, for example it passes through the stomach largely unchanged.

$\beta$ -Lactoglobulin exists as a dimer (Fig. 1) at pH 2.5 to 7.5 as long as salts are present, which means in all foods and almost all manufacturing conditions (Mercadante et al., 2012). It should be noted that the dimer is in equilibrium with a small amount of the monomer (Fig. 1) and so it is conceivable, although unlikely, that it could interact with other food components as the monomer.

The second thing to note is pectin comes in a variety of forms. Here we are concerned with commercial pectins and not the far more complex pectins that occur in plant cell walls (dietary fibre). The basic structure is a polymer made up of  $\alpha$ -D-galacturonic acid



**Figure 1** (A) The structure of the  $\beta$ -lactoglobulin dimer (B) monomers of  $\beta$ -lactoglobulin,  $\alpha$ -helices are red and  $\beta$ -strands are green. From Mercadante, D., et al., 2012. Biophysical J. 103, 303–312. Reproduced with permission The Biophysical Society.



**Numbers** = Degree of Methylesterification, determines overall charge density

**'R' or 'B'** = Random or Block-wise distribution of charged groups, determines local charge density

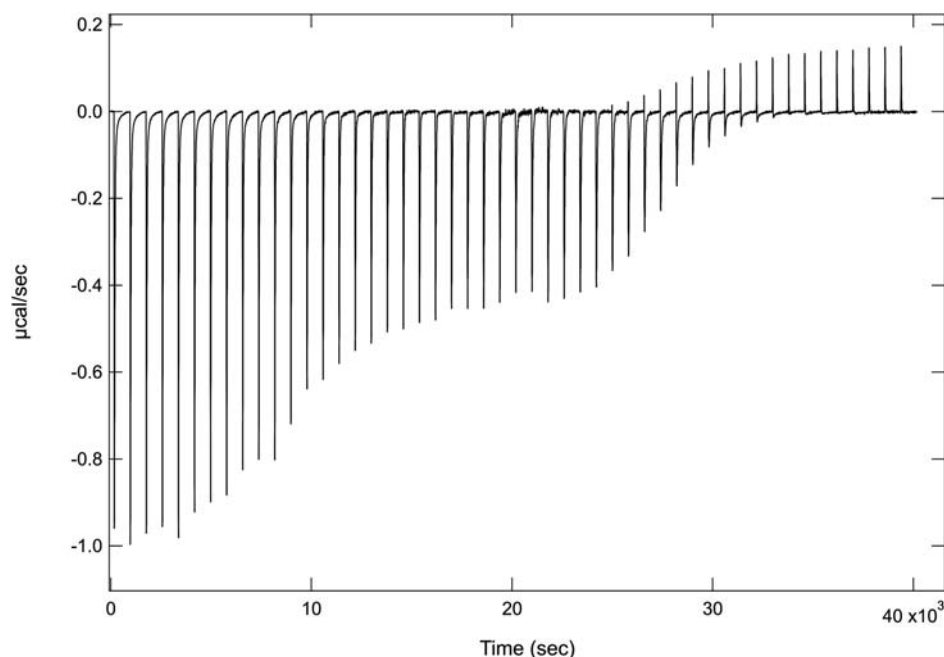
**Figure 2** Pectins samples used in this research. The molecular structure of pectin backbone: 1,4-linked  $\alpha$ -D-galacturonic acid (GalA) is in the top panel. When the solution pH is sufficiently high, the GalA units are negatively charged while the methyl-esterified GalA units are neutral. The pectin samples are named based on the degree and distribution of methyl-esterified groups: the numbers indicate the degree of methyl-esterification in percentage, and the letters 'R' and 'B' indicate random and blockwise distribution patterns of the uncharged methyl-ester groups respectively. Xu, A.Y., Melton, L.D., Ryan, T.M., Mata, J.P., Rekas, A., Williams, M.A.K., McGillivray, D.J. 2018. Effects of polysaccharide charge pattern on the microstructures of  $\beta$ -lactoglobulin-pectin complex coacervates, studied by SAXS and SANS. *Food Hydrocolloids* 77, 952–963. <https://doi.org/10.1016/j.foodhyd.2017.11.045>, plus supplementary data. Reproduced with permission from Elsevier.

units (Fig. 2). Hence it will have a negative charge. Not only the length of the polymer can vary but also the carboxylic acids in the galacturonic acids can be esterified by methyl groups so they have no charge. Not only does the degree of methyl esterification varies (typically from 80% to 40%) but also the arrangement of the methyl esters which occur in blocks or at random (Fig. 2). As you will see whether blocky or random can have a considerable influence on how the pectin interacts.

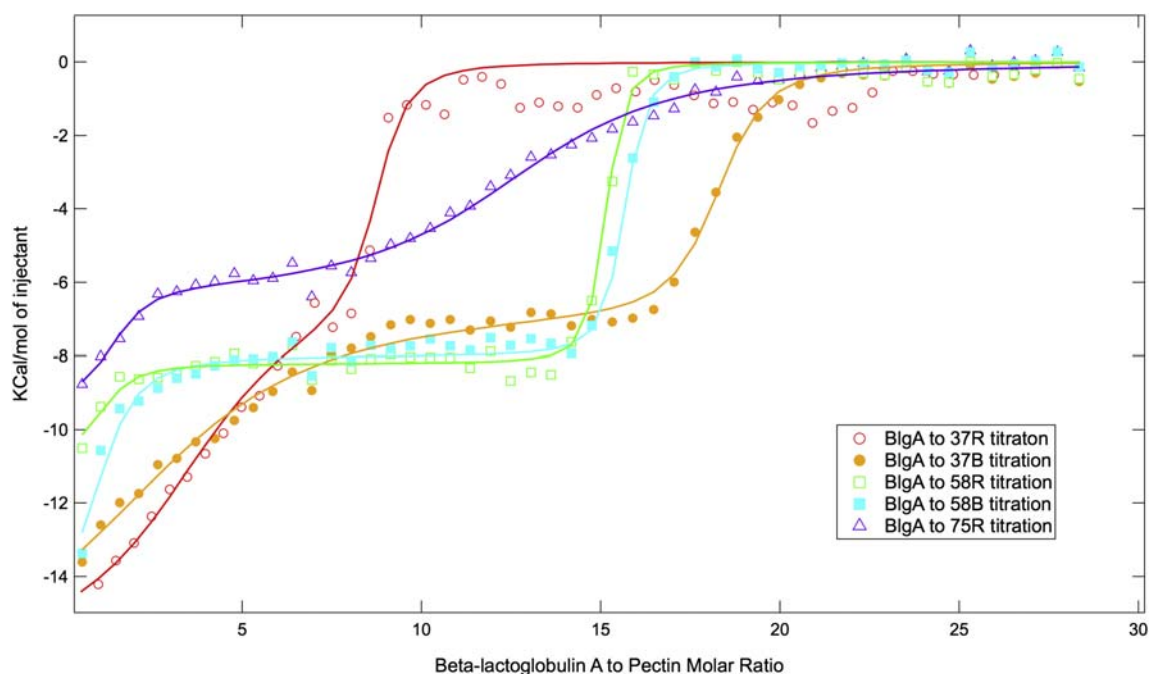
We chose to work at pH 4, so the positively charged  $\beta$ -lactoglobulin ( $\beta$ Lg) will interact electrostatically with negatively charged pectins. Isothermal titration calorimetry (ITC) showed they do indeed interact (Fig. 3) but it is complex interaction which varies according to the type of pectin (Xu et al., 2015). There are two stages of interaction between  $\beta$ -lactoglobulin and pectins which have an adequate amount of charge distribution (e.g. pectins with DM between 37 and 75) (Fig. 4). Starting at about  $-14$  Kcal and finishing at  $-8$  Kcal the interaction sites are quickly exhausted. Then in the second stage the same interaction is recurring because the amount of heat produced remains constant at  $-8$  Kcal until the  $\beta$ Lg to pectin molar ratio is 16 when the interaction quickly ends with the amount of heat given off declining to zero. This is explained in Fig. 5. The sites on the pectin for binding  $\beta$ Lg are rapidly used up in the first step then the  $\beta$ Lg molecules interact with  $\beta$ Lg molecules that are already bound to the pectin. In this way protein rich domains are formed, which serve as crosslinks to bring individual pectin chains together to form a compact structure. Circular dichroism showed the  $\beta$ Lg loses  $\alpha$ -helix structure and increases  $\beta$ -strands when it interacts with pectin or itself (Xu et al., 2015). In other words, the change in the secondary structure may be the same in the protein-pectin interaction as in the protein-protein interaction.

To understand what is the minimum block length required for interaction with a single  $\beta$ Lg to occur, we adopted another approach (Xu et al., 2017a). We studied how oligosaccharides composed of galacturonic acids interacted with a  $\beta$ Lg. They ranged in size from a trisaccharide to decasaccharide. In other words the degree of polymerisation was DP3 to DP10. All of the galacturonic acids in each oligosaccharide are negatively charged; none being methyl esterified. Isothermal titration calorimetry showed the trisaccharide (DP3) and hexasaccharide (DP6) did not interact with  $\beta$ Lg but dramatically the heptasaccharide (DP7) did. One





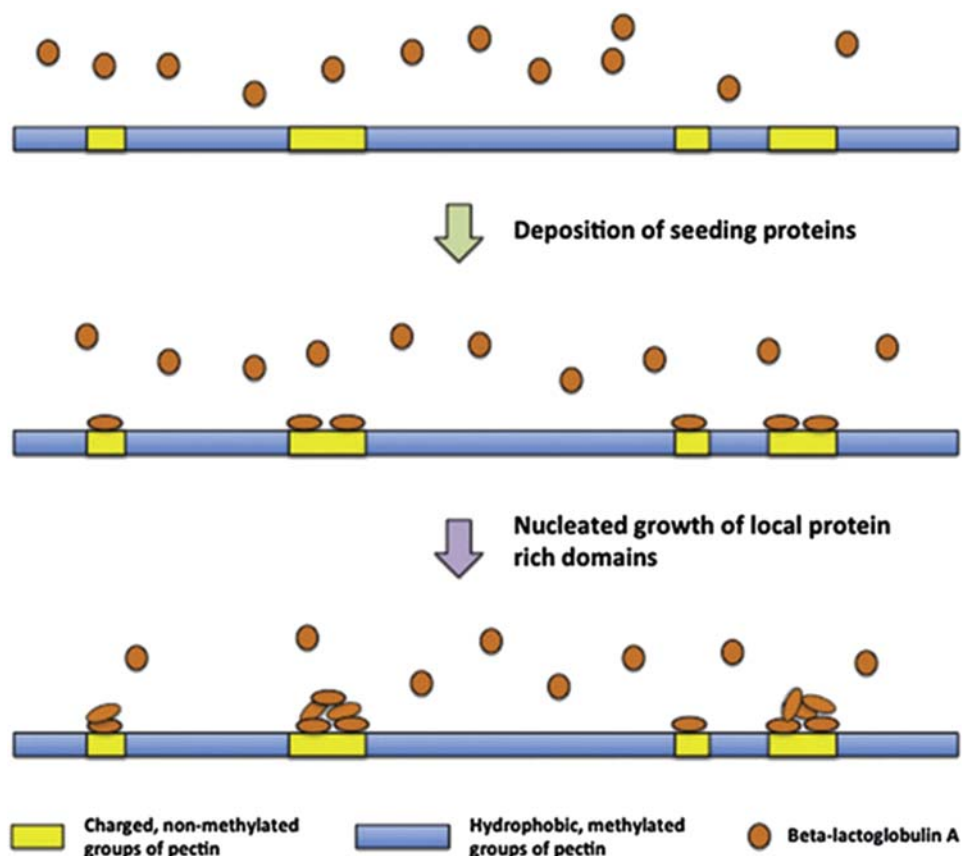
**Figure 3** Typical ITC thermogram of the titration of  $\beta$ -lactoglobulin and pectin 37B showing a two stage interaction. From Xu, A.Y., et al., 2015. *Soft Matter* 11, 6790–6799. Reproduced by permission of the Royal Society of Chemistry.



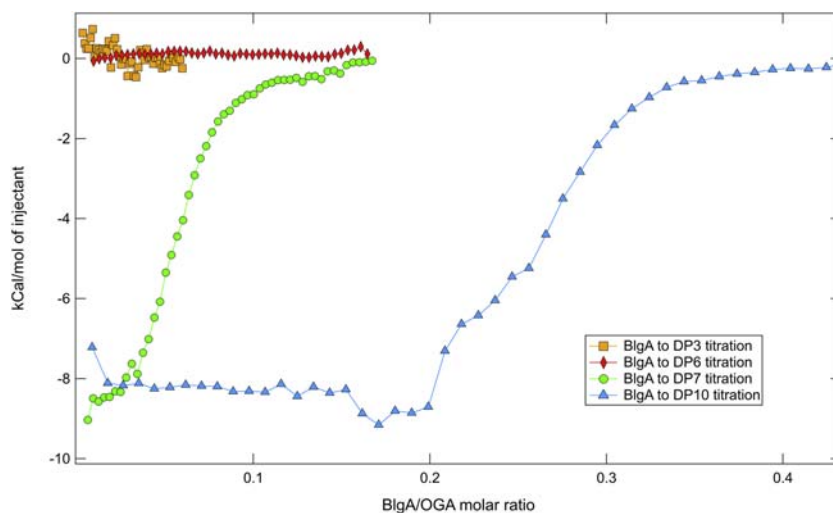
**Figure 4** ITC integrated binding isotherms for  $\beta$ -lactoglobulin and various pectins:  $\beta$ -lactoglobulin to 37R titration (red), 37B (orange), 58R (green), 58B (blue), 75R (purple). From Xu, A.Y., et al., 2015. *Soft Matter* 11, 6790–6799. Reproduced by permission of the Royal Society of Chemistry.

more galacturonic acid makes a huge difference. As one might expect the deca-saccharide (DP10) also interacted (Fig. 6). Circular dichroism showed that the  $\alpha$ -helix content decreased and the  $\beta$ -strand content increased when  $\beta$ Lg interacted with the heptasaccharide and deca-saccharide but there was no change with the tri- or hexa-saccharide as one would expect if there was no interaction. SAXS and SANS confirmed the interaction with DP7 and DP10 oligo-galacturonic acids and determined the size of the protein clusters to be about 8 nm in cross-section and the primary particles about 80 nm. This allowed us to generate a new model of the interaction (Fig. 7). Clearly a minimum of 7 adjacent galacturonic acids are need for an interaction with a  $\beta$ Lg. However, it does not follow that all 7 negative charges are needed; it may be the length of the oligosaccharide is essential to reach positively charged



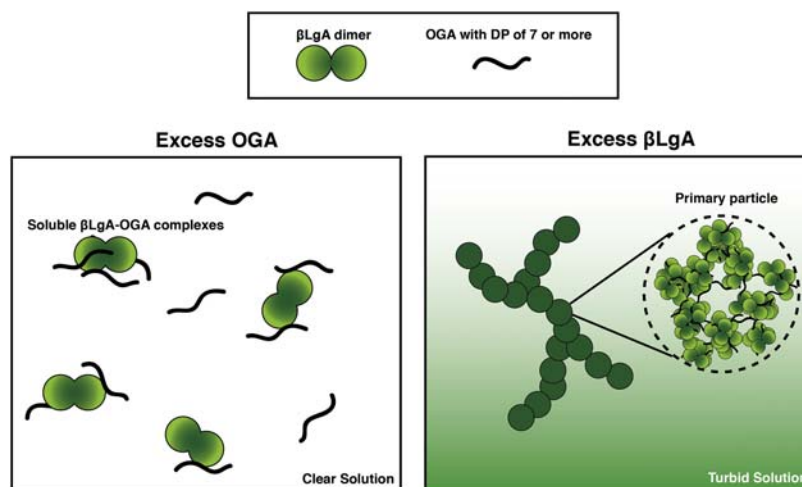


**Figure 5** Model of the two-step interaction of  $\beta$ Lg and pectin. In the first step “seeding proteins” interact with negatively charged sites on the pectin. In the second step the seeding proteins trigger interaction with other proteins leading to the formation of local  $\beta$ Lg rich domains. Native  $\beta$ Lg is shown as orange circles and the changed conformation of  $\beta$ Lg is shown as ellipses. From Xu, A.Y., et al., 2015. *Soft Matter* 11, 6790–6799. Reproduced by permission of the Royal Society of Chemistry.

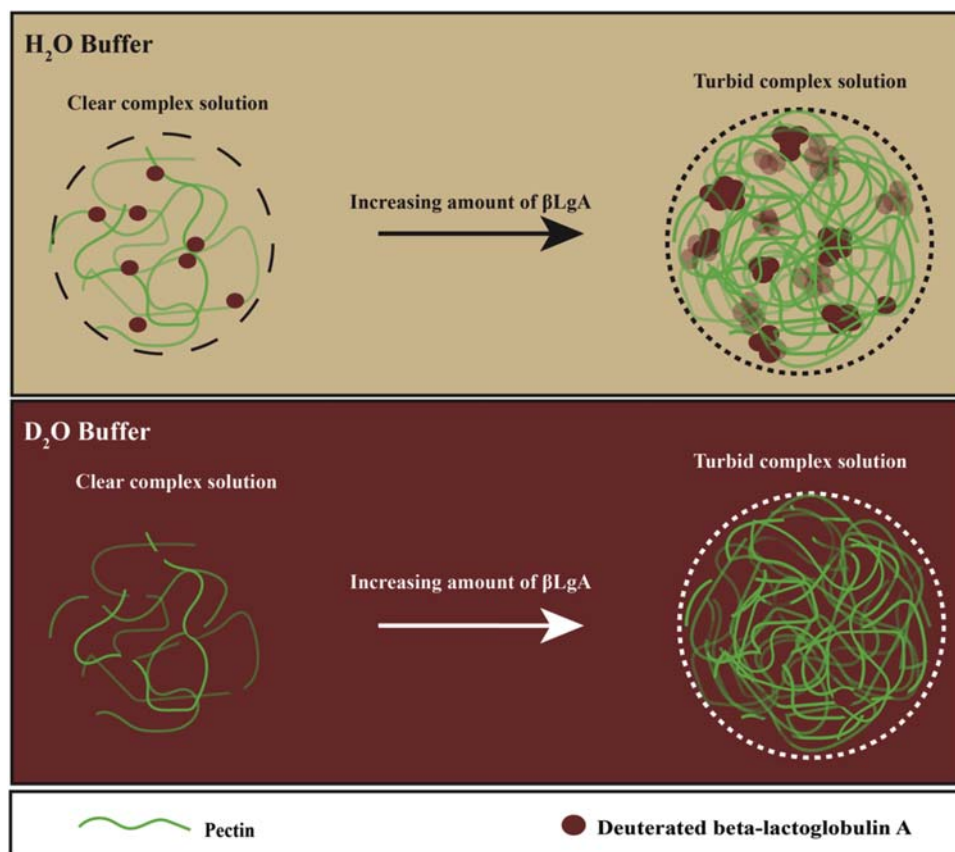


**Figure 6** ITC integrated binding isotherms for  $\beta$ Lg titration with galacturonic acid oligosaccharides: DP3 (orange), DP6 (red), DP7 (green) and DP10 (blue). Xu, A.Y., et al., 2017. *Soft Matter* 13, 2698–2707. Reproduced by permission of the Royal Society of Chemistry.

binding sites on the  $\beta$ Lg. Initially when there is an excess of DP7 or DP10 relative to  $\beta$ Lg the reaction products are soluble. However, when  $\beta$ Lg is in excess the samples are turbid. We believe the  $\beta$ Lg -oligosaccharide complexes interact with each other to form primary particles (Fig. 7) that further aggregate into larger branched fractal structures. The primary particles act as precursors for the formation of the larger structure.

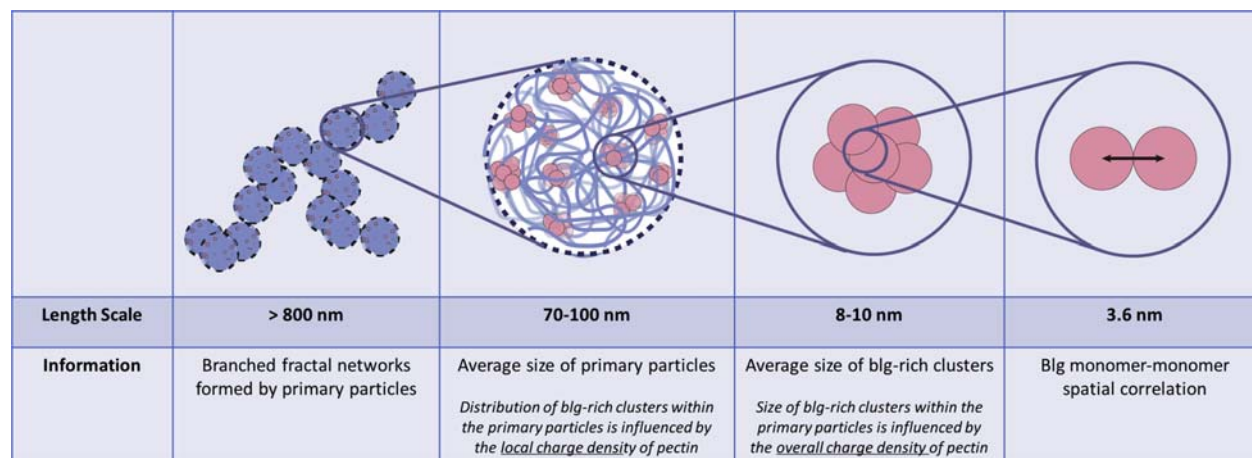


**Figure 7** Two systems during  $\beta$ Lg titration into OGAs (DP greater than 6). In the first system (left), observed when OGA is in excess,  $\beta$ Lg binds to OGAs forming soluble complexes. In the second stage when  $\beta$ Lg is in excess, the  $\beta$ Lg-OGA complexes form primary particles which aggregate, resulting in a turbid sample. Xu, A.Y., et al., 2017. *Soft Matter*. 13, 2698–2707. Reproduced by permission of the Royal Society of Chemistry.



**Figure 8** SANS of  $\beta$ -lactoglobulin-pectin complexes in  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$ . Formation of primary particles during  $\beta$ Lg to pectin titration. Deuterated proteins are brown circles while pectins are green lines. Dotted circles show the primary particles.  $\text{H}_2\text{O}$  is light brown and  $\text{D}_2\text{O}$  is brown. Xu, A.Y., et al. 2018. *Food Hydrocoll.* 77, 952–963. Reproduced with permission from Elsevier.

The final stage in understanding the interaction of  $\beta$ Lg with pectins is to apply the knowledge gained from the oligosaccharide interactions to interpret the interactions of the larger pectic polymers when they form complex coacervates. SAXS and SANS were used to study the complexes formed by  $\beta$ Lg and the pectins with different degrees of methylation and blockiness (Xu et al., 2018). This showed the coacervates consist of compact primary particles which are composed of adjacent pectin chains cross-linked by



**Figure 9** Models showing the size of the structural components that make up the  $\beta$ -lactoglobulin-pectin complex coacervates based on SAXS and SANS measurements. Xu, A.Y., Melton, L.D., Ryan, T.M., Mata, J.P., Rekas, A., Williams, M.A.K., McGillivray, D.J. 2018. Effects of polysaccharide charge pattern on the microstructures of  $\beta$ -lactoglobulin-pectin complex coacervates, studied by SAXS and SANS. *Food Hydrocolloids* 77, 952–963. <https://doi.org/10.1016/j.foodhyd.2017.11.045>, plus supplementary data. Reproduced with permission from Elsevier.

clusters of  $\beta$ Lg molecules (see Fig. 8). The overall and local charge densities of pectin influence the size and distribution of  $\beta$ Lg domains within the primary particles. The results confirmed that the greater the charge density of the pectin the smaller the  $\beta$ Lg-rich domains in the primary particles. When the primary particles are arranged in an open fractal network structure a complex coacervate results (Fig. 9). However, if the primary particles associate extensively the result is a precipitate.

## Conclusion

Overall we have shown the amount and arrangement of charges on the pectin influences the structure of the protein-polysaccharide complexes. This knowledge can be applied to improve the structure and texture of food products and delivery systems.

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# Milk Protein–Polysaccharide Interactions in Food Systems

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## Introduction

Proteins and polysaccharides are commonly used as ingredients in food formulations. These biopolymers confer vital functionality, such as thickening, stabilizing, gelling, and emulsifying properties, to food products. The functional properties of individual biopolymers (either used naturally or added as ingredients) add to the complexity of the multi-component food system. The overall stability and the microstructure of the food system depend on the individual functionalities of the proteins and polysaccharides and also on the nature and the strength of interactions between them. Milk proteins, namely caseins and whey proteins, have different characteristics depending on the type of system in which they are present. Casein exists in different states, ranging from small dissolved macromolecules to stable colloidal particles. It exists mostly in the colloidal state in commercial dairy products, whereas whey proteins are present as dissolved polymers of low molecular weight unless temperature/high pressure treatments are induced, which lead to unfolding and aggregation. Aggregates of whey proteins that are formed by thermal and high pressure processing have their own versatile functional properties. The interaction of polysaccharides with milk proteins leads to the formation of a ternary 'milk protein–polysaccharide–water' polyelectrolyte solution. An understanding of the basic concepts of the mixing behavior of biopolymer solutions and the factors contributing to their formation and their various functionalities is important in the elucidation of this rather complicated system.

## Theoretical Concepts on Mixing Behavior of Biopolymers

When aqueous solutions containing proteins and polysaccharides are mixed, one of four phenomena can occur: (1) co-solubility, i.e., the components mix well and the solution is stable; (2) thermodynamic incompatibility, i.e., the polymers repel each other and two phases appear, one abundant in polysaccharide and the other abundant in protein; (3) depletion interaction, which occurs in colloidal dispersions in the presence of non-interacting polymers (e.g., polysaccharides in an emulsion, or polysaccharides and colloidal casein micelles); (4) complex coacervation, i.e., electrostatic interaction occurs, giving rise to a concentrated protein–polysaccharide phase and a dilute phase.

It is important to consider the influence of the biopolymer concentration on the enthalpic interactions occurring in the system. For mixed polymer systems at high dilutions, the system becomes homogeneous regardless of whether thermodynamic incompatibility or coacervation occurs. However, coacervation can occur in dilute solutions whereas thermodynamic incompatibility cannot.

Some terms associated with mixed biopolymer systems are listed below.

### Co-solubility

This phenomenon is based on the thermodynamic principle that the Gibbs free energy of mixing ( $\Delta G_{\text{mixing}}$ ) is negative when the entropy of mixing favorably exceeds the enthalpy term. The highest entropy is achieved when different kinds of molecules are randomly distributed throughout the system. The expression for the Gibbs free energy accompanying mixing under standard conditions is given by

$$\Delta G_{\text{mixing}} = \Delta H_{\text{mixing}} - T\Delta S_{\text{mixing}}$$

where  $\Delta G_{\text{mixing}}$ ,  $\Delta H_{\text{mixing}}$ , and  $T\Delta S_{\text{mixing}}$  are the free energy, enthalpy (interaction energy), and entropy changes, respectively, between the mixed and unmixed states.

The interaction of smaller molecules (monomers, hydrophilic amino acids) on mixing results in a co-soluble system. As the entropy of a mixed biopolymer system is much lower than that for smaller molecules, the system tends to become less co-soluble because of thermodynamic incompatibility. Also, given the polymeric nature of proteins and polysaccharides and the presence of various functional groups in their macromolecules, co-solubility is the least typical situation.

### Thermodynamic Incompatibility

This occurs when two dissimilar noninteracting macromolecular species separate into two different phases because the enthalpy of mixing exceeds the entropy difference. Such phenomena are also observed when the two biopolymers have different affinities towards the solvent. For mixed systems, molecular and structural differences between the same biopolymers also give rise to thermodynamic incompatibility (polysaccharides with different structures, proteins of different classes, e.g., proteins of different solubilities, native and denatured forms of the protein, and aggregated and nonaggregated forms of the same protein).

### Depletion Interaction

Depletion interaction occurs in systems containing depletants such as nonadsorbing polysaccharides. It occurs by entropically driven exclusion of the depletant by an osmotic pressure gradient, ultimately leading to flocculation of the system. This type of flocculation is considered to be weak or reversible as the flocs are easily broken down on remixing. However, in most food systems, this weak flocculation leads to extensive aggregation, resulting in creaming and instability of the system.

### Complex Coacervation

This phenomenon occurs because of the formation of electrostatic complexes between the protein molecules and the polysaccharide molecules, resulting in a two-phase system. One phase is rich in both of the biopolymers that represent the complex, whereas the other phase contains the excluded solvent and is depleted in biopolymers. This is observed when two oppositely charged biopolymers interact. These reactions are highly dependent on the ionic nature of the system. Coacervates are formed when proteins interact with polysaccharides that have a low charge density and/or a very elastic backbone, e.g., gum arabic, gelatin, dextran, pectin etc.

## Mixing Behavior of Milk Proteins and Polysaccharides

### Casein–Polysaccharide Systems

Casein micelles are assembled in large supramolecular entities that may be considered to be spherical particles. The surface of casein micelles is covered by  $\kappa$ -casein, which helps in suspension stabilization by means of steric and electrostatic repulsions. Its hairy surface restricts the adsorption of neutral polymers on to the micelles. Inherent problems of casein micelles include association over a wide pH range, especially in the presence of calcium ions, and insolubility at the isoelectric point (pI). When casein micelles are added in a mixed food system, in which excessive pH adjustments are not made and the environmental ionic changes are minimal, the behavior of the system can be assessed in terms of the adsorption or nonadsorption of polysaccharides on to the colloidal casein micelles.

Dairy products are abundant in surface-active colloidal particles on to which polysaccharides can be adsorbed or nonadsorbed. Both adsorbing and nonadsorbing polysaccharides are capable of increasing or decreasing the stability of the mixed system, depending on their concentrations and the ratio of protein to polysaccharide. For pectin–casein systems, it has been observed that the pectin does not adsorb on to the micelles at neutral pH (Maroziene and de Kruif, 2000). Nonadsorbing pectin causes a segregative phase separation. Adsorption leads to bridging flocculation in casein–pectin systems; for example, by lowering the pH, pectin and casein micelle complexes are formed. A casein–polysaccharide mixture results in depletion flocculation because of the difference in osmotic pressure between the bulk solvent containing the polysaccharide and the solvent entrapped between the casein micelles, from which the polysaccharide is excluded because of steric hindrance. This leads to the casein micelles being pushed together and finally aggregating. Polysaccharides with a lower intrinsic viscosity or hydrodynamic molecular volume tend to occupy lesser volumes, leading to a lesser extent of their exclusion. This leads to a reduction in the phase separation because the aggregation of casein is slowed. Phase separation in dairy systems results in visual separation of a clear serum (wheying off) and loss of the desired quality in the product. Hence, the type of polysaccharide added to a casein-containing system can be tailored depending on the desired functionality of the system.

### Whey Protein–Polysaccharide Systems

Whey proteins in their native state behave like low molecular weight dissolved polymers (mainly in the form of  $\beta$ -lactoglobulin present as pH-dependent oligomers). Processing factors such as heat and high pressure lead to the unfolding of whey proteins and their aggregation. Generally, when whey proteins are mixed with polysaccharides, thermodynamic incompatibility occurs. If one of these polymers is highly viscous or forms a gel, phase separation is retarded. That is, the rate and the extent of phase separation may be severely retarded when one phase gels and the other remains dispersed in the gel. The addition of xanthan gum to whey protein with 200 mM sodium chloride (salt) resulted in the formation of a protein gel network in which xanthan-gum-rich regions were embedded. However, without the addition of salt, the system formed two separate phases (Bryant and McClements, 2000). Mixtures of whey protein isolate (WPI)/pectin and WPI/guar gum showed phase separation, but a mixture of WPI/iota-carrageenan showed no visual separation because of the high gelling capacity of iota-carrageenan (Erçelebi and İbanoğlu, 2007). In some cases, when the polysaccharide concentration is low, phase inversion causes dispersed whey protein gel particles in a dilute polysaccharide solution to form whey protein gels with polysaccharide-rich regions (Syrbe et al., 1998). Electrostatic interactions between whey proteins and polysaccharides at a pH slightly above their pI, lead to the formation of soluble complexes. This mechanism enhances the interfacial properties of whey protein emulsions, because a thicker homogeneous layer is formed around the oil droplets or air bubbles (Benichou et al., 2002), and also helps in modifying the heat-induced gelation of the whey protein. For complexation, the ratio of protein to polysaccharide and the pH should be regulated because a high concentration of polysaccharide causes the complexes to re-solubilize, whereas a lower concentration leads to un-complexed protein remaining in the solution. On mixing with whey protein, nonionic polymers can show thermodynamic incompatibility.

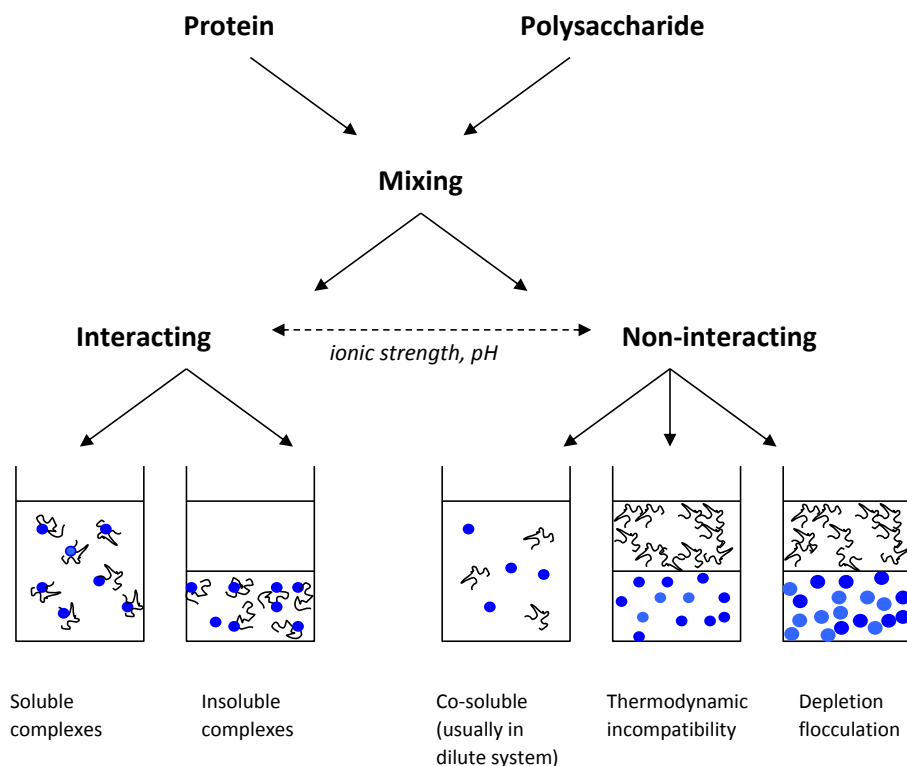


## Phase Diagram

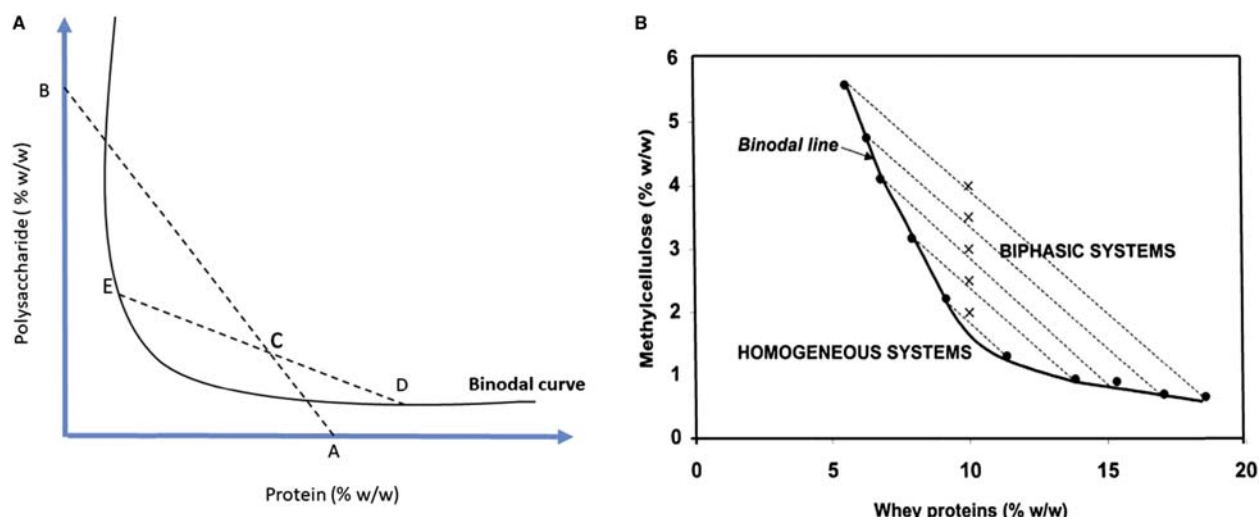
The mixing together of aqueous solutions of proteins and polysaccharides results in either a one-phase system or a two-phase system, depending on the composition of the solution and the environmental conditions, as depicted in Fig. 1. In one-phase systems, the biopolymers exist as individual molecules or as soluble complexes that are uniformly dispersed throughout the entire system. However, if the molecular weight and the concentration of the biopolymers are increased, the system starts to become less co-soluble and this results in the formation of two distinct phases, in which each phase has different biopolymer concentrations. A typical phase diagram demonstrating the segregating biopolymer system that is caused by thermodynamic incompatibility is shown in Fig. 2A and a phase diagram of a milk protein–polysaccharide system is given in Fig. 2B. Phase diagrams aid in determining the effective concentrations of the biopolymers in the two phases and the concentration at which maximal co-solubility of the biopolymers is achieved.

## Interactions Involved in Protein–Polysaccharide Complex Formation

Tolstoguzov (1997) classified the macromolecular interactions required for complex formation into three types: interactions between charged biopolymers, interactions between oppositely charged functional groups, and interactions between other available side groups of poly-ions, i.e., charged regions on polymers called 'patches'. Repulsive interactions exist transiently and are considered to be weak (unless the molecules exist at very close range or at low ionic strengths). They are considered to be nonspecific and to arise because of electrostatic interactions and/or excluded volume effects. Attractive interactions may be weak or strong and specific or nonspecific. Whereas electrostatic forces are commonly involved in complex formation, non-Columbic interactions contribute to nonspecific net attractive protein–polysaccharide interactions. Non-electrostatic interactions are usually less sensitive to pH and ionic strength changes and the formation and the stabilization of complexes occur by hydrogen bonds, hydrophobic interactions, and van der Waals' forces. These forces generally depend on the physico-chemical properties of the biopolymers and the system. The overall interaction that occurs between proteins and polysaccharides is the average of the multitude of intermolecular forces that occur between the biopolymers.



**Figure 1** Different types of interactions between protein and polysaccharide in aqueous solutions. Reproduced with permission from Elsevier, from Goh, K.K.T., Sarkar, A., Singh, H., 2014. Milk protein–polysaccharide interactions. In: Boland, M., Singh, H., Thompson, A. (Eds.), *Milk Proteins: From Expression to Food*, second ed. Academic Press, London, pp. 387–419.



**Figure 2** (A) Phase diagram of a typical protein–polysaccharide system showing thermodynamic incompatibility. On mixing concentrations of protein (A) and polysaccharide (B), a mixture of C is formed. After phase separation, the composition of the polysaccharide-rich phase is given by point E and that of the protein-rich phase is given by point D. The lengths of lines EC and CD are proportional to the volume fractions of the protein-rich phase and the polysaccharide-rich phase. (B) Phase diagram of the ternary system whey protein/methylcellulose/water at pH 7: (x) mixing points (●) equilibrium points. Reproduced with permission from Elsevier, from Reiffers-Magnani, C.K., Cuq, J.L., Watzke, H.J., 2000. Depletion flocculation and thermodynamic incompatibility in whey protein stabilised O/W emulsions. *Food Hydrocolloids* 14, 521–530.

### Factors that Influence Protein–Polysaccharide Interactions

Factors that affect the nature of protein–polysaccharide interactions and the corresponding structures formed on the basis of these interactions can be classified as extrinsic, intrinsic, and processing factors. These factors can be varied to obtain desired functionalities for tailored applications (Table 1).

Syrbe et al. (1998) laid down some basic rules for understanding the combined effect of pH and ionic strength on the mixing behavior of protein–polysaccharide systems. For protein–nonionic polysaccharide systems, pH and ionic strength affect only

**Table 1** Factors that influence milk protein–polysaccharide interactions

	Factor	Observations
Extrinsic	Mixing ratio ( $r$ )	Affects structure of complexes or coacervates formed. Lower $r$ results in the formation of coacervates that coalesce quickly, whereas higher $r$ results in more stable coacervates.
	Molecular concentration	Coacervation can occur in very dilute solutions, whereas thermodynamic incompatibility is favored in more concentrated systems.
	pH	At a pH > the isoelectric point (pI) of the protein, there is repulsion between the protein and the polysaccharide. Near the pI, soluble complexes are formed, whereas a further reduction in the pH results in the formation of coacervates.
	Ionic strength	At high salt concentration, micro-ions cause a decrease in electrostatic interaction, whereas, at low ionic strength, interference to the formation of the protein–polysaccharide complex is minimal.
	Charge density	Coacervation does not occur at low charge densities, whereas precipitation of the inter-polymeric complexes occurs at very high charge densities. The nature of the charge group (carbonyl versus sulfated) influences the interaction strength.
Intrinsic	Molecular conformation	Increased chain flexibility and increased charge mobility result in stronger binding.
	Charge distribution	Evenly charged chains form large homogeneous phases, whereas an uneven charge distribution leads to the formation of a meso-phase with a micellar structure.
	Molecular weight	The internal structure and the final size of the complexes/coacervates can be controlled by adjusting the molecular weight of the polyelectrolyte.
Processing	Shear	Shear either can cause restructuring of the complexes formed or can stabilize coacervates against flocculation.
	Pressure	Pressure causes partial denaturation of the protein, which strengthens the interaction during complex formation.
	Temperature	Heat denaturation increases the molecular flexibility, which leads to stronger electrostatic interactions, yielding more stable complexes.
	Acidification	The type of acidification method used, e.g., hydrochloric acid or glucono-delta-lactone, influences the structure of the complexes formed.

protein self-association. Incompatibility is directly correlated with protein self-association, which is highest at the pI of the protein and decreases towards acidic and alkaline pHs. In mixed systems, incompatibility in the pH range close to the pI is linked to low ionic strengths, where both biopolymers are uncharged, the difference in charge densities is zero, and the presence of salt suppresses protein self-association. The presence of charged carboxylated or sulfated groups causes ionic polysaccharides to have an anionic nature in the pH ranges that are relevant to food, where complexation occurs with the positively charged protein.

### Effect of Protein–Polysaccharide Interactions on the Interfacial Behavior of Food Systems

The interfacial property of emulsions and foams is attributed mainly to the proteins that adsorb at the air–water and oil–water interfaces and enable stabilization of these systems. Although not all food proteins are surface active, all are amphiphilic and most contain similar amounts of polar and nonpolar amino acid residues. The surface activity of a protein depends on its chain flexibility, its thermodynamic stability, its charge, its molecular size, and the presence of polar and nonpolar domains. Milk proteins are highly surface active in both their soluble form and their dispersed form. Extensive literature on milk-protein-based emulsions is available. Flexible caseins have no tertiary structure whereas compact globular whey proteins possess a tertiary structure and preserve their globular shape even after adsorption on to an interface. As most high molecular weight polysaccharides are hydrophilic, they are not considered to be surface active. These include polysaccharides such as xanthan gum, pectin, and their derivatives. Surface-active polysaccharides include gum arabic, gum tragacanth, cellulose-derivative polysaccharides such as methylcellulose, carboxymethylcellulose, and hydroxypropyl methylcellulose, and propylene glycol esters of alginic acid. However, in the literature, their surface activity is attributed to the presence of protein impurities.

The stability of protein foams during storage can be improved by complexation with polysaccharides. The stability increases because of the thick interfacial layers of protein–polysaccharide complexes that stabilize the bubbles and prevent coalescence. These interfacial networks also tend to show a ‘jamming effect’, which reduces foam drainage and maintains the structural integrity of the system. The main factors governing the surface pressure, dilatational, and surface shear rheological behavior of protein–polysaccharide interfacial films under associative (i.e., net attractive) conditions depend on the electrostatic charge of the complexes in the bulk solution and the order of adsorption of the biopolymers to the interface (simultaneous or sequential). Under conditions of limited thermodynamic compatibility, the polysaccharides adsorb to the interface by competing with the protein or by complexing with the adsorbed protein. This leads to excluded volume effects that cause the protein at the interface to perform as a more concentrated film, leading to an increase in the surface pressure.

### Effects of Milk Protein–Polysaccharide Interactions on Rheological Properties

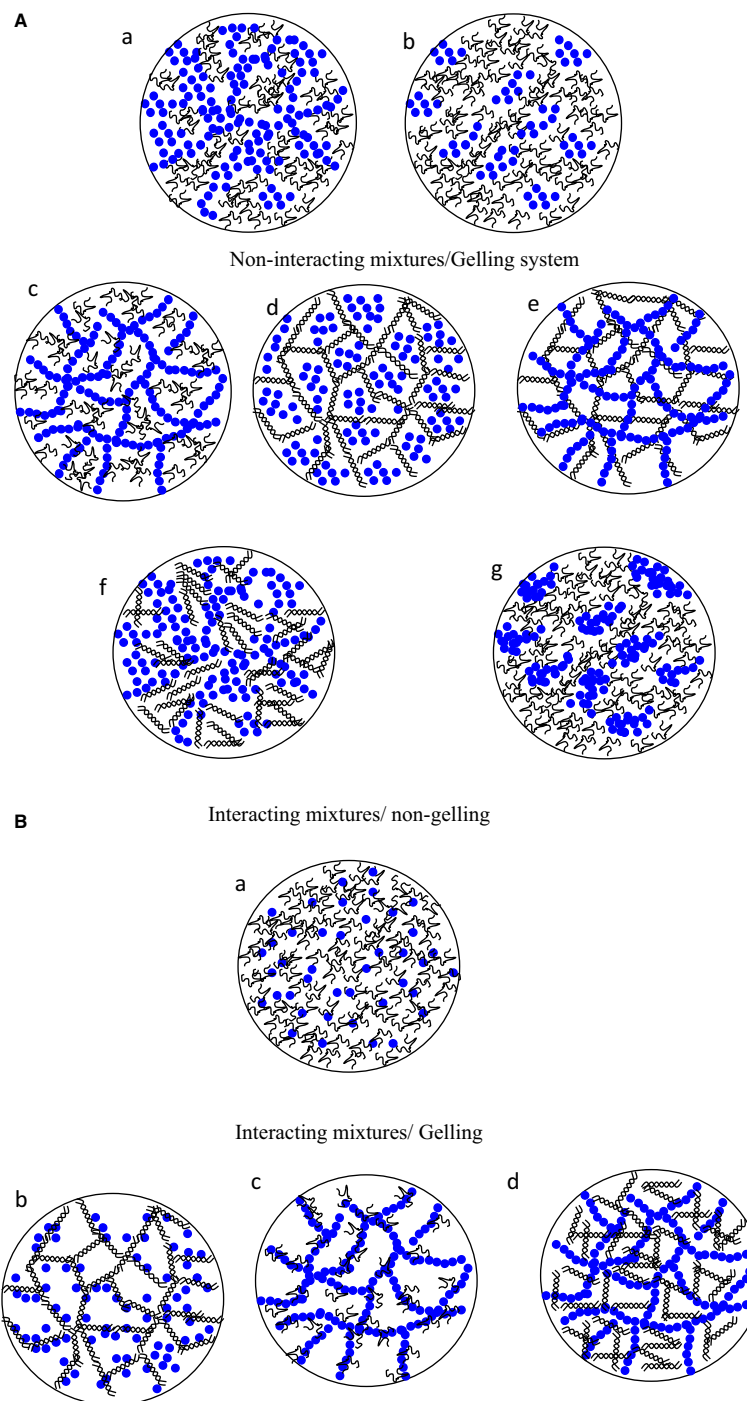
The rheology of a substance is defined as the behavior of a solid or liquid under shear. For mixed systems, the rheology of the bulk system is similar to those occurring at the interface. The functional properties of biopolymer mixtures that confer stability and texture-modifying effects generally include aggregation and gelation of the biopolymers. Aggregation is a generic term for a process in which two or more colloidal particles associate under the influence of attractive colloidal interactions. Gelation is a multistage reaction that gives rise to an extended three-dimensional network that entraps a large amount of solvent molecules. Changing the processing conditions that induce aggregation and gelation by denaturing the structure of the biopolymers helps to create novel microstructures with versatile functionalities.

Goh et al. (2008) classified milk protein–polysaccharide systems into two broad categories, i.e., interacting mixtures and noninteracting mixtures Fig. 3A and B. These categories can be further classified according to the ability of these mixtures to form gels, i.e. gelling or nongelling. Mechanisms resulting in the formation of these microstructures are listed below.

- For interacting mixtures, complexes are formed if electrostatic attraction occurs between the biopolymers. If one of the biopolymers in the interacting mixture is gelling, the other phase directly interacts with the gelled network. If both biopolymers form gels, then a coupled gel network is formed.
- Phase-separated networks are formed when de-mixing of the system occurs prior to gelation. Phase separation comes to a halt when gelation occurs. The final structure of the system depends on the relative rates of phase separation and gelation. Subsequent gelation of the other phase (i.e., if the phase is made up of a gelling polymer) will result in a second gel phase. If the first network is weak, enthalpic advantage results in the second network forming a stronger gel and becoming the continuous phase, with the first network being broken down into dispersed particles.
- Some polysaccharides do not interact with the protein but instead modify the viscosity of a gelling system by limiting the fluidity of the gel. When mixed with proteins, neutral hydrocolloids such as guar gum impart a bulking effect to the gelling system and are not affected by the ionic strength or pH of the system.

### Applications of Milk Protein–Polysaccharide Systems in the Food Industry

Protein–polysaccharide interactions can give rise to a multitude of functional colloids that can have numerous applications in the food industry. The functional properties of protein–polysaccharide complexes and the rationale behind their application are listed



**Figure 3** (A) Schematic diagrams of some possible microstructures formed between noninteracting protein–polysaccharide mixtures. Circle (●) represents protein; coil structure represents polysaccharide molecules. (a) Flocculated protein network formed with polysaccharide filling the space in the network; (b) polysaccharide molecules overlap and form a continuous 'network' with protein filling the space; (c) particulate protein gel network formed with polysaccharide filling the space; (d) polysaccharide gel network formed with protein filling the space; (e) bi-continuous network formed from protein and polysaccharide; (f) polysaccharide gels dispersed among weakly flocculated protein network; (g) protein gels dispersed among entangled polysaccharide molecules. (B) Schematic diagrams of some possible microstructures formed between interacting protein–polysaccharide mixtures. Circle (●) represents protein; coil structure represents polysaccharide molecules. (a) Protein–polysaccharide complexes formed; (b) protein interacting with gelling polysaccharide helices; (c) polysaccharide interacting with protein particulate gel network; (d) polysaccharide gel helices interacting with protein particulate gel network. Reproduced with permission from Elsevier, from Goh, K.K.T., Sarkar, A., Singh, H., 2014. Milk protein–polysaccharide interactions. In: Boland, M., Singh, H., Thompson, A. (Eds.), *Milk Proteins: From Expression to Food*, second ed. Academic Press, London, pp. 387–419.

**Table 2** Functional properties of protein–polysaccharide complexes and rationale behind use of protein–polysaccharide complexes

Functionality	Molecular interactions involved
Fat substitutes/Prevention of excess protein aggregation	Thermal aggregation of proteins leads to gritty textures, which can be prevented by the addition of polysaccharides, which minimize excessive protein aggregation by minimizing protein–protein interactions. Their hydrophilic characters confer plasticity, lubricity, and creaminess, which improves the mouthfeel of the complexes, making them excellent fat mimetics.
Meat analogs	Thermal treatment is carried out to destabilize regions of a protein–polysaccharide complex, which stabilizes the interaction of divalent metal ions and the polysaccharides, resulting in a thermostable polysaccharide gel that entraps proteins and resemble minced meat.
Improvement in protein solubility	The complexation of a polysaccharide with a protein increases the solubility of the protein at its isoelectric point (pI). Insoluble complexes that are formed are solubilized by acid titration. The main principle is to modify conditions such as pH and ionic strength to obtain maximum complex coacervation for maximum efficiency.
Encapsulation	Complexes can be utilized to encapsulate bioactive compounds against processing and storage conditions. The complexes can be tailored for controlled release to specific target sites. Release can be controlled by modifying the pH or changing the enzymatic conditions.
Emulsification and foam stabilization	Complexes can be formed by electrostatic interaction or Maillard reaction to stabilize oil-in-water emulsions. Polysaccharides can be added to a protein-stabilized emulsion so that complexation can take place at the interface and stability is achieved by the formation of a stronger steric-stabilizing layer. Smaller droplet sizes are obtained when emulsions are homogenized with the polysaccharides and, as long as no bridging flocculation occurs, the rate of creaming may be reduced.
Biodegradable film formation	The formation of a protein–polysaccharide complex improves the emulsifying and solubility properties of the biopolymers, which results in better mechanical and moisture sorption properties of the composite film formed.
Stability in dairy products	Dairy products contain dispersed particles in the form of emulsions and other dairy ingredients (e.g., cocoa) coated with caseins. Polysaccharides can act as adsorbing polymers and can incorporate these dispersed particles as fillers in their network, thus stabilizing the system. Further gelation of this system will result in additional stability.
Protection of probiotic bacteria in the gastrointestinal tract	Complexes act as good protective matrices to protect probiotic bacteria from various stresses encountered during processing, storage, and passage through the gastrointestinal tract, preventing loss of viability of the bacteria.

**Table 3** Some applications of milk proteins with commonly used polysaccharides in the food industry

Milk protein	Polysaccharide	Mechanism	Application
Casein	$\kappa$ -Carrageenan	Electrostatic interaction OR suspension of casein micelles in a weak $\kappa$ -carrageenan gel.	Stabilization of milk gels, dairy emulsions, and suspensions. Also acts as a secondary stabilizer in ice cream mixes.
	Carboxymethylcellulose (CMC)	Electrostatic interaction OR, when added in excess, depletion flocculation of the system by excess CMC.	Prevents milk protein flocculation in acidified milk drinks and stabilizes oil-in-water emulsions.
	High methoxyl pectin	Prevents settling out of casein aggregates (whey off) in acid milk drinks by electrostatic complexation.	Stabilization of low pH milk drinks, preventing protein aggregation at low pH, and stabilizing emulsions.
	Dextran	Complexation by Maillard reaction.	To improve casein solubility at acidic pH.
	Chitosan	Induces destabilization and coagulation of casein micelles without changes in the milk pH or the stability of whey proteins OR thermodynamic incompatibility OR depletion flocculation.	Casein micelle precipitation, biodegradable packing materials, and stabilizing emulsions.
Whey protein	Xanthan gum	Coacervation.	Used to produce fibrous complexes that are used as meat mimetics.
	Gum arabic	Coacervation.	Encapsulation.
	Exopolysaccharides	Electrostatic complexes are formed under acidic conditions whereas segregative interactions are observed at neutral pH.	Stabilize the texture of dairy food products.
	Dextran	Conjugation by Maillard reaction.	Improved emulsification properties.
	Maltodextrin	Conjugation by Maillard reaction.	Improved emulsification properties.
	Pectin	Conjugation by electro-synthesis.	Film formation.

in Table 2. Milk protein–polysaccharide systems can have innumerable applications as many variables can be exploited to create colloids with specific functionalities. Some common applications of milk proteins with food-grade polysaccharides are given in Table 3.

## Conclusions

Over the last few decades, extensive work has been carried out on the rather complicated protein–polysaccharide system to deduce the fundamental molecular interactions that occur. As numerous factors are involved in determining the formation and the stability of the system, it becomes difficult to develop colloids with specific functionalities. Also, for many food applications, the digestive fate of these systems is unknown, which creates a huge gap in the successful application of the system. Bridging this gap will help to create food systems and ingredients that are of high value to the food industry.

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# Interaction Between the Polysaccharides and Proteins in Semisolid Food Systems

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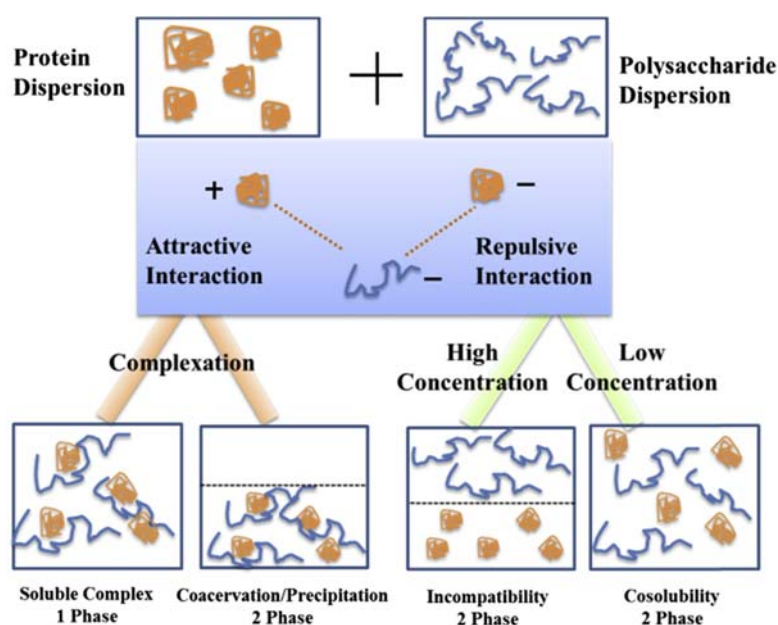
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## Introduction

Semi-solid food, i.e. amorphous solid food, is a viscous rather than solid-like substance which possesses both solid and liquid characters (Laverne et al., 2015). Different from conventional crystalline solids, the microscopic structures of semi-solid foods are disordered (i.e. lacks long range ordered arrangement of atoms, molecules, or ions within the structure). For semi-solid food, structure, texture and stability are important and fundamental attributes which depend on the rational design of food structure and complex assembly of various food ingredients such as polysaccharides, proteins, lipids, water and others (Ai et al., 2015). Among the food components, the nature of biopolymers and their interactions (e.g. those between polysaccharides and proteins) are essential to the structuration and stabilization of semi-solid food systems owing to their influences on gel-forming, thickening, hydration and surface/interface stabilizing properties (Cgde and Tuinier, 2001). A number of recent reviews and research publications (Jain et al., 2015; Li et al., 2016b; Timilsena et al., 2016) describe thermodynamic considerations and dominant molecular properties underlying the interactions of biopolymers, and emphasize the importance of polysaccharide-protein interactions in developing food products with desired structure, texture and stability.

## Overview of Polysaccharide-Protein Interaction

Interestingly, the simultaneous addition of protein and polysaccharides may induce intermolecular interactions that modify or generate functional properties more desirable than those derived from proteins or polysaccharides alone (Souza and Garcia-Rojas, 2016). The overall polysaccharide-protein interaction, depending mainly on the functional groups and environmental conditions, is made up from an average over the large number of different intermolecular forces arising between the various segments and side-chains on the two macromolecules including electrostatic forces, excluded volume effects, hydrogen bonding, hydrophobic interactions, ion-bridging, and Van der Waals interactions (Dickinson, 1998). Under the influence of the aqueous environmental conditions (e.g. pH, charge density, solvent quality, biopolymer ratios, ionic type and strength, etc.) and the distribution of various functional groups for molecular bonding (charged, hydrophobic, hydrogen bonding, etc.), protein/polysaccharide mixtures in an aqueous dispersion is often accompanied by either segregative/associative phase separation (Fig. 1) through thermodynamic incompatibility or compatibility (Le et al., 2016).



**Figure 1** Mixed biopolymer solutions may encounter phase separation under conditions where either attractive or repulsive interactions take place between the biopolymer molecules. Adapted from McClements, D.J., 2006. Non-covalent interactions between proteins and polysaccharides. *Biotechnol. Adv.* 24 (6), 621–625.

The strength and specificity of attractive interactions (including covalent and non-covalent bonding) vary widely (Benichou et al., 2002). Covalent bonding can be considered as the simplest extreme case of thermodynamic compatibility with “highly specific” and very “strong” characteristics. Non-covalent bonding is often derived from specific and non-specific ionic or non-ionic interactions, resulting in weak (reversible) or strong (difficult to reverse) protein-polysaccharide attraction. The aqueous environmental conditions (solvent quality, pH, ionic content, etc.) and macromolecular structure characteristics (net charge, shape, size, length, flexibility) (Dong et al., 2013; Sabliov et al., 2015) all influence the nature of non-covalent bonding. Complexation can take place when the overall net attraction exists between the oppositely charged protein and polysaccharide macromolecular chains. Most proteins (e.g. acidic and basic species) can form complexation with anionic polysaccharides in the region of pH ( $pK_{a\text{polysaccharide}} < \text{pH} < pI_{\text{protein}}$ ).

At low concentrations, biopolymers with the same type of charge can co-exist in a solvent solution (i.e. co-solubility) (Khalesi et al., 2016), whereas, at higher concentrations, phase separation into two phases, protein phase and polysaccharide phase, is possible. Thermodynamic incompatibility is the driving force of the phase separation between proteins and polysaccharides under repulsive conditions ( $\text{pH} < pI_{\text{protein}}, pK_{a\text{polysaccharide}}$ ).

## Polysaccharide-Protein Interaction in Semi-Solid Food Systems

Polysaccharides and proteins are conducive to the textural and structural features of semi-solid food owing to their aggregation and gelation behaviors. In comparison with the polysaccharide-protein interactions in solutions and at the interfaces (which have been studied extensively), the interactions in food colloids still are less examined and remain challenging topics (Semenova, 2017). Accordingly, this article is focused on the polysaccharide-protein interactions in semi-solid foods, two typical systems, hydrogels and wheat doughs.

### Hydrogels

Interactions between polysaccharides and proteins allow efficient approaches to construct food structures and improve the stability and textural properties of semi-solid food colloids (such as viscoelastic emulsions, gels and their mixtures) (Le et al., 2016; Semenova, 2017) through their water-holding capacity, interfacial properties and gelation behavior (Ghosh and Bandyopadhyay, 2012). Mixed polysaccharide-protein hydrogels are ideal model for the studies on the influence of microstructure on the textural and other properties (Stieger and Velde, 2013).

The network of the mixed hydrogels can be classified into three types: interpenetrating, phase-separated and coupled networks (Turgeon and Beaulieu, 2001). Donato et al. (2005) observed an interpenetrated network of BSA (4 and 8 wt%) and low-methoxyl pectin (0.21 wt%) gels in the presence of  $\text{CaCl}_2$  after a heat treatment (80 °C, 30 min) using Confocal Laser Scanning Microscope (Bastien et al., 2007). reported an interpenetrated structure i.e. gelatin helices (at a low gelation concentration like 1.5%) elongated inside a pre-existing calcium-alginate network.

Phase-separated networks are obtained under segregative conditions in aqueous mixed protein-polysaccharide systems. During the formation of the gel network using gelling molecules, phase segregation in an aqueous medium may be trapped to generate a two-phase co-gel containing continuous and dispersed phases. Spotti et al. (2012) reported higher gel strength and solid character in phase-separated networks of proteins and polysaccharides. Coupled networks are formed under associative conditions when proteins and polysaccharides are linked together through electrostatic attraction (Le et al., 2016). To date, there are only a few reports on the formation of coupled network gels between proteins and polysaccharides. Laneuville et al. (2006) firstly reported a coupled gel formed between oppositely charged proteins (native  $\beta$ -lactoglobulin) and polysaccharides (xanthan gum) at an extremely low concentration (0.1 wt %). Through tailoring the composition and hydrophilic-hydrophobic balance of the internal and surface structure, the polysaccharide-protein hydrogels can be used as encapsulants for bioactives, fat mimetics, and oil–water or air–water interfacial stabilizers (Dickinson, 2015; Mao et al., 2015). The roles of polysaccharide-protein hydrogels as fat mimetics and interfacial stabilizers are described as follows.

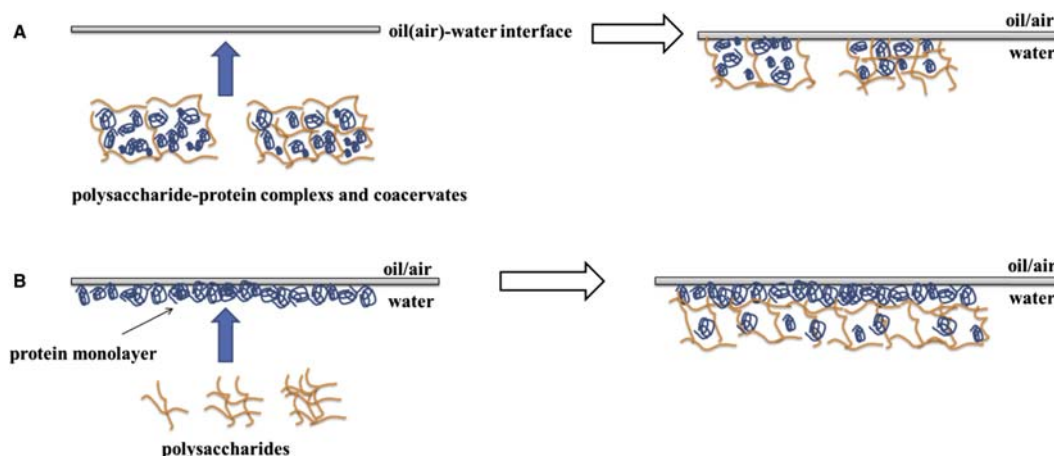
### Polysaccharide-Protein Electrostatic Hydrogels as Stabilizers of Emulsions and Foams

Polysaccharide-protein complexes and coacervates play important roles in the preparation of emulsions/foams and control of product shelf-life, e.g. in soft drinks and a large number of semi-solid foods such as ice-cream, sauces and dressings etc. Owing to their abilities to facilitate the formation and stabilization of oil droplets or air bubbles during and after emulsification, polysaccharides and proteins can be used as stabilizers and emulsifiers at oil–water and air–water interfaces (Dickinson, 2009; Wagoner et al., 2016). In the past decade, there have been increasing investigations on the adsorption behaviors, rheological properties and stability of polysaccharide-protein at the oil–water and air–water interfaces (Table 1).

Monitoring influencing factors such as pH, ionic strength, solvent quality and experimental procedure can facilitate delicate dynamic balance of competitive and cooperative (associative) processes (Dickinson, 2008; Hernández-Marín et al., 2013) between proteins and polysaccharides at the oil–water or air–water interface. Pre-formed protein-polysaccharide complex may adsorb together on the interface to form a single complex layer (Fig. 2A). Alternatively, a protein monolayer formed first, with protein-polysaccharide complex underneath after subsequent addition of polysaccharide (Fig. 2B).

**Table 1** Physicochemical factors studied for polysaccharide-protein interaction at the oil (air)-water interface

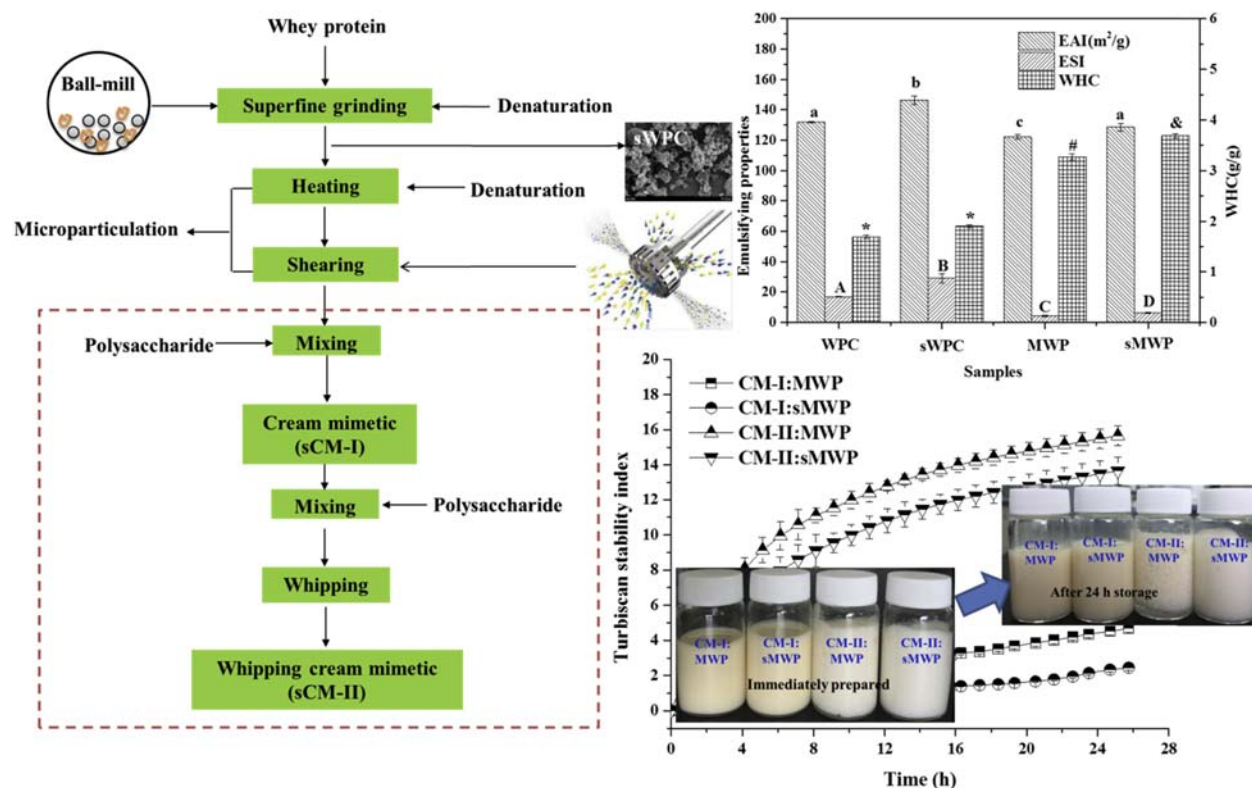
Protein(s)	Polysaccharide(s)	Parameters under consideration	References
Egg white protein	Gum arabic	pH 3.5–7.5 Protein concentration 0.05–0.50 wt%	Padala et al. (2009)
Whey protein	Carboxymethylcellulose	pH 3.3–4.3 Protein: polysaccharide 2:1–1:6 Experimental procedure (layer-by-layer or pre-formed complex)	Hernández-Marín et al. (2013)
	Gum arabic	pH 2.0–8.0 Salt concentrations 100–500 mM Temperature 30–90 °C	Ozturk et al. (2015)
	Pure gum	pH (3.0–7.0)	Li et al. (2016b)
Lentil legumin-like protein	Gum arabic	Polysaccharide concentration 0–12 wt%	Jarpa et al. (2016)
	Guar gum	pH 3.0–7.0	
	Xanthan gum		
Egg white protein	Pectin		Asghari et al. (2016)
	Rice starch	Protein concentration 0.5, 1 wt % Polysaccharide concentration 0.5–5 wt %	
Pea protein isolate	Xanthan	pH 5.4–6.4	Hanazawa and Murray (2014)
Sodium caseinate		Calcium ion concentration 1–64 mM	
Wheat protein	Pectin	pH 3.5–7.0	Qiu et al. (2015)
	Xanthan gum	Polysaccharide concentration 0–0.3 wt%	
Crayfish protein	Xanthan gum	Protein concentration 0.5–5.0 wt%	Felix et al. (2017)
	Xanthan gum	pH 3.0–8.0	

**Figure 2** Schematic representation of two experimental procedures of protein and polysaccharide at the oil (air)-water interface: (A) a mixed layer formed by pre-formed protein-polysaccharide complexes or coacervates; (B) adsorption of polysaccharide onto the protein layer. Adapted from Dickinson, E., 2009. Hydrocolloids as emulsifiers and emulsion stabilizers. *Food Hydrocolloids* 23 (6), 1473–1482.

### Biopolymer-Based Hydrogel Particles as Fat Mimetics

Small spheroidal protein particles (0.1–10 µm in diameter) as fat mimetics have similar textural, rheological and sensory properties to those of fats (Liu et al., 2016). Polysaccharides act as fat replacements mainly through stabilizing a large quantity of water in the gel-like matrix to generate creamy and flow attributes in a way similar to that of fat (Tiwari et al., 2015). Single carbohydrate-based and protein-based fat mimetics suffer from several sensory and functional limitations such as poor stability and undesirable mouth-feel. Thus, gelled polysaccharide-protein based particles as fat mimetics have attracted growing attention (Lee and Chin, 2017; Toto-saus and Rojas-Nery, 2017). For example, microparticulated whey protein (MWP) in combination with either modified starch or locust bean gum (LBG), with or without fat droplets (5%), could be used as fat mimetics to modulate the texture, appearance and stability of emulsion-based food products with reduced calorie such as sauces, mayonnaise, dressings and dips (Chung et al., 2014).

In recent years, our research team has done a lot of investigations on the interaction between polysaccharides and proteins at the interface and the rational design of food colloidal structures using various food ingredients such as polysaccharides, proteins, fat and water etc. Our studies reveal that protein molecular configuration, protein aggregation and protein particle size as well as



**Figure 3** The regulatory roles of protein molecular configuration and particle size in the interactions between polysaccharides and proteins and associated application in the design of a composite fat mimetic. Adapted from Sun, C., Wu, T., Liu, R., Liang, B., Tian, Z., Zhang, E., Zhang, M., 2015. Effects of superfine grinding and microparticulation on the surface hydrophobicity of whey protein concentrate and its relation to emulsions stability. *Food Hydrocolloids* 51, 512–518 and Sun, C., Liu, R., Wu, T., Liang, B., Shi, C., Cong, X., Hou, T., Zhang, M., 2016. Combined superfine grinding and heat-shearing treatment for the microparticulation of whey proteins. *Food Bioprocess Technol.* 9 (2), 1–9.

the aqueous environmental conditions could modulated macromolecular structure characteristics, balance between phase separation and gelation and heating/cooling kinetics of polysaccharide-protein systems.

Compared with microparticulated whey protein (MWP), superfine and microparticulated whey protein (sMWP) exhibited more stable liquid behaviors (Sun et al., 2015) and could maintain creamy mouth-feel better due to high dispersion stability of sMWP-pectin-xanthan gum gel mixtures (Sun et al., 2016). Accordingly, a composite fat mimetic (microparticulated whey protein-pectin-xanthan gum gel complex system) (Fig. 3) and a texture-controllable fat mimetic can be designed and applied in semi-solid emulsion-based food systems such as mayonnaise, ice cream and Chinese sausage.

Our most recent focus is on the regulation of protein molecular conformation to allow oil–water interfacial compatibility of protein-polysaccharide electrostatic complexes, and the characterization of the emulsions in terms of the stability, lipid oxidation and in vitro digestibility.

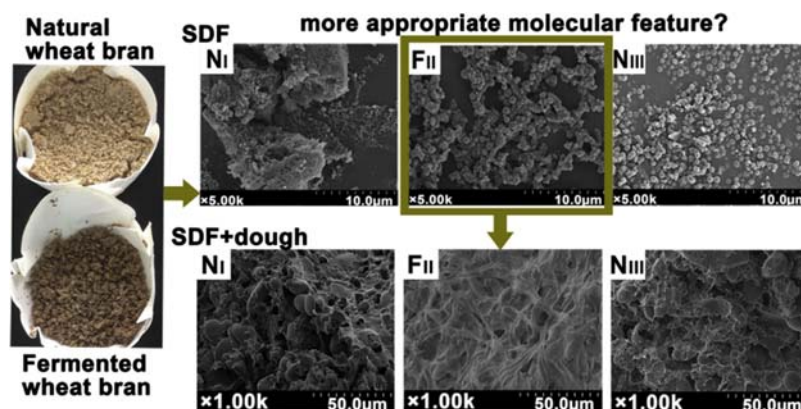
## Dough

Bread, steamed bread, biscuits and other wheat flour products are popular consumer foods worldwide. Therefore, the properties of wheat dough including rheological, textural and nutritional attributes have attracted considerable attention. Effects of various hydrophilic gums on the properties of dough and gluten proteins have been widely investigated (Burešová et al., 2017).

Among the hydrophilic gums, soluble dietary fiber (SDF) represents a valuable constituent for health-promoting foods (Garrett, 2015). SDF participates in the regulation of cholesterol in blood and offers prevention from cardiovascular disorders such as atherosclerosis. Further, SDF acts as prebiotics in the gastrointestinal tract. SDF can exert more significant physiological functions due to its hydrophilic characteristics and availability to beneficial microorganisms (Chawla, 2010). Thus, consumption of foods with a high SDF content would promote human health.

Wheat is a global staple food thus wheat flour-based consumer foods provide ideal matrices for SDF delivery. The processing adaptability of wheat dough during mixing, kneading and rising is largely dependent on the complex interactions among natural constituents in flour and other added ingredients or additives in dough. Many studies have been carried out and both positive and negative effects of SDF addition on the properties of dough were found (Flander et al., 2008; Saeed et al., 2011). These contradictory findings are usually governed by the molecular characteristics of SDF such as composition, molecular weight distribution,



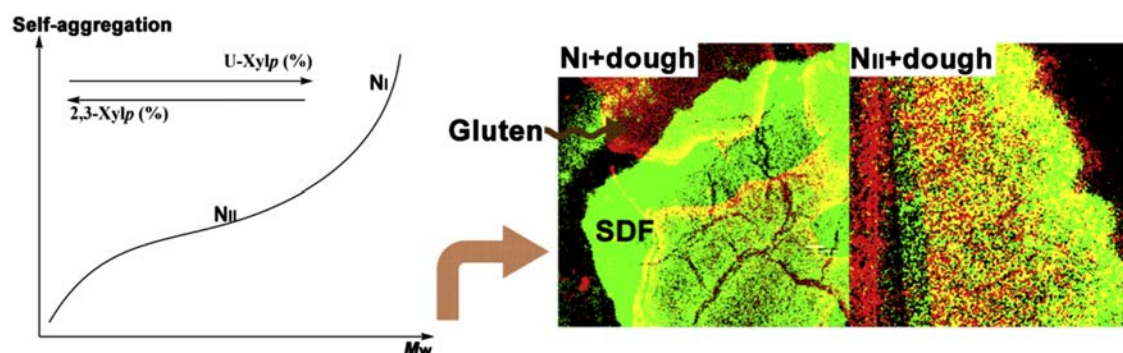


**Figure 4** The scanning electron micrographs of doughs fortified with different SDF samples. SDF refers to water-soluble dietary fiber. NI, NII and NIII are SDF fractions from the natural wheat bran precipitated with <40%, 40%–60%, and 60%–80% ethanol. FI, FII and FIII are SDF fractions from the fermented wheat bran precipitated with <35%, 35%–65%, and 65%–80% ethanol.

substitution degree and substitution position, etc. (Scheller and Ulvskov, 2010). For instance, the arabinofuranose (Araf) and other substituents can cause steric hindrance and prevent the aggregation of xylan backbone structure of the molecular chains, leading to the formation of an extended polysaccharide molecular structure and changes in corresponding functions. Thus, besides the structural characteristics of SDF, complicated interactions between SDF and individual constituents or whole matrix of wheat dough may play significant roles in dough quality.

The addition of SDF can not only improve the nutritional value of flour-based products, but also alter the rheological properties of wheat dough because of the influence of SDF on dough microstructure (Izydorczyk et al., 2001) e.g. addition of arabinoxylans or  $\beta$ -glucans to wheat flour. The interactions between SDF molecules and gluten may lead to desirable outcomes such as increased SDF addition and improved processing adaptability or dietary function.

It is of high interest to study the structural features and physico-chemical properties of SDF preparations that have been sub-fractionated via stepwise ethanol precipitation from natural and fermented SDF materials (Li et al., 2016a). The relationship between the structural features and physicochemical properties of isolated and purified SDF fractions were also investigated. Analysis of the distribution of SDF fractions (major components: Arabinoxylans (AXs)) from natural and fermented wheat bran in the wheat dough network by confocal laser scanning microscopy (CLSM) allows the examination on the effects of SDF addition on the microstructure and morphology (as shown by scanning electron microscope (SEM), Fig. 4), texture, extension property, and thermodynamic stability of wheat dough (Li et al., 2017). The relationship between the self-aggregation of SDF molecules and the interactions of SDF fractions with gluten network is shown in Fig. 5. To our best knowledge, the use of double staining for protein and polysaccharide fractions in the dough system has not been published previously. The following parameters, such as average size ( $\Phi_A$ ) and fractal dimension (FD) parameters of SDFs and the protein, are useful for elucidating the correlation between the structural factors affecting SDF molecular entanglement and the distribution of SDF in the dough found by CLSM. The correlations among the physicochemical properties of SDF, and the results of CLSM, texture analysis, tensile tests and differential scanning calorimetry (DSC) analysis can also be determined for displaying the effects of SDF addition on the properties of wheat dough. The interactions of SDF fractions with wheat gluten network can be demonstrated by CLSM with the dynamic and minimally invasive observation of dough microstructure, and the collected CLSM images can be converted into statistical data (Jekle and



**Figure 5** The self-aggregation of SDF molecules and the interaction between SDF fractions and gluten network. SDF, NI, NII, U-Xylp and 2, 3-Xylp refer to water-soluble dietary fiber, natural SDF fractions precipitated with <40%, 40%–60% ethanol, un-substituted and di-substituted at O-3 and O-2 xylose residues.

Becker, 2011). This technique does not require complicated sample preparations for microscopy (e.g. the fixing and dehydrating steps in SEM), and provides images of dough's native structure in an original environment (Jekle and Becker, 2015). Based on the theory of the wormlike model of tangled macromolecule chains (a very large molecule, such as polysaccharide, protein) in a soft condensed state, the different molecular weights and stiffness of the molecular chains affect the morphology and dynamics of the molecules (Egorov et al., 2003; Wei et al., 2016). Therefore, it is possible to estimate interactions between SDF fractions and wheat gluten network based on  $\Phi A$  and FD of SDFs and corresponding protein CLSM images, as a functions of  $M_w$  and the  $\rho$  parameter (which estimates the rigidity of dough) of SDFs using the SPSS software (Li et al., 2017).

## Conclusions

As interactive polysaccharide-protein polymers can be assembled into functional particles or gels, designed polysaccharide-protein interactions are used to control the structure, texture and shelf-life of semi-solid foods. The emphasis in this article has been on segregative or associative phase separation of polysaccharide-protein mixtures in two semi-solid food systems, such as hydrogel and dough, through thermodynamic incompatibility or compatibility. Modulation of polysaccharide-protein hydrogels structure and functionality can be controlled through fabrication conditions (biopolymer type, concentration, pH, biopolymer ratios, ionic type and strength). These polysaccharide-protein hydrogels have the potential to act as emulsion/foam stabilizer and fat mimetics. Thus, more investigations are needed to better understand the structure and processing characteristics of such polysaccharide-protein complexes in real multicomponent semisolid foods during their processing, storage, and digestion.

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# Protein-Starch Interactions in Cereal Grains and Pulses

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## Introduction

Grains are the edible seeds of specific grasses belonging to the *Poaceae* family, which includes wheat and its types (farro, freekeh, emmer and spelt), oats, rice, corn, barley, sorghum, rye and millet (Grains & Legumes Nutrition Council, 2017). Pulses comprise the dried seeds of the legume family, which includes pea, lentil, bean and chickpea. In the western civilization, cereal grains have been a major food source for centuries but more recently, pulses are also drawing a lot of attention due to their higher protein, fibre and mineral contents. Cereal grains and pulses together could theoretically make up for daily human intake of all essential nutrients, i.e. carbohydrates, protein, lipids, vitamins and minerals. Determining the chemical composition and knowing the microstructure of cereal grains and pulse crops allow the understanding of desirable traits in their derived products. Various interactions occur within the food systems on a molecular level to bring about changes in food properties. The interactions among the chemical constituents (such as water, starch, protein, etc.) and their functional properties within grains are critical to several ongoing chemical and biological reactions. This chapter primarily focuses on the interactions between protein and starch, their relevance to the functional properties of foods, processes that cause alteration in the protein-starch matrix, and the chemical imaging methods typically used to understand these interactions.

## Functional Attributes of Cereal Grains and Pulses

Cereal grains and pulses comprise starch, protein, lipids, carbohydrates, fibre, ash, etc. Starch is primarily composed of amylose and amylopectin whereas proteins are categorized into albumins, globulins, gliadins and glutenins, based on their solubility (Kiosseoglou and Paraskevopoulou, 2011; Lásztity, 1996). Starch granules of cereal grains vary in size (2–35  $\mu\text{m}$ ), size distribution (normal, bi-modal or tri-modal), gelatinization onset temperature, and amylose content (0%–70%) (Wrigley, 2010). Similarly, pulse starch granules vary in shape (disk, elliptical, irregular, oval, round and spherical) and size (0.4–103  $\mu\text{m}$  depending on the botanical source) (Singh, 2011). Difference in starch granule size affects the physico-chemical properties such as swelling power, light transmittance and water-binding capacity (Singh, 2011). Moreover, the functional properties of individual starch granules and protein bodies such as swelling, solubility, digestibility, water-holding and fat-holding capacities affect the overall protein-starch matrix in food products.

Swelling of starch granules happens as a result of molecular disruption caused by heating in excess water. This change in structure is generally characterized by parameters such as swelling power or swelling factor (Singh, 2011). Swelling power is a measure of inter- and intra-granular water (Leach et al., 1959), whereas swelling factor only measures the former (Tester and Morrison, 1990). As a function of temperature, the swelling power of pulse starches have been found to increase considerably above 70 °C with no change observed below 60 °C (Hoover et al., 2010). Li and Yeh (2001) found that the swelling power of starches from cereals (corn and rice) and legumes (peas and mung bean) increased with temperature beyond 55 °C, but starch granular size, amylose content and heat of gelatinization were not statistically correlated with the swelling power of starches. However, Sasaki and Matsuki (1998) demonstrated that the swelling power of wheat starch was positively interrelated with the structure, gelatinization temperature and enthalpy whilst negatively related to the starch-amylose content. Cereal starches show higher swelling power and amylose leaching than pulses due to lower amylose content and higher bound lipid content (Hoover et al., 2010).

Protein solubility reflects the balance between protein-solvent (hydrophilic) and protein-protein (hydrophobic) interactions (Schwenke, 2001), and it depends on the method of extraction, stirring and configuration conditions, and pH (Kiosseoglou and Paraskevopoulou, 2011; Tiwari and Singh, 2012). High solubility of legume proteins is typically found at low acidic (pH 4–6) and high alkaline (pH 8–9) conditions (Boye et al., 2010). For instance, higher solubility values (61%–96%) have been found for certain legume protein isolates obtained via isoelectric precipitation compared to those via salt extraction (38%–96%) (Karaca et al., 2011). Starch solubility is associated with the swelling power as it increases with elevated granular swelling depending on the amylose content. Starch solubility (leaching of soluble species) also depends on the starch-lipid content and formation of amylose-lipid complexes (Singh, 2011). Higher amylose compacts the starch granules thus restricting their overflow, which causes lower solubility (Wani et al., 2012).

Water- and oil-absorption capacities (WAC, OAC) are defined as the amount of water or oil absorbed per gram of the respective protein material. WAC and OAC values for pulse proteins were reported in the ranges of 0.6 to 4.9 and 1.0 to 3.96 g/g, respectively. These functional properties are dependent on pulse type, variety and method of extraction (Kiosseoglou and Paraskevopoulou, 2011). Water binding capacity (WBC, similar to WAC) of several cereal and tuberous starches decreases with an increase in amylose content (Wootton and Bamunuarachchi, 1978). Therefore, pulse starches that are known for its low amylose content would likely have lower WBC values than cereal grains. However, high WBC values are also possible for pulse starches e.g. 73%–84% for starches of black gram varieties (Kaur et al., 2004).

Interactions between the foods can be binary and ternary (more complex) comprising main food components (starch, proteins and lipids). The interactions between starch and biopolymer mixtures within food systems have been studied extensively (Yang et al., 2004). Tang and Copeland (2007) provide a comprehensive review of the binary starch-lipid interactions in wheat. Efforts have also been made to explore the ternary interactions of protein-starch-lipids (Zhang et al., 2003; Zhang and Hamaker, 2003). It is worth noting that information on protein-starch interactions in foods is scattered throughout the literature and it is only studied with respect to the effect of certain functional (viz. swelling, OAC, WAC, thermal, and rheological) and physico-chemical properties in food systems.

## Protein-Starch Matrix

Study of protein-starch matrix is very important for understanding the various biochemical interactions that occur in foods. Eliasson and Larsson (1993) discussed two possible interactions: proteins interacting with amylose/amylopectin in solution or proteins interacting with the solid starch granule. The latter type of interaction is described elsewhere (Eliasson and Tjerneld, 1990). In this section, we will discuss the protein-starch interaction at a broader level.

Protein-starch interaction is purely electrostatic in nature given the facts that protein bodies are negatively charged and starch granules are positively charged. Takeuchi (1969), Dahle (1971), and Dahle et al. (1975) conducted extensive experiments to unravel the protein-starch interaction in foods. It was found that the protein-starch interaction is pH dependent, with the degree of wheat protein-starch interaction being the highest at pH 6.5 (corresponding to the lowest absorption). Additionally, the interaction of gelatinized wheat starch and protein was much greater at low and neutral pH; heat denaturation and an alkaline pH could further reduce the degree of interaction (Dahle, 1971). The author further reported that sustaining the native structure of proteins is essential for the formation of protein-starch complexes (Dahle et al., 1975). This interaction plays a major role in food products typically obtained via grain processing. For instance, the interaction of starch with gluten proteins determines bread baking performance while staling of bread is also influenced by the degree of protein-starch interaction (Martin et al., 1991; Martin and Hoseney, 1991). The effect of protein-starch interaction on the thermal and rheological properties of food systems has been summarized by Jamilah et al. (2009).

Protein-starch interactions change with the amount of moisture and processing temperature in the initial grain pre-processing. For instance, protein-starch interactions may stimulate an increase in viscosity upon surpassing the starch gelatinization temperature in wheat and maize (Madeka and Kokini, 1992), which was also shown upon protein addition to corn starches by Chedid and Kokini (1992). An inverse relationship was found between the moisture content and the threshold temperature (temperature at which the viscosity increases) as a result of the protein-starch interactions (Madeka and Kokini, 1992). Furthermore, the interaction of amylopectin with gliadin (wheat protein) could lead to a lower threshold temperature than the interaction with zein (maize protein).

Dry and mature endosperm of grain cereals have a matrix of protein bodies fused around the starch granules that also engulf other cellular structures (Shewry and Halford, 2002). The gluten proteins form the same continuous protein matrix, which allows the flour to form a dough and enhances visco-elastic properties to enable the incorporation into fermented, baked, or pasta-based products (Shewry and Halford, 2002). Weaker cross-links between starch and gluten produce less firm bread while greater amylose-protein interactions would enhance the staling process (Martin et al., 1991). The shape and size of starch granules play major roles in bread quality (Kang et al., 2015; Sahlström et al., 1998). Further, the protein-starch interaction may present nutritional advantages. Protein-starch interaction in bread made from white flour has been demonstrated responsible for a decrease in glycemic rate and digestion rate as compared to gluten-free bread or gluten-free bread with gluten added later (Jenkins et al., 1987).

Texture (hardness or softness) of the wheat kernel has been attributed as either the degree (Barlow et al., 1973; Simmonds et al., 1973) or strength (Stenvert and Kingswood, 1977) of adhesion between starch granules and the protein matrix. Positive relationship between the grain hardness and the presence/adsorption of certain proteins on starch granules has been established (Eliasson, 2016). In hard wheats, greater adhesion may also be due to the large amount of water-soluble material of uniform composition among the starch granules surrounded by the protein matrix (Simmonds et al., 1973), which was later revealed to be the presence of protein (fibrillin) on the surface of starch granules (Greenwell and Schofield, 1986).

The functional properties of cereals and pulses depend on the interaction of starch and protein. For instance, tight adhesion of proteins (obtained from the flour of the outer portion of rice kernels) to starch granules inhibits the crystalline and pasting properties of rice, and starch swelling is restricted as a result of the interactions between starch and the protein matrix (Yang and Chang, 1999). Using scanning electron microscopy (SEM), Saleh (2017) found that the rigidity of the closely-bound protein-starch matrix could be attributed to the higher pasting viscosity of rice flour. The behavior of starch changes from linear viscoelastic to nonlinear due to its introduction into gluten protein (Smith et al., 1970).

## Alteration in Protein-Starch Matrix

Processing operations (viz. baking, cooking, dough formation, drying, fermentation, extrusion, milling, thermal processing, etc.) to create food products from pulses and cereal grains affect the protein-starch network. The resultant alteration of protein-starch matrix then changes food properties such as texture, flavor and aroma.

The microstructural changes during dough development and baking processes are highly dependent on the interactions between starch and protein, and to a lesser extent, between other carbohydrates and protein. The interactions between gluten protein and starch lead to phase separation that makes uniform alignment of starch granules in the continuous gluten phase crucial for the closely-packed structure of dough (Eliasson and Larsson, 1993). Dough microstructure is described as a bicontinuous protein-starch system (Eliasson and Larsson, 1993). Regarding the effects of processing steps such as mixing, proofing and reshaping during dough development, Kieffer and Stein (1997) showed how protein and starch segregate during dough reshaping after a rest period. Sadowska et al. (2003) reported the progressive changes (swelling of the starch granule and increased protein content) resulting from the significantly higher protein to starch ratio in pea-flour supplemented dough. The pasta made from soft wheat was found to have a "less extensive protein framework with more diffuse starch particles" (Resmini and Pagani, 1983).

Milling of grain into flour decreases the particle size while increasing the surface area thereby causing microstructural changes. More importantly, as starch damage occurs due to extensive milling of cereal grains and pulses, the effect of milling on the protein-starch matrix is inevitable. Micrography has revealed compactly packed elliptical starch granules within the protein matrix of coarse-ground green gram flour whilst higher starch damage and disentangled aggregates of protein matrix with single, unaggregated starch granules were found in finely-ground flour (Sakhare et al., 2014). Moreover, red gram, green gram, and black gram have elliptical starch granules ingrained in the protein matrix, similar to other well-known legumes except for the shape and size differences of starch granules between legumes (Joseph et al., 1993). During dry milling of barley and likely other cereals and pulses, endosperm cells could be drawn out where starch was embedded within the protein matrix (McAllister et al., 1993). In comparison, wet milling may detach the starch granules from the matrix.

Thermal processing of raw food materials is essential to introduce desirable organoleptic and textural qualities in end-products (e.g. gelatinization of starch caused by thermal treatment can lead to mechanical changes in extrudes). Gelatinization of starch is attributed to water interrupting crystallinity, swelling of granules, amylose leaching from starch granule with intact amylopectin, and eventually the formation of an amylose-matrix gel with disintegrated granule (Remsen and Clark, 1978). During cooking, the protein matrix offers rigidity to the starch granules resulting in enhanced visco-elastic properties (Derycke et al., 2005). Cooking quality of pasta is dependent on the formation of continuous gluten protein network rather than the physio-chemical properties of gelatinized starch (Sung and Stone, 2005). Cooking of rice at 120 °C for a short duration (e.g. 20 minutes) led to starch granules still attached to the protein matrix and adjacent protein areas (Srikaeo et al., 2006). Further cooking caused starch gelatinization and fusing of the molten granules. Disintegration of the surface layers, cells and granules via cooking evokes the change in the interactions between protein bodies and starch granules (Chrastil, 1990). Likewise, the degree of binding of rice protein (oryzenin) to starch proportionally increases the stickiness of rice (as a result of cooking and cooling) (Chrastil, 1990).

Roasting, micronization, hydrothermal treatment and high-temperature drying are commonly used thermal operations, which can induce changes in the microstructure of starch and protein. Roasting of barley resulted in a reduced bulk density due to the potential loss of structural integrity between starch-starch and protein-starch matrices and void (pore) creation in the endosperm (Chandrasekhar and Chattopadhyay, 1990; Sharma and Gujral, 2011). Micronization (infrared heat processing) of barley (hulless and pearled) kernels caused more expansion due to starch gelatinization as shown in the micrographs of hulless barley (Fasina et al., 1999). McAllister and Sultana (2011) also showed that micronization of three wheat varieties (Kansas, Laura, and Sceptre) modified the protein matrix and slowed the *in situ* starch digestion. Micronization-induced changes in the mechanical properties of different types of barley were attributed to starch gelatinization, and alterations in protein-starch and starch-lipid interactions (McAllister and Sultana, 2011). SEM analysis revealed that hydrothermal treatment (parboiling) of rice grain caused the formation of a peripheral thin layer due to the stronger insoluble protein matrix escaping the starch granules (Chandrasekhar and Chattopadhyay, 1990). High temperature-low moisture (HT-LM) drying treatment of raw pasta resulted in a continuous protein network while improving pasta quality (Resmini and Pagani, 1983). This cooking treatment stimulates protein-starch interactions in the outer part and linear or branched fibrils in the central area by creating a coagulated protein network and restricting starch swelling. However, high temperature-high moisture (HT-HM) treatment of raw pasta (made from soft wheat flour) led to expanded volume and restricted protein coagulation and network formation. This resulting pasta was of poor quality with no protein-starch interaction observed.

Extrusion is another common process for grains that utilizes high moisture and high temperature to allow product expansion or puffiness by which breakfast cereals, pasta products and snacks, etc. are produced. Extrusion modifies the structural configuration of food products by which these foods can be enriched with resistant starch through controlled swelling and boiling during cooking (Vaz Patto et al., 2015). A review by Moraru and Kokini (2003) on extrusion of cereal foods revealed the influence of starch in product expansion; protein matrix also affects water distribution and promotes extensive framework formation because of covalent and non-bonding interaction in the extruded product. Protein-starch interactions also prevent molecular starch from degradation as a result of the formation of amylose-protein complex (Matthey and Hanna, 1997). Alkali addition during extrusion promotes higher degrees of protein-protein and protein-starch interactions leading to a cohesive extrudate product (Allen et al., 2007).

## Chemical Imaging

Visual characterization allows illustration of how the presence, and more importantly distribution, of chemical components within grain kernels influence food properties. Additionally, it can illustrate the spatial and structural alteration of the chemical constituents during various processes. With the advances in imaging and microscopy technology, real-time and accurate analyses of the distribution of food components are conceivable. Numerous imaging techniques, such as scanning probe microscopy e.g.

atomic force microscopy (ATF), SEM, scanning tunneling microscopy (STM), and optical/spectral imaging e.g. light microscopy (LM), near-infrared (NIR) spectroscopy, Fourier Transform Infrared (FTIR) spectro-microscopy, hyperspectral imaging (HSI), and X-ray microcomputed tomography ( $\mu$ -CT), have been utilized to study the chemical changes and microstructure of food products. Herein, we focus on the imaging techniques such as SEM, FTIR and NIR spectroscopy, HSI, and X-ray  $\mu$ -CT that have been utilized to understand the allocation and distribution of the protein-starch matrix during processing.

SEM involves a focused beam of electrons to produce images encompassing the topographical and compositional information of the scanned material. This technique allows us to study the microstructure of food products in a way that was previously impossible. For example, a SEM study could show a higher degree of protein-starch interaction and protein matrix continuity in the hard kernels than in soft kernels of barley (Nair et al., 2011). By which, the authors explained why milling of hard barley kernels produced coarse particles compared to soft kernels that produced fine particles (Nair et al., 2011). The strong protein-starch interaction and continuous endosperm protein matrix in hard kernels would require much more energy during milling to release the strongly bound starch particles. In addition, SEM also showed starch granules not connected to the disorganized protein matrix in mature soft wheat kernels as opposed to the mature hard wheat kernels (in which a uniform matrix and stronger adhesion of starch and protein bodies were found) (Turnbull and Rahman, 2002).

Difference in wheat cultivars corresponding to the degree of adhesion between starch and protein matrix has already been reported (Moss et al., 1980). A more homogenous and less porous structure along with a disorganized matrix composed of partially "fused" starch granules and proteins was found in SEM images of puffed wheat, emmer wheat, and barley as compared to rice (Mariotti et al., 2006). Further magnification of SEM images of puffed wheat and barley revealed a non-homogeneous structure with compact matrix in some areas and large cavities elsewhere. In a different study, SEM analysis was able to demonstrate more starch agglomeration in pearl millet flour than finger millet suggesting a greater protein-starch interaction, whereas, FTIR analysis failed to reveal significant differences as similar stretching and bending vibrations were detected for both flours (Gull et al., 2016).

SEM has also been used to explore the structural changes that take place during various unit operations involved in the production of processed food products. Matsuo et al. (1978) used SEM to image each step during the development of spaghetti. Preliminary mixing of the semolina and water resulted in visible starch granules held within the protein matrix. With the dough entering the extrusion auger, protein particles were clearly visible with different shapes (platelets and jagged edges rather than sheet and fibril-form). The pasta exiting the auger still had irregular protein matrix; however, starch particles were aligned along the direction of flow. SEM of freshly extruded spaghetti exhibited starch granules embedded deep within the protein matrix. High-absorption doughs tended to have fibrillar network with smooth protein matrix, whilst low-absorption doughs had jagged network with the outer surface containing pores with starch granules loosely held within a discontinuous protein matrix.

FTIR spectroscopy can show the presence or absence of a functional group in a sample by measuring the absorption of light at different wavelengths. The wavelengths are typically measured in wavenumbers ( $1/\lambda$ , expressed in  $\text{cm}^{-1}$ ) over the range from 4000 to  $400\text{ cm}^{-1}$ . The absorption of the infrared radiation produces a fingerprint region for a particular functional group. FTIR defines the natural chemical variability of *in situ* structures (to elucidate their differences) (Wellner, 2013). The technique is readily utilized to characterize the chemical composition of materials (cereal grains, gels, and vegetables), and the wavenumbers corresponding to the functional groups and their bonding of certain plant components such as starch, protein, fibre, etc. are well understood and documented (Barron and Rouau, 2008; Starr and Paliwal, 2012; Vlădoiu et al., 2017). The presence or dominance of functional groups associated with constitutional components is gleaned by manipulating the spectral data. For example, the distribution or component ratio (such as protein-starch or protein-carbohydrate ratio) within a sample can be obtained by dividing the area (or height) under a functional group band by the area (or height) under another functional group (Yu, 2007; Yu et al., 2004a). Accordingly, the protein to starch ratio for cereal grain (i.e. barley) could be obtained by utilizing the protein (amide I at  $1650\text{ cm}^{-1}$ ) and starch (at  $1025\text{ cm}^{-1}$ ) peaks, which indicates the adhesion between starch and protein matrix (Yu et al., 2004a). Following this method, extensive differences in the absorbance intensities of starch and protein were found between two barley varieties ("Valier" and "Harrington"). A lower ratio of protein absorbance intensity for "Valier" variety suggested a closer association of starch granules with the protein than "Harrington" variety (Yu et al., 2004b).

NIR spectroscopy is a non-invasive method that predicts the chemical composition of a sample through indirect measurements of its optical characteristics based on light absorption in the wavelength range of 750–2500 nm (Jayas et al., 2016). NIR spectra of wheat flour indicated a decrease in free water corresponding to the reduced absorbance of the O–H stretch at 1160 nm (Wesley and Blankeney, 2001). Further explanation of the differences in the interactions of gluten components (glutenin and gliadin) with starch is provided but only with respect to the changes in bound water with temperature.

HSI provides direct identification of different components and their distribution in a sample using a 2-D spatial array of vectors representing the spectrum at each pixel location (ElMasry and Sun, 2010; Erkinbaev et al., 2017). HSI has been utilized for estimating moisture, kernel hardness, pre-germination status, etc. in grains and legumes (Fox and Manley, 2014). HSI of wheat kernels showed clear spectral differences around 1450–1550 nm for starch and protein; relocation of these peaks would demonstrate differences in starch/protein mixture (Arngren et al., 2011). The authors proposed a Bayesian method for spectral analysis of wheat kernel (back and front) to reveal differences in protein-starch matrix, and believed this method was similar or better than other volume constrained methods based on a projected gradient non-negative matrix factorization framework. In another study, NIR-HSI of whole yellow maize showed positive peaks at 1220, 1405, 1690 and 1870 nm resulting from the differences in starch composition for vitreous endosperm signifying tight adhesion (or density) of starch granules and the protein matrix (Manley et al., 2009). Different peaks and thus different interactions occurred in floury endosperm.



X-ray  $\mu$ -CT is a non-destructive and non-invasive technique that takes 3-D sectional images of a cross-section with micrometer pixel sizes based on the differences in absorbance of X-rays by the constituents in a sample. With the advent of benchtop instrumentation,  $\mu$ -CT is becoming extensively popular for characterizing food microstructure (Schoeman et al., 2016). This technique is useful for determining various microstructure properties of starch-based extruded products (average diameter, cell wall thickness, true void fraction, cell number density, etc.) (Horvat et al., 2014; Trater et al., 2005). The effects of different processing methods (milling, parboiling, wet-processing and extrusion) have been shown via  $\mu$ -CT by examining porosity, pore size and mean pore diameters (Mohorič et al., 2009). The information about the cavities and pores allows predicting the milling characteristics (Guelpa et al., 2016). Based on the wealth of microstructural information generated using this technique, one can easily provide in-depth interpretation of the interactions between the building blocks (e.g. protein and starch) in food products.

## Conclusions

The importance of understanding protein-starch interactions in raw and processed grains and pulses has been discussed. These interactions have been studied using a variety of tools and it is established that the interactions are strongly correlated to moisture, pH, kernel hardness, processing operations (such as dough and bread making, pasta formation, etc.), and other functional properties of food products. A number of modern imaging modalities have been used to unravel the structure (and structural alterations due to changes in the aforementioned factors) and so far, SEM has been found to be the best technique to unravel the protein-starch interactions. However, implementation of other promising non-destructive and non-invasive 3-D imaging techniques, especially X-ray  $\mu$ -CT, for studying protein-starch interactions has not gained much attention primarily due to the rigorous analyses required post image acquisition. Further progress in imaging research would require publication of standards and protocols for easy quantification and analyses of 3-D images. These techniques can provide exhaustive characterization of food materials that can assist in the development and formulation of new products and processes to enhance the overall product quality.

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# The Use of Spin-Label ESR Spectroscopy to Study Protein-Lipid Interactions

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## Glossary

**Electrostatic interaction** Electrostatic interactions are the interactions between charged species. Electrostatic interactions between unlike charges are attractive, and repulsive between like charges.

**Head group** In phospholipids, the polar moiety that is attached to the phosphate is referred to as the head group. For example, in phosphatidylcholine and phosphatidylethanolamine, the choline moiety and ethanolamine moiety, respectively are the head groups.

**Hydrophobic interaction** The tendency of nonpolar molecules in a polar solvent (usually water) to interact with one another is called the hydrophobic effect. The interactions between the nonpolar molecules are called hydrophobic interactions. Hydrophobic interactions play an important role in the clustering of amphiphilic lipid molecules such as phospholipids to form bilayers and micelles.

**Integral membrane protein** An integral membrane protein is a protein that is permanently attached to the biological membrane. All transmembrane proteins are integral membrane proteins. In addition, proteins which are permanently associated with the membrane, for example, by a lipid anchor such as glycosylphosphatidylinositol, are also considered as integral membrane proteins. Glycophorin in the erythrocyte membrane, cytochrome oxidase in the mitochondrial membrane are some of the well characterized integral membrane proteins.

**Outer hyperfine splitting ( $2A_{\max}$ )** The spectral width between the first maximum and the last minimum in the ESR spectrum of a nitroxide spin label.

**Peripheral protein** Peripheral membrane proteins are proteins that adhere only temporarily to the biological membrane with which they are associated. These proteins may attach to the membrane by electrostatic interaction with the negatively charged lipids of the membrane, or by interaction with integral membrane proteins. Some peripheral protein may exhibit partial penetration into the hydrophobic interior of the lipid bilayer. Cytochrome c, which binds to cytochrome oxidase in the mitochondrial membranes and myelin basic protein, which binds to negatively charged lipids in membranes and forms a part of the myelin sheath in the spinal cord, are well characterized peripheral proteins.

**Segmental motion** Due to the rotation around the C—C bonds in the hydrocarbon chains of lipid molecules, the chains exhibit lateral movement, which increases as one goes further away from the head group region. This is referred to as segmental motion.

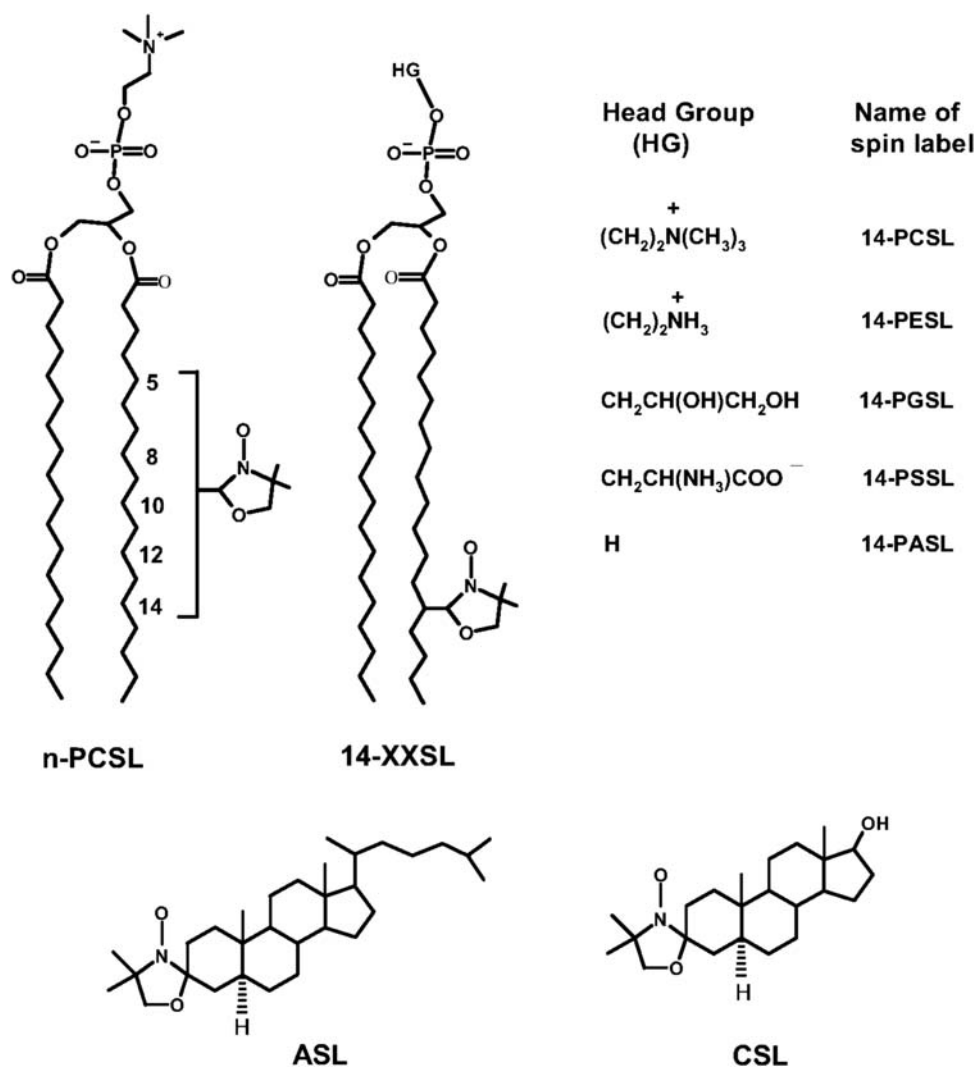
**Transmembrane protein** An integral membrane protein that spans the entire thickness of the membrane at least once. Glycophorin is a classic example of a transmembrane protein that spans the membrane once. Rhodopsin, the transmembrane present in the retinal rods has 7 transmembrane segments.

## Introduction/Overview

Biological membranes are made up of lipids and proteins in nearly comparable quantities by weight. While the lipids are essentially organized in a bilayer format, membrane proteins can be associated with the lipid bilayer in different ways. Peripheral (or extrinsic) proteins are attached to the membrane surface predominantly by electrostatic interactions whereas the driving force for the association of transmembrane (or intrinsic membrane) proteins, which are embedded in the membrane and traverse its thickness, is primarily hydrophobic in nature. Their association with the lipid membrane is primarily driven by the hydrophobic effect. A third category of protein association with membranes involves initial association by either electrostatic attraction or specific recognition of the lipid head group, followed by partial insertion of protein segment(s) into the hydrophobic core of the membrane.

## Spin Labelled Lipids

Since neither membrane proteins nor membrane lipids normally possess paramagnetism, biomembranes do not give rise to an ESR spectrum. This apparent limitation, can be turned into an advantage by attaching stable nitroxide spin labels to membrane lipids or proteins at specific sites. The attached spin label then can act as a probe or reporter and give information about the dynamics of the surrounding environment. The doxyl (4,4'-dimethyl-oxazolidine-*N*-oxyl-) and proxyl (2,2,5,5-tetramethyl-1-pyrrolidinyloxy) groups are the most commonly employed spin labels, and they contain the nitroxide moiety bearing an unpaired electron. In order to probe the environment at different depths of the bilayer lipid membrane, the spin label is attached at different positions of the lipid acyl chains. A host of lipid derivatives bearing these stable free radical probes are commercially available. In addition, a number



**Figure 1** Structures of spin-labelled phospholipids used for investigating lipid-protein interactions by ESR spectroscopy. n-PCSL are phosphatidylcholines spin-labelled on the sn-2 acyl chain at various positions (C-5, C-8, C-10, C-12 and C-14). 14-XXSL are spin-labelled phospholipids bearing different head groups, but the same acyl chain structure, with the nitroxide moiety located on the 14th C-atom of the sn-2 acyl chain. Androstanol spin label (ASL) and cholestane spin label (CSL) are sterol based spin labels. In ASL the hydroxyl group of cholesterol is derivatized to introduce the spin label into the structure, resulting in a loss of the hydrogen bonding capability of the steroid. On the otherhand, in CSL, the 8 carbon tail is replaced by a hydroxy group; hence this molecule is capable of forming H-bonds, but is devoid of the 8-carbon long hydrocarbon tail present in cholesterol. Reprinted from Swamy, M.J., 2004. Interaction of bovine seminal plasma proteins with model membranes and sperm plasma membranes. *Curr. Sci.* 87, 203–211.

of small molecules bearing functional groups that can react with the amino or thiol side chains of proteins are also commercially available for labelling proteins. Structures of selected spin labelled phospholipids are given in [Fig. 1](#). Besides these, spin labelled fatty acids and gangliosides as well as spin labelled lipids bearing other head groups have been used to investigate lipid-protein interactions. For the structures of a variety of such spin labelled lipid probes used in membrane research, the reader is referred to [Marsh \(1982, 2010\)](#).

### ESR Spectrum of the Nitroxide Spin Labels

In the commonly used spin labels, such as those shown in [Fig. 1](#), the nitroxide radical is incorporated into a heterocyclic ring. In such a structure the unpaired electron is predominantly localized on the N–O bond and ESR signal arising from the unpaired electron is split into three lines since the nuclear spin,  $I$  of the nitrogen atom ( $^{14}\text{N}$ ) is unity. If the molecule bearing the nitroxide group is tumbling rapidly in solution then the spectral anisotropy is averaged and three sharp, narrowly spaced lines of nearly comparable width and intensity. However, the extent of averaging is influenced by molecular size, temperature, viscosity of the medium or other



**Figure 2** ESR spectra of 5-PCSL in DMPC membranes in the gel phase at 8 °C (1) and in the liquid crystalline phase at 28 °C (2). The spectral width is 100 G. The spacing between the first maximum and the last minimum is referred to as the outer hyperfine splitting,  $2A_{\text{max}}$ .

parameters that can affect the molecular motion. In general, as motion slows down the spectral lines become broader and appear farther apart from each other. The ESR spectrum of a phosphatidylcholine spin label, bearing the doxyl spin label on the fifth C-atom of the sn-2 acyl chain, incorporated in the gel phase of dimyristoylphosphatidylcholine (DMPC) membranes is shown in Fig. 2 (spectrum 1). The molecular motion is strongly reduced in the lipid gel phase and the spectral lines are broad, with large separation between the outer wings (first maximum and last minimum). In the liquid crystalline phase, increased rotational motion and segmental mobility result in a sharpening of the spectral lines and the spacing between the outer wings of the spectrum is decreased (see Fig. 2, spectrum 2). However, significant anisotropy is still retained in the spectrum, reflecting that although the molecular motion increases when the lipid membrane undergoes gel-liquid crystalline phase transition, the motion is still significantly anisotropic.

## Effect of Membrane Proteins on the ESR Spectra of Spin Labelled Lipids

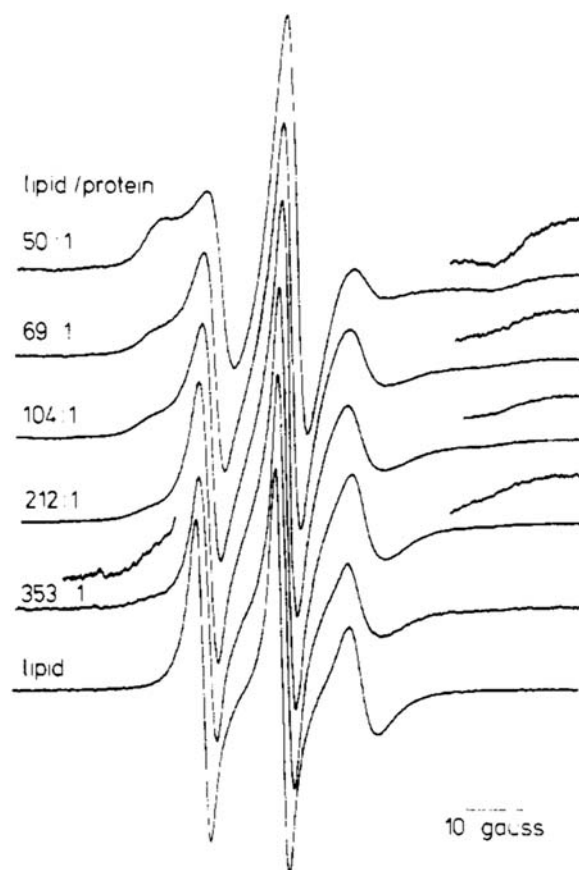
### Intrinsic Membrane Proteins

The effect of transmembrane proteins on the motional properties of membrane lipids is investigated by ESR spectroscopy employing spin-labelled lipid probes shown in Fig. 1. Most useful information is obtained when such studies are carried out with individual purified and reconstituted proteins incorporated either in lipid extracts obtained from the native membranes or in pure synthetic lipids. An extensively characterized system is that of cytochrome oxidase, and the results obtained with this system are described below to illustrate the type of information that can be obtained in such studies.

Studies employing either 16-doxylstearic acid (Jost et al., 1973) or 14-PCSL (Knowles et al., 1979) as the spin label probe incorporated into reconstituted cytochrome oxidase membranes yielded similar results. The study of Jost et al. (1973) is a seminal work that gave the first evidence for the existence of boundary lipid around transmembrane proteins. However, the studies by Knowles et al. (1979, 1981) provided a more extensive characterization of the system and their results will be discussed below to illustrate the application of spin-label ESR spectroscopy for investigating the interaction between transmembrane proteins and membrane lipids.

Knowles et al. (1979, 1981) employed a variety of phospholipid spin labels bearing the doxyl probe on the 14th C-atom of the sn-2 acyl chain. ESR spectra of 14-PCSL incorporated into cytochrome oxidase reconstituted in dimyristoylphosphatidylcholine (DMPC) membranes of different lipid/protein ratios are shown in Fig. 3. A spectrum of the spin label in pure DMPC membranes is also shown in this figure. All the spectra recorded in lipid/protein complexes could be resolved into two components, an immobilized component and a more mobile, fluid like component. Analysis of the relative proportions of these two components revealed that a constant number of lipid molecules ( $55 \pm 5$ ) per protein are immobilized. This was attributed to a single shell of boundary lipid molecules associated with the protein, which is then surrounded by fluid bilayer. The motional correlation times from the immobilized lipid difference spectrum gave values of  $\geq 50$  ns, suggesting that the rate of exchange between bilayer and boundary lipids is probably at least 1 order of magnitude slower than the rate of lateral diffusion of unperturbed bilayer lipids in pure DMPC membranes.

The preference of transmembrane proteins to interact with different membrane lipids would be expected to be different based on the lipid structure. ESR spectroscopic studies employing spin-labelled phospholipids bearing different head groups provide a sensitive and elegant approach to investigate this. ESR spectra of various spin labelled phospholipids incorporated into DMPC membranes containing reconstituted cytochrome oxidase are shown in Fig. 4A and spectra obtained in pure DMPC membranes are shown in Fig. 4B (Knowles et al., 1981). A careful observation of these spectra reveals that the proportion of the motionally restricted and more mobile components varies with the different spin-label head groups for complexes of the same lipid/protein ratio. Similar proportions of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol



**Figure 3** ESR spectra of 14-PCSL spin-label in cytochrome oxidase-DMPC complexes of various lipid/protein ratios at 32 °C. Spectra from all the lipid/protein complexes exhibit two-components; however, in samples with increasing lipid/protein ratio the immobilized component becomes relatively weaker. In some spectra, the immobilized component is highlighted by showing a vertically expanded segment of the outer wings. Adapted with permission from Knowles, P.F., Watts, A., Marsh, D., 1979. Spin-label studies of lipid immobilization in dimyristoylphosphatidylcholine-substituted cytochrome oxidase. *Biochemistry* 18, 4480–4487. Copyright (1979) American Chemical Society.

have been found to have restricted chain motion. On the other hand a distinct preference was seen for cardiolipin (CL) and phosphatidic acid (PA), with the greatest preference being seen for cardiolipin. From an analysis of the relative proportions of the immobilized components seen in their spectra, the selectivity for cardiolipin and phosphatidic acid was expressed as increased relative binding constant:  $K_r^{CL} \sim 5.5$  and  $K_r^{PA} \sim 2$ , respectively (Knowles et al., 1981).

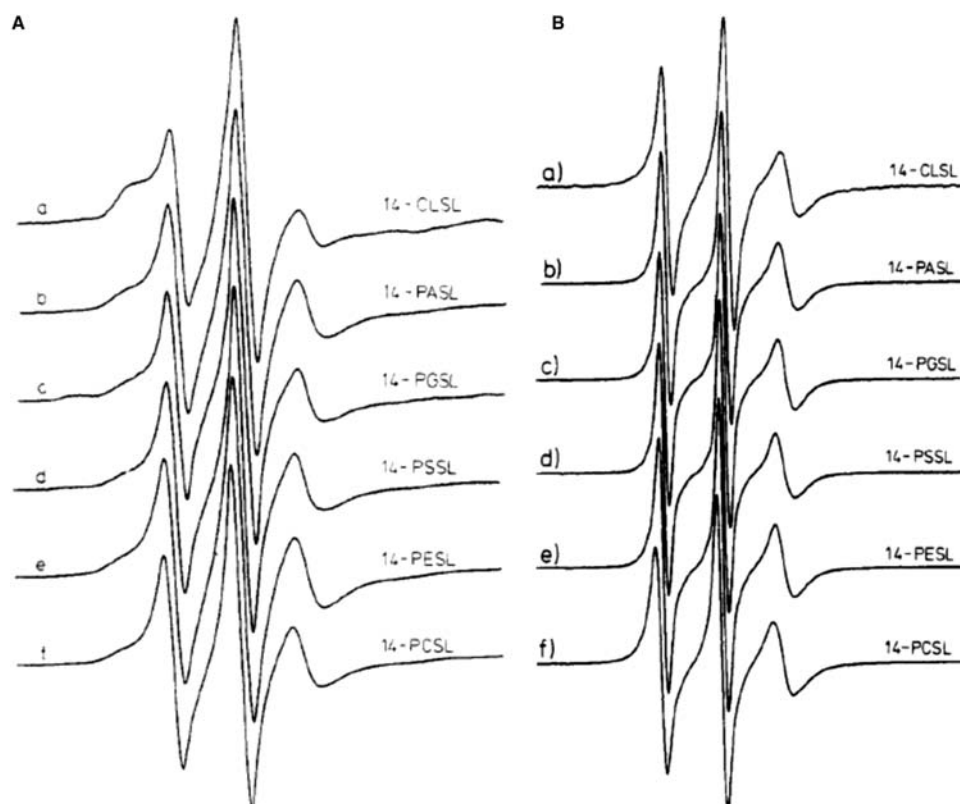
### Peripheral Proteins

A well-studied example of the application of spin label ESR spectroscopy to the study of peripheral protein interaction with membrane lipids is that of cytochrome c. This association is mediated predominantly by electrostatic interaction of the protein with anionic lipids such as phosphatidylglycerol and phosphatidylserine (Görrissen et al., 1986). On the other hand, the major protein of bovine seminal plasma, PDC-109, associates with lipid membranes by a specific association with the head group of the zwitterionic phospholipid, phosphatidylcholine and sphingomyelin (Ramakrishnan et al., 2001). These two examples are discussed in the following sections with the objective of highlighting the application of ESR spectroscopy to the study of such diverse modes of protein association with lipid membranes.

### Interaction of Apocytochrome c With Lipid Membranes

ESR spectra of spin-labelled phosphatidylglycerol (n-PGSL,  $n = 5, 8, 10$  and  $14$ ) incorporated into bovine phosphatidylserine membranes in the absence and in the presence of apocytochrome c are shown in Fig. 5. For all positional isomers of the spin labelled lipid (up to  $n = 10$ ), the spectral lines are broader in the presence of the protein as compared to those obtained in lipid alone and values of the outer hyperfine splitting increase. For 12-PGSL and 14-PGSL, in which the spin label is closer to the terminal methyl group of the acyl chain, the spectra contain two components with one component that is motionally restricted, whereas the other component is more mobile and fluid-like. The two components in the spectrum of 12-PGSL could be resolved by spectral subtractions (see Fig. 6), and the resulting immobilized component had an outer hyperfine splitting ( $2A_{max}$ ) of 56 G. This value is considerably smaller than the value of ca. 60 G observed for the motionally restricted component found with typical integral





**Figure 4** (A) ESR spectra of phospholipids with different head groups spin-labelled on the 14th C-atom of the sn-2 acyl chain, introduced exogenously into separate aliquots taken from the same cytochrome oxidase-dimyristoylphosphatidylcholine complex of lipid/protein ratio 95:1, at  $T = 32^\circ\text{C}$ . Corresponding spectra of the spin labelled phospholipids in DMPC alone are shown in (B). Total scan width = 100 G. Adapted with permission from Knowles, P.F., Watts, A., Marsh, D., 1981. Spin-label studies of head-group specificity in the interaction of phospholipids with yeast cytochrome oxidase. *Biochemistry* 20, 5888–5894. Copyright (1981) American Chemical Society.

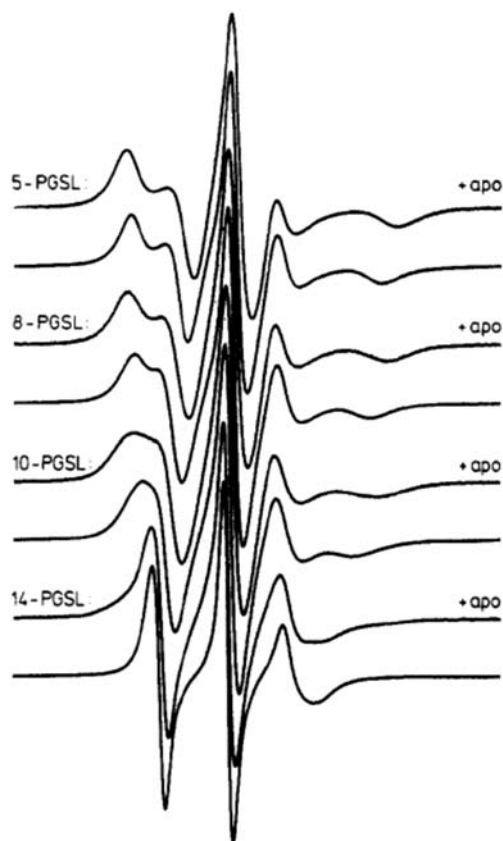
membrane proteins (Marsh and Watts, 1982). This observation is consistent with only a partial penetration of the protein segment into the hydrophobic core of the membrane. Further, at low lipid/protein ratios (that is, in the presence of excess lipid), approximately 5 lipids per protein were motionally restricted. This number compares reasonably well with the number of lipids that could be arranged around a cylinder of  $10\text{ \AA}$  diameter that penetrates only half of the bilayer. These dimensions are similar to those of an  $\alpha$ -helix and suggest that most likely a single  $\alpha$ -helical segment of the protein inserts into the lipid membrane.

### Interaction of PDC-109 With Lipid Membranes

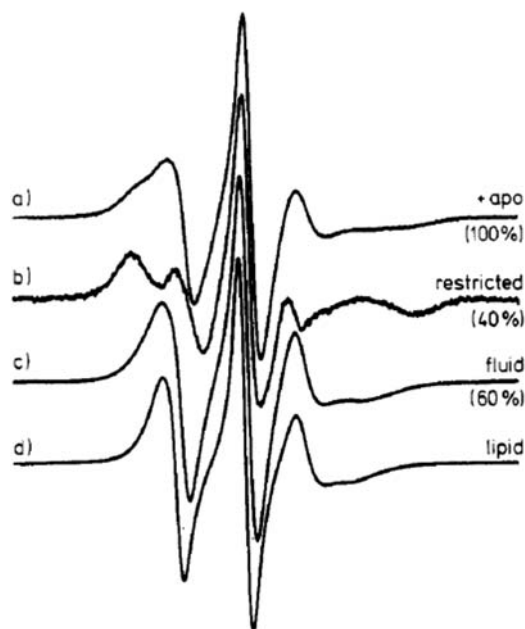
As indicated above, association of the major bovine seminal plasma protein, PDC-109 with phospholipid membranes is mediated by a specific binding of the protein with choline phospholipids such as phosphatidylcholine and sphingomyelin. Binding of PDC-109 to sperm plasma membranes results in an efflux of choline phospholipids and cholesterol. This process, referred to as cholesterol efflux, is crucial for priming the sperm cells for fertilizing the egg (reviewed in Swamy, 2004). In view of this, the interaction of this protein with membranes made up of DMPC and DMPC/cholesterol mixtures was investigated in the absence and in the presence of cholesterol by spin-label ESR spectroscopy (Ramakrishnan et al., 2001; Swamy et al., 2002).

ESR spectra of 5-PCSL in DMPC membranes in the absence and in the presence of PDC-109 are shown in Fig. 7A. Plots of the outer hyperfine splittings ( $2A_{\text{max}}$ ) for the two samples are shown in Fig. 7B. The spectra recorded in the absence of protein (dashed lines) exhibit a sharp change in the line shapes and outer hyperfine splittings ( $2A_{\text{max}}$ ) at  $\sim 24^\circ\text{C}$ , resulting from the increase in lipid chain mobility at the chain-melting phase transition. On the other hand, spectra recorded in the presence of PDC-109 (solid lines) show more gradual changes with increase in temperature. Importantly, at all temperatures the  $2A_{\text{max}}$  values obtained in the presence of the protein are higher than those obtained with lipid alone, suggesting that binding of PDC-109 makes the membrane more rigid.

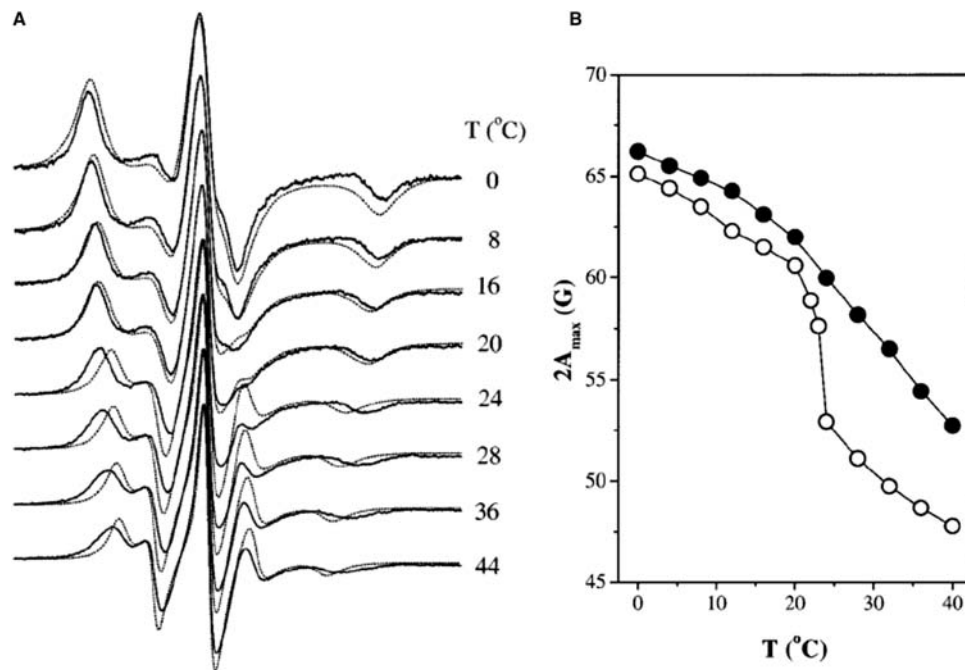
Since PDC-109 was able to induce efflux of lipids from sperm plasma membranes, it was expected that the protein most likely penetrates into the hydrophobic interior of the bilayer membrane. This was probed by employing spin labelled phosphatidylcholines (n-PCSL) and sterol probes (ASL and CSL) shown in Fig. 1. Spectra of these probes obtained at  $28^\circ\text{C}$  in the fluid phase of DMPC host membranes in the absence and in the presence of PDC-109 are shown in Fig. 8A and plots of the  $2A_{\text{max}}$  values obtained as a function of the chain position of the spin label are shown in Fig. 8B. Whereas the ESR spectra of spin-labels near the head group ( $n = 5$  and  $8$ ) are broadened by protein binding, possibly indicating the presence of overlapping components, those that are near



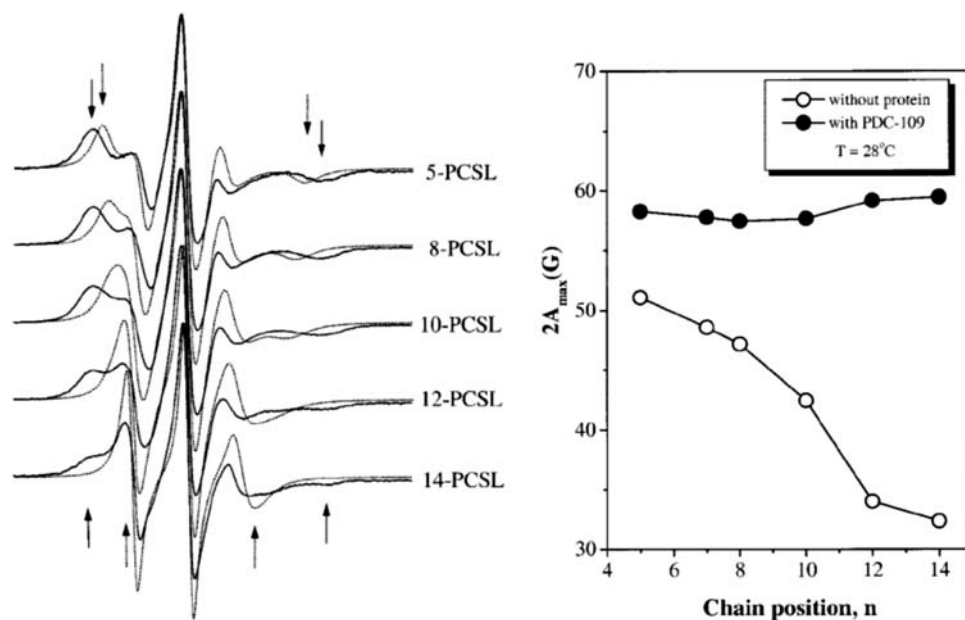
**Figure 5** ESR spectra of phosphatidylglycerol spin-label positional isomers n-PGSL in bovine phosphatidylserine dispersions in the presence and absence of apocytochrome c. The upper spectrum of each pair (+apo) is with a saturating amount of apocytochrome c bound (4:1 w/w added apocytochrome c), and the lower spectrum of each pair is from the lipid alone. Total scan width = 100 G;  $T = 30^\circ\text{C}$ . Adapted with permission from Görrissen, H., Marsh, D., Rietveld, A., de Kruijff, B., 1986. Apocytochrome c binding to negatively charged lipid dispersions studied by spin-label electron spin resonance. *Biochemistry* 25, 2904–2910. Copyright (1986) American Chemical Society.



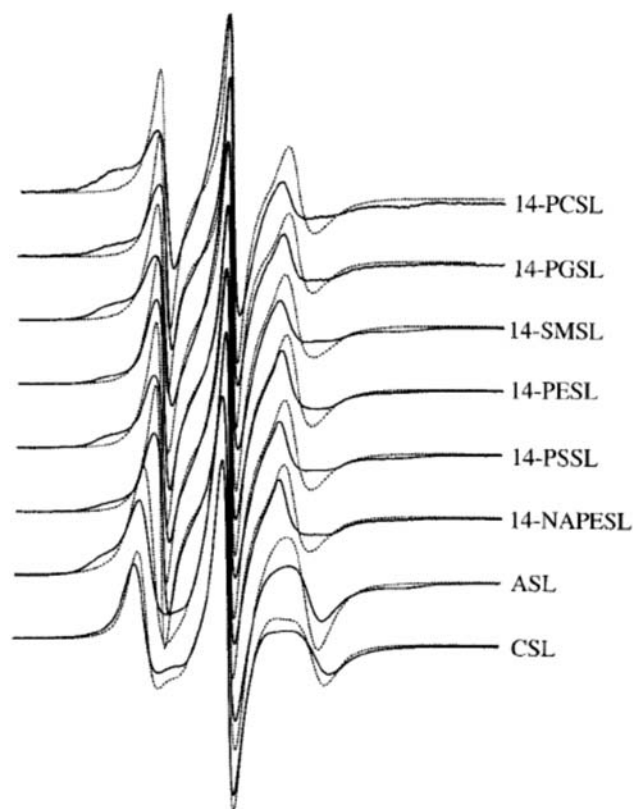
**Figure 6** Spectral subtraction of the composite ESR spectrum of the 12-PGSL spin-label in bovine PS dispersions in buffer in the presence of a saturating amount of apocytochrome c: (A) 12-PGSL in bovine PS + apocytochrome c,  $30^\circ\text{C}$ ; (B) difference spectrum obtained by subtracting 60% from the double-integrated spectral intensity of composite spectrum a, using spectrum c for subtraction; (C) fluid spectral component used for the subtraction from composite spectrum a (12-PGSL in bovine PS at  $22^\circ\text{C}$ ); (D) 12-PGSL in bovine PS alone.  $30^\circ\text{C}$ . Total scan width = 100 G. Adapted with permission from Görrissen, H., Marsh, D., Rietveld, A., de Kruijff, B., 1986. Apocytochrome c binding to negatively charged lipid dispersions studied by spin-label electron spin resonance. *Biochemistry* 25, 2904–2910. Copyright (1986) American Chemical Society. For a more detailed description of spectral subtraction method, see Section 5.1 in [Marsh \(1982\)](#).



**Figure 7** (A) ESR spectra of the phosphatidylcholine spin label, 5-PCSL, bearing the nitroxide moiety at the C-5 position of the sn-2 acyl chain, in DMPC membranes in the presence and absence of PDC-109 (lipid:protein, 1:2 w/w). Solid lines correspond to spectra recorded from DMPC/PDC-109 recombinants, and dashed lines correspond to the spectra recorded from DMPC membranes alone. The temperature at which the spectra were recorded is indicated in the figure. The spectral width is 100 G. (B) Temperature dependence of the outer hyperfine splitting,  $2A_{\max}$ , for the 5-PCSL spin label in DMPC membranes (○) and in DMPC/PDC-109 recombinants (●). Reprinted from Ramakrishnan M., Anbazhagan, V., Pratap, T.V., Marsh, D., Swamy, M.J., 2001. Membrane insertion and lipid-protein interactions of bovine seminal plasma protein PDC-109 investigated by spin-label electron spin resonance spectroscopy. *Biophys. J.* 81, 2215–2225. Copyright (2001), with permission from Elsevier.



**Figure 8** (A) ESR spectra of phosphatidylcholine spin labels, n-PCSL, at 28 °C in the fluid phase of DMPC membranes alone (••••) and in the presence of PDC-109 (—, lipid:protein, 1:2 w/w). Arrows indicate the outer hyperfine splitting,  $2A_{\max}$ . The spectral width is 100 G. (B) Positional dependence of  $2A_{\max}$  values in DMPC membranes (○) and in DMPC/PDC-109 (lipid:protein, 1:2 w/w) recombinants (●), at 28 °C in the fluid phase. The data given for n = 12 and 14 in DMPC/PDC-109 recombinants correspond to the motionally restricted component of the two-component spectra. Reprinted from Ramakrishnan, M., Anbazhagan, V., Pratap, T.V., Marsh, D., Swamy, M.J., 2001. Membrane insertion and lipid-protein interactions of bovine seminal plasma protein PDC-109 investigated by spin-label electron spin resonance spectroscopy. *Biophys. J.* 81, 2215–2225. Copyright (2001), with permission from Elsevier.



**Figure 9** ESR spectra of different phospholipid spin labels, 14-XXSL, bearing the spin label on the 14th C atom of the sn-2 chain, as well as the cholestane spin label (CSL) and androstanol spin label (ASL), in the fluid phase of DMPC membranes (.....) and of DMPC/PDC-109 (lipid:protein, 1:2 w/w) recombinants (—). Phospholipid spin labels are: 14-PCSL (phosphatidylcholine), 14-PGSL (phosphatidylglycerol), 14-SMSL (sphingomyelin), 14-PESL (phosphatidylethanolamine), 14-PSSL (phosphatidylserine), and 14-NAPESL (N-acyl phosphatidylethanolamine). Spectra were recorded at 28 °C. The spectral width is 100 G. Reprinted from Ramakrishnan, M., Anbazhagan, V., Pratap, T.V., Marsh, D., Swamy, M.J., 2001. Membrane insertion and lipid-protein interactions of bovine seminal plasma protein PDC-109 investigated by spin-label electron spin resonance spectroscopy. *Biophys. J.* 81, 2215–2225. Copyright (2001), with permission from Elsevier.

the chain end ( $n = 12$  and  $14$ ) clearly consist of two components. In particular, the spectrum of 14-PCSL closely resembles the spectra obtained with integral transmembrane proteins reconstituted into lipid membranes. The plots in Fig. 8B show that the values of  $2A_{\max}$  obtained for the lipid membranes alone decrease as the spin-label position is shifted down the sn-2 chain of the lipid, reflecting the chain flexibility gradient characteristic of fluid lipid membranes, whereas values of  $2A_{\max}$  for the motionally restricted component remain approximately constant, and are consistently larger than those obtained in pure lipid membranes, throughout the chain. These observations are consistent with a penetration of the protein segments into the membrane interior and their direct interaction with the lipid chains.

ESR spectra of different spin labelled phospholipids (14-XXSL, see Fig. 1) and sterol probes (CSL and ASL) in DMPC host membranes in the absence and in the presence of PDC-109 are shown in Fig. 9. Similar to what is observed with cytochrome oxidase, all spectra obtained in the presence of the protein show two components, a motionally restricted component and a more mobile, fluid-like component. However, analysis of the relative proportions of the two components (obtained by spectral subtraction) revealed that the motionally restricted component is highest for 14-PCSL and 14-SMSL, both of which have the choline moiety in the headgroup. The overall lipid selectivity of PDC-109 was found to be in the following order: phosphatidylcholine  $\approx$  sphingomyelin  $\geq$  phosphatidic acid (pH 6.0)  $>$  phosphatidylglycerol  $\approx$  phosphatidylserine  $\approx$  androstanol  $>$  phosphatidylethanolamine  $\geq$  N-acyl phosphatidylethanolamine  $\gg$  cholestane.

## Conclusions

Spin label ESR spectroscopy is a powerful technique which provided versatile information on protein-lipid interactions in membranes. In this chapter, several examples employing lipid spin labels are discussed which show that this technique can yield valuable information from rather simple and elegant experiments. The major results obtained in the studies discussed include: i) evidence for boundary lipid in the case of transmembrane proteins and the corresponding stoichiometry (i.e. the number of lipids surrounding each protein molecule), ii) lipid selectivity of different membrane proteins (both transmembrane and peripheral), iii)

evidence for partial membrane penetration by peripheral proteins, and iv) evidence for the abolition of lipid chain-melting phase transition by protein binding in specific cases. To get more detailed information about employing spin label ESR spectroscopy for the investigating protein-lipid interactions the reader is referred to the articles listed in the end of the chapter under 'Further Reading'.

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# Lipoprotein Lipase and Its Interactions With Phospholipids

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## Introduction

### Overview of the Developmental History of LPL

Lipoprotein lipase (LPL, EC 3.1.1.34) is an extracellular and multifunctional water-soluble enzyme that is produced by many tissues and plays a key role in lipid metabolism and transport especially in the catabolism of triglyceride-rich lipoproteins. It catalyzes the hydrolysis of a variety of acyl esters including the triacylglycerols (TAGs) of circulating very low-density lipoproteins (VLDL) and chylomicrons to provide free fatty acids and 2-monoacylglycerols for tissue utilization (e.g. for energy production or synthesis of other lipids) (Bensadoun, 1991). The discovery of the first LPL dated back to 1943 has been believed to be made by Paul Hanh who found that an intravenous injection of heparin abolished the lipaemia associated with a fatty meal (Hahn, 1943). Subsequent studies revealed the critical role of LPL as a clearing factor for the overall lipid transport and metabolism with apolipoprotein-CII (apoC-II) as the activator (Scanu, 1966), and the interactions of apolipoprotein-AV with the LPL to accelerate the hydrolysis process through guiding VLDL and chylomicrons towards LPL. To date, a lot of important roles of LPL, either catalytic or non-catalytic function, have been identified, and the consequences of LPL abnormalities including LPL overexpression or deficiency have been well documented such as the close association with a number of pathophysiological conditions such as atherosclerosis, chylomicronemia, obesity, Alzheimer's disease, diabetes, infection, and impairment in bone marrow myelopoiesis. Most of the disorders caused by LPL are associated with its genetic mutations. Currently, gene therapy places a focus on treating LPL deficiency e.g. the AAV1-LPLS447X gene therapy that has been found positive in humans (Gaudet et al., 2012).

### Occurrence and Structure of LPL

The activity of LPL has been reported in adipose, lung, cardiac, and skeletal muscle tissues as well as lactating mammary glands, brain, kidney, and macrophages of humans (Chajek-Shaul et al., 1985; Stegmayr, 2014) and animals such as rabbit, duck, goat, cattle, rat, chicken, bovine and sheep (Mahoney et al., 1982; Borensztajn, 1987).

LPL belongs to the lipase gene family of proteins comprising endothelial lipase, hepatic lipase, pancreatic lipase, and *Drosophila* yolk proteins (i.e., YP1, YP2, and YP3). This enzyme family has a high TAG esterase activity with varied phospholipase activity, although *Drosophila* yolk proteins lack lipase activity and obvious LPL functionality (even though share sequence similarity with LPL) (Pistillo et al., 1998).

LPL catalyzes the hydrolysis of TAGs, diacylglycerols (DAGs), phosphatidylcholines (PCs), and phosphatidylethanolamines (PEs) in plasma TAG-rich lipoproteins (Jackson et al., 1983). LPL is a polypeptide with 839 amino acids with four functional sites: (1) a glycosaminoglycan binding site that immobilizes LPL to the endothelial cell surfaces; (2) a binding site for the physiological activator of LPL catalysis, apoC-II; (3) an active or catalytic site; (4) a lipoprotein interfacial lipid-binding site. The lipolytic active site with conserved Ser-132, Asp-156 and His-241 triad inside the large N-terminus domain is important for lipolysis (McIlhargey et al., 2003). Advanced characterization techniques such as X-ray crystallography, nuclear magnetic resonance (NMR), analytical ultracentrifugation and isothermal titration calorimetry have been used to study the structure–function relationship of LPL and LPL's interaction with regulatory factors. Most recently, new models of LPL structure have been generated using disulfide mapping, computational modeling, and the data derived from the single-molecule Forster resonance energy transfer (sm-FRET) (Hayne et al., 2018).

### LPL Synthesis and Translocation

#### The Synthesis, Activation, and Secretion of LPL

In general, LPL is synthesized and secreted in the form of a glycosylated homodimer within the parenchymal cells of various tissues, and then translocated through the extracellular matrix to the luminal surface of vascular endothelial cells. LPL is first synthesized as an inactive monomer proenzyme in the rough endoplasmic reticulum (ER) and then turns into its active form after glycosylation in the presence of lipase maturation factor 1 (LMF1). That is, native LPL monomers in a head-to-tail subunit orientation are commonly converted to the noncovalent active homodimer prior to transport. The mechanism of glycosylation-related LPL activation is still under investigation, and different inhibitors seemed to exert different impacts: using tunicamycin as the inhibitor to block completely glycosylation resulted in an inactive non-secretable form of LPL whilst using menonsine as the inhibitor to glycosylation resulted in a slightly active non-secretable form of LPL (Chajek-Shaul et al., 1985). The monomers tend to aggregate rather than re-associate e.g. through rapid dissociation to misfolded monomers in the absence of LMF1 in the ER, and undesirable dissociation was found to cause loss of enzyme activity (Ben-Zeev et al., 2011). The active LPL enters the Golgi apparatus, with a part of the active LPL binding to a sortilin-related receptor with type-A repeats (SorLA) for further modifications, sorting, and packaging, and the rest of the active LPL binding to heparan sulfate-proteoglycans (HSPG) on the parenchymal cell surface. Some of the synthesized LPL



undergo degradation e.g. the lysosomal degradation prior to their secretion, probably as a result of the cell/tissue-specific differences in the turnover rate of LPL.

### Translocation of LPL

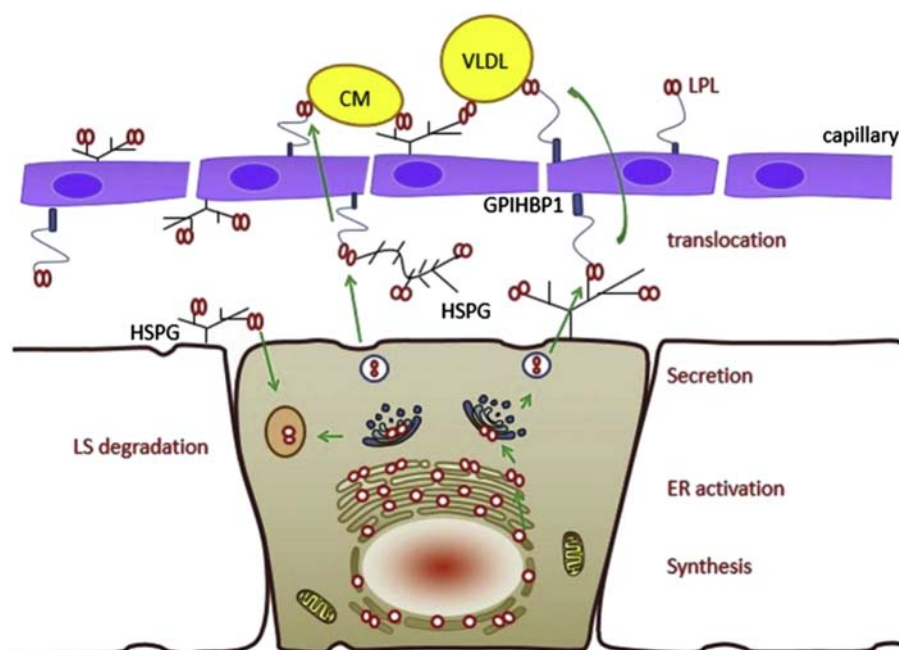
LPL has been thought to bind to the HSPGs on the luminal surface of the endothelial cells where the hydrolysis of TAG-rich lipoproteins normally takes place (Obunike et al., 2001). Recent studies indicated that the role of a glycosylphosphatidylinositol-anchored protein named “glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1” (GPIHBP1) as the binding site for LPL in the capillary lumen, a “platform for lipolysis”, should also deserve attention (Meneghetti et al., 2015). The exact mechanism of LPL transport remains unclear, although transcytosis of LPL across endothelial cells involving parenchymal heparan sulfate-proteoglycans (HSPGs) and the VLDL receptor has been believed the characteristic process. LPL can be released from the parenchymal HSPGs via the catalysis by the heparanase from the endothelial cells, which may either enter the circulation or bind to the oligosaccharides and transport to the endothelial cells due to the polarity of endothelial cells. The endothelial cells release two forms of heparanase: active and the latent form. The active form releases LPL from the myocyte surface whilst the latent form stimulates intracellular LPL through RhoA activation mediated by HSPGs (Li et al., 2014). Sub-endothelial matrix isolates and stabilizes the LPL, Fig. 1 summarizes the synthesis, activation, secretion, and translocation of LPL.

### Functions of LPL

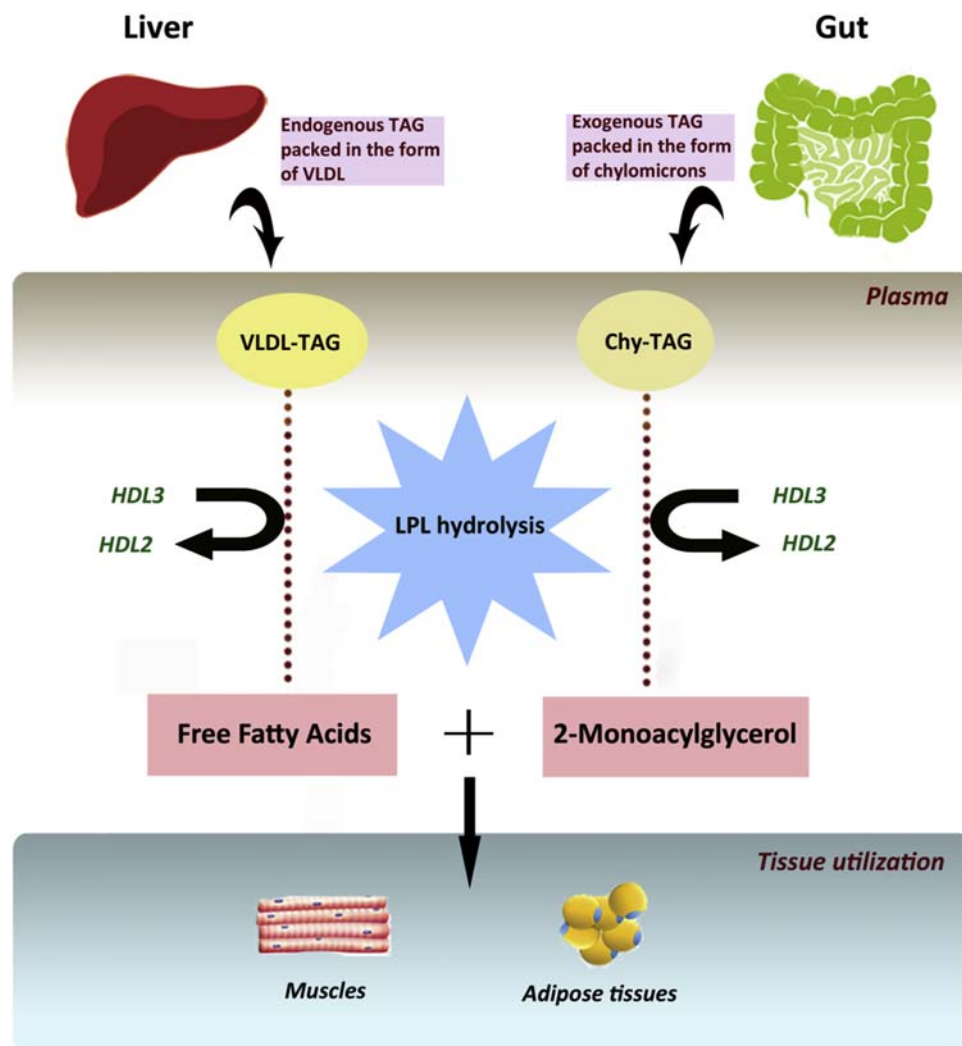
LPL is a rate-limiting enzyme that plays a major role in lipid metabolism and transport e.g. VLDL and chylomicron catabolism and transport of phospholipids, apolipoproteins, and cholesterol among lipoprotein particles. LPL exerts dual functions as triglyceride hydrolase and ligand/bridging factor for receptor-mediated lipoprotein uptake. VLDL can be converted to IDL and then to LDL via LPL catalysis. The hydrolysis of the TAG component of chylomicrons and VLDL catalyzed by LPL results in non-esterified fatty acids and 2-monoacylglycerol, which are utilized by different tissues in many ways e.g. the non-esterified fatty acids produced can be re-esterified for storing energy in the form of the TAG. LPL can simultaneously bind to both the lipoproteins and the cell surface receptors or proteoglycans to form bridges in the absence of another catalyst, allowing accumulation and cellular uptake of lipoproteins.

### LPL-Catalyzed Hydrolysis of VLDL-TAG and Chy-TAG

The exogenous and endogenous TAGs are transported and distributed in the circulation in the form of large multi-molecular lipoprotein particles. The exogenous dietary TAGs and fat-soluble vitamins are packed into the chylomicrons synthesized by the mucosal cells of the intestine, while the endogenous TAGs secreted by the liver are packed in the form of VLDL. Chylomicrons



**Figure 1** A diagram representing the synthesis, activation, secretion, and translocation of LPL. After synthesis and activation in the ER, LPL enters the Golgi apparatus where it is either secreted or undergo lysosomal degradation following which LPL binds to the HSPGs and translocated to the abluminal side of endothelial cells. Finally, LPL is transported to the luminal surface via transcytosis process where it carries out hydrolysis with the help of GPIHBP1. Adapted from Li et al. (2014).



**Figure 2** The metabolism of lipoproteins involving LPL and the resultant formation of free fatty acids and 2-monoacylglycerol for tissue utilization.

and VLDL enter the blood circulation by which TAGs are distributed to tissues (Fig. 2). The lipoproteins can deliver lipids to specifically target tissues in which apolipoproteins can act as either a cofactor for cell surface lipases (such as LPL) or a ligand for cell surface receptors. Thereby, monitoring lipoproteins can prevent unnecessary dispersal of lipids during transport through exchange or diffusion.

Besides the well-known catalyst role for TAG hydrolysis, LPL has a range of other physiological functions: LPL expressed in heart and skeletal muscles mainly catalyzes the lipolysis of plasma lipoproteins and produces free fatty acids for oxidation (providing energy); LPL also transports cholesterol and vitamin E to the neurons thus assists in recycling and scavenging of lipids released from the degenerating nerve; More recently, hypothalamic LPL has been found to assist in ceramide distribution, glucose metabolism and regulation of body weight.

### Mechanistic Action of LPL on Lipid Interface or Phospholipids

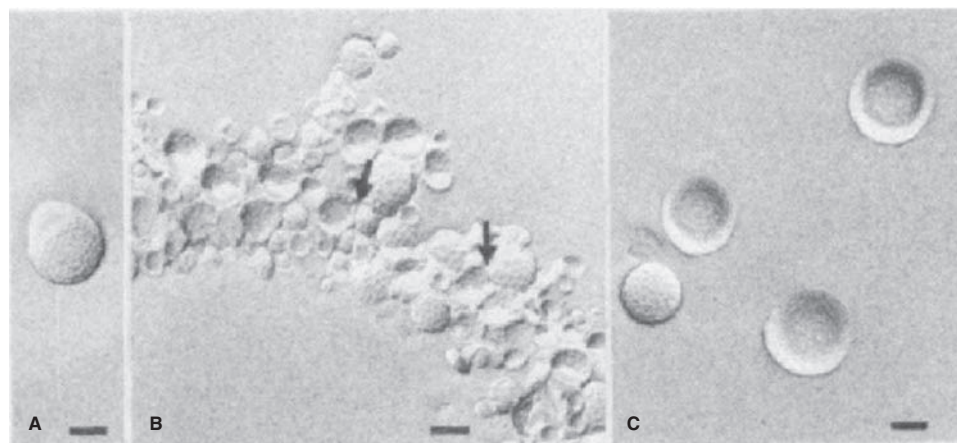
Phospholipids consist of two fatty acids chain and are the key component of almost all the biological membranes (including cells and cellular components). The intake of PL is estimated to be 1%–10% of total fat consumption. Foods such as eggs, fish, shellfish, animal organs, lean meats, dairy products, cereal grains and oilseeds contain a high PL content, while some leafy vegetables, fruits, and tubers also contain small amounts of PL. The fatty acid composition of PLs varies with their origin. PC is the most common PL in foods with other PLs such as PE, phosphatidylserine (PS) and phosphatidylinositol (PI) present at much lower levels. Moreover, PLs are commonly used ingredients/additives in food, pharmaceutical, nutraceutical and cosmetic industries for manufacturing products like dairy foods, baked goods, chocolate, and confectionery.

PLs are digested and absorbed in the small intestine in ways different from TAGs. Unlike TAG, PLs do not need bile salts for emulsification and lingual or gastric lipases for hydrolysis, and can form micelles by themselves in the digesta and be hydrolyzed

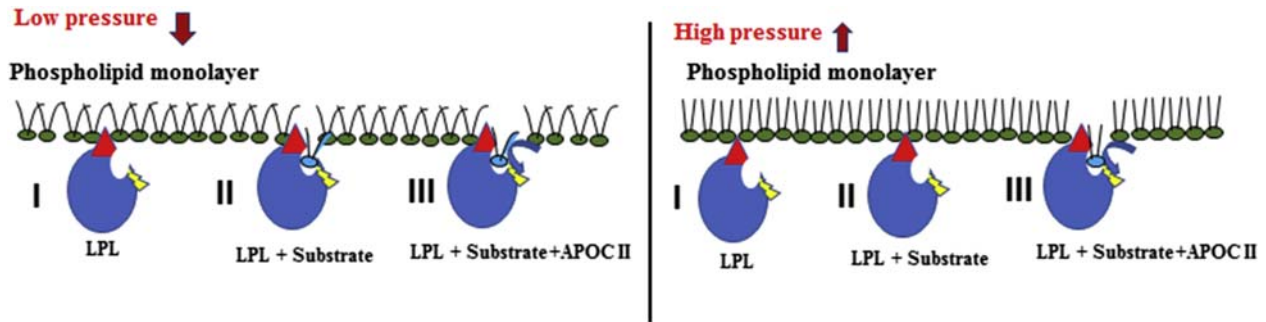
directly by enzymes in the small intestine (where they are almost absorbed and then enter in plasma lipoproteins and red blood cells). PC is the most common PL present in the intestinal lumen and represent the second most abundant lipid in the digestive tract after TAGs, with other PLs like PE, PS, and PI of much lower contents (Murru et al., 2013). In the lumen, chemical breakdown of most PLs via hydrolysis at the sn-2 position is mainly catalyzed by pancreatic phospholipase A2 (PLA<sub>2</sub>) and other lipases secreted by the pancreas, to yield free fatty acids and lyso-PLs. Both components are then taken up by mucosal cells. The type of PL including fatty acid chain length and unsaturation number determines PL's digestion, absorption, transport, and metabolism e.g. PLs containing medium-chain fatty acids tend to be absorbed better than those with long chain fatty acids (Ramirez et al., 2001). Lyso-PLs and some free-FAs may be re-esterified to newly-formed PLs (while some free FAs may bind to for new TAGs) and enter the bloodstream incorporated into the surface layer of chylomicrons. A small proportion of PLs may also incorporate into very low-density lipoproteins (VLDL). In the meantime, co-occurring TAG-rich particles are incorporated into the core of chylomicrons and then degraded, resulting in a proportion of chylomicron PLs especially PC to be transferred to the high-density lipoprotein (HDL) (a process takes place within 5–6 hours of PLs ingestion with about 20% of intestinal PLs being incorporated directly into HDL) (Burri et al., 2012) (Burri et al., 2012). Via HDL, PLs are then transferred into cells of different tissues and organs such as brain, liver, muscle, kidneys, heart, and lung including blood cells such as platelets and erythrocytes (Burri et al., 2012; Hussain, 2014). Accordingly, PLs may deliver health-beneficial polyunsaturated fatty acids more efficiently than TAGs.

Phospholipids are capable of shield other lipid species from aqueous environments via the formation of a mixed monolayer with proteins such as apolipoproteins. Moreover, LPL can catalyze the hydrolysis of TAGs and DAGs, PCs, and PEs in plasma triacylglycerol-rich lipoproteins, as well as in emulsion-like systems (McLean et al., 1986). Thus, it is of high interest to examine the interactions with LPL with PLs. However, very few studies have been performed to examine the interaction of LPL with PLs, because the detailed molecular organization of the lipoprotein substrate for LPL has not been elicited. Only a few studies investigated this aspect and in which PC was used singly as the major lipid component of the surface of the lipoprotein. McLean, Larsen et al. (1986) studied the interaction of LPL with a nonhydrolyzable PC, 1,2-ditetradecyl-rac-glycero-3-phosphocholine (C14-ether-PC) using physical methods: Analysis of LPL by circular dichroism spectroscopy revealed that its conformation contained 35%  $\alpha$ -helix, 30%  $\beta$ -pleated sheet and 45% other structures, and no significant change was found in LPL's conformation upon addition of C14-ether-PC vesicles. Addition of LPL to sonicated C14-ether-PC vesicles containing entrapped carboxy fluorescein caused the release of less than 15% of the vesicle contents in 20 min, indicating that LPL hardly disrupt the bilayer structure of the lipid (as observed by freeze-fracture electron microscopy, Fig. 3). However, the addition of apolipoprotein A-I to such a vesicle preparation caused the release of more than 80% of the vesicle contents. Further, at a low ionic concentration (0.1–0.25 M NaCl) significant aggregation of intact vesicles was observed by light scattering and electron microscopy (Fig. 3B (as indicated by arrows)). Other studies also found that vesicle aggregation could be prevented and reversed by 1 M NaCl or heparin, and LPL may bind as a dimer to the surface of a lipid interface e.g. the endothelial cell surface (Garfinkel et al., 1983) and to heparin and lipid droplet (Olivecrona et al., 1985), without causing dramatic changes in lipid bilayer or protein structure.

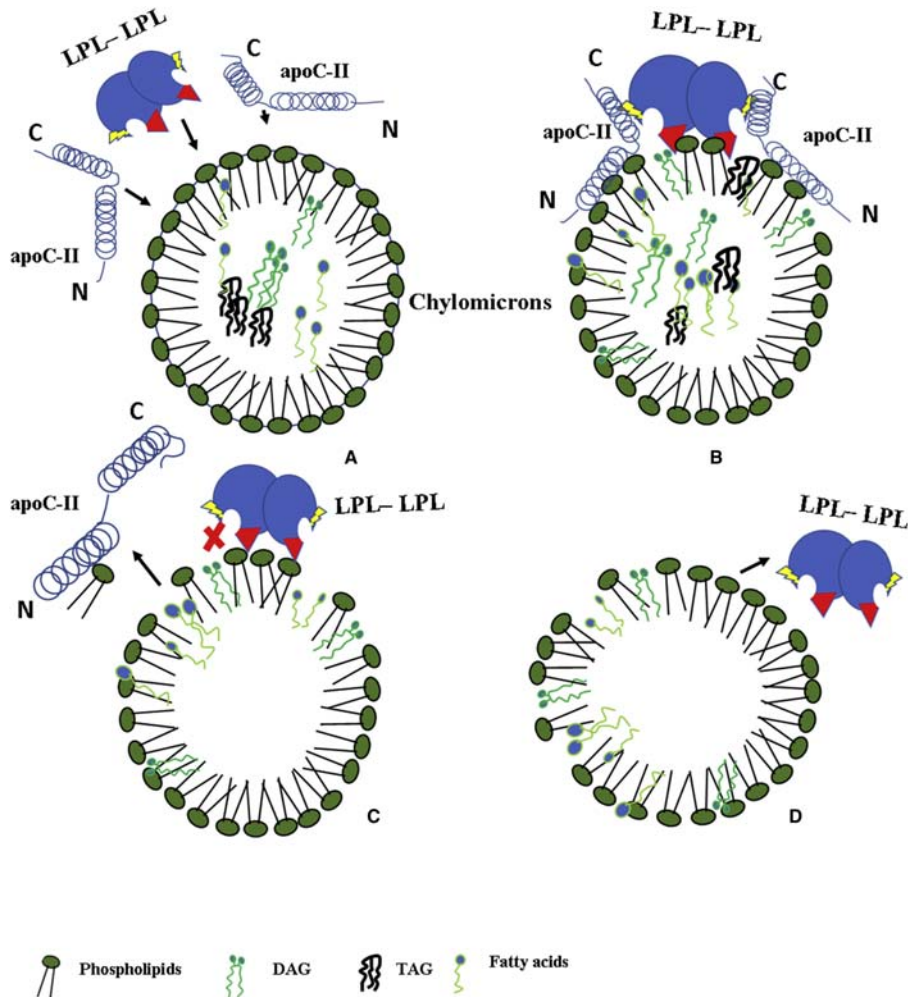
The function of LPL and its activation by apoC-II were studied with monomolecular films of 1,2-didodecanoyl-*sn*-glycero-3-phosphoglycerol as a substrate. The enzymatic velocity and specific activity of the interface-bound enzyme showed a bell-shaped curve as a function of lipid packing, in the presence and absence of apoC-II. Above the critical surface pressure of 20 dyn cm<sup>-1</sup>, LPL alone was found not able to hydrolyze a monolayer of 1,2-didodecanoyl-*sn*-glycero-3-phosphoglycerol, but could readily penetrate the phospholipid interface up to surface pressures exceeding 40 dyn cm<sup>-1</sup>, without any effect by apoC-II (Fig. 4). Below the critical surface pressure of 20 dyn cm<sup>-1</sup>, apoC-II merely increased the turnover number of LPL by 4-fold. Lambert et al. (2000) reported that the longer the carboxyl-terminal fragment of apo C–II was, the greater was the activation. The PL hydrolysis activity



**Figure 3** Freeze-fracture electron microscopy of C14-ether-PC vesicles with and without LPL: (a) C14-ether-PC vesicles in 0.25 M NaCl (500  $\mu$ g of PC/mL); (b) C14-ether-PC vesicles (500  $\mu$ g of PC/mL) plus LPL (250  $\mu$ g/mL) in 0.25 M NaCl; (c) sample B in 1 M NaCl. The final glycerol concentration was 25%. Magnification: (a) and (b) 30840  $\times$ ; (c) 31480  $\times$ ; scale bar = 0.1  $\mu$ m. Adapted from McLean et al. (1986).



**Figure 4** Action of lipoprotein lipase (LPL) on monolayer of phospholipids (PLs), in the presence of low or high pressure, in low-pressure monolayer packing is loose, easily access the substrate without apolipoprotein C-II (apoC-II), and in high pressure, difficult access the substrate without apoC-II, and apoC-II assists in the penetration of PL monolayer layer. Lipid binding sites: red triangles; apoC-II binding sites: yellow Zig-Zag; substrate: Cyan; apoC-II activator: Blue, curved arrow.



**Figure 5** A model action of lipoprotein lipase (LPL)-dimer on chylomicrons: (A) LPL-dimer and apoC-II activator approaching the substrate; (B) LPL-dimer act on the phospholipid (PL) monolayer, but cannot access the substrate, when apoC-II binds to LPL and gets access to the substrate; (C) when activator apoC-II dissociates to the LPL, it loses activity or the hydrolysis stops; (D) Finally, LPL-dimer leaves the substrate.



represented, in the presence of apo C-II, 36% of the TAG hydrolysis activity. PL hydrolysis is less dependent on the activator than TAG hydrolysis (100% and 300% of increase with apo C-II for PC and TAGs, respectively). The mechanism of hydrolysis activated by apoC-II or the synthetic apo C-II fragments seems to be the same as that for TAGs, as each synthetic apo C-II fragment present the same percentage activation in comparison with the whole apoC-II, irrespective of the substrates. The carboxyl-terminal 30 amino-acid fragment of intact apo CII can activate LPL, suggesting that other elements beyond the carboxyl-terminal 30 amino-acid fragment are necessary for the binding of apo C-II to LPL and its full activation. Moreover, expression of LPL with catalytic activity can be regulated by the physical state of the substrate interface. ApoC-II can affect this regulation. The requirement for the presence of PL-associating residues in apoC-II would be due to that the activator either alters PL structure or changes the orientation of LPL at the interface. Further, if apoC-II could change PL structure, the presence of the lipid binding region between residues 43 and 50 would probably affect LPL activation at low lipid packing. The two proposed activation mechanisms — increased turnover and assisted penetration are by no means mutually exclusive. Under certain conditions, they may well be working simultaneously, with the latter likely being a prerequisite for the former to occur. It is worth noting the hypothesis that LPL is not interacting directly to PLs, LPL assisted by lipoprotein apoC-II which gives more grip on lipoprotein particles (such as chylomicrons) during hydrolysis of substrate and apoC-II interacts with PLs via amphipathic interactions, which would afford greater stability to LPL (Fig. 5). In summary, the presence of two cooperative activation mechanisms would secure the catalytic efficiency of LPL under dynamically changing interfacial conditions during the hydrolysis (Vainio et al., 1983)

## Conclusions

LPL is an important and versatile enzyme that influences human metabolism and health. The understanding of the synthesis, structural characteristics, physiological and physical behaviors and modes of catalytic action (including substrate specificity, cofactor requirements, and working system/location) of LPL would support LPL monitoring and utilization. Although PLs are generally considered a minor component of foods, PLs are key components of cellular membranes. The interactions of both bioactive substances, LPL, and phospholipids, thus represent an important research topic, and the obtained knowledge would benefit the food, pharmaceutical, nutraceutical and cosmetic industries.

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# Reactivity of Lipid Oxidation Products in Foods – Is Malondialdehyde a Reliable Marker?

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## Introduction

Lipid oxidation is one of the major causes of food quality deterioration responsible for the generation of off-flavors (rancidity) and undesirable changes of color, texture and nutritive value. In some foods, a limited amount of lipid oxidation is desirable because it leads to the generation of desirable aroma substances; such as 2,4-decadienal, hept-4-enal and non-2-enal (Zehentbauer and Rein-eccius, 2002). Furthermore, the oxidation of unsaturated lipids results in the generation of toxic compounds which have been associated with cardiovascular diseases, as well as cancer, atherosclerosis, aging, etc (Negre-Salvayre et al., 2008; Ayala et al., 2014).

It is generally accepted that lipid oxidation starts with the transformation of unsaturated lipids by oxygen-active species to lipid hydroperoxides, which are considered as the primary lipid oxidation products. These lipid hydroperoxides can be generated via three different pathways: (1) via a free radical chain mechanism or autooxidation; (2) via photooxidation, requiring the presence of visible light and a photosensitizer (e.g. riboflavin, chlorophyll); or (3) via an enzymatic reaction requiring the presence of lip-oxygenases. Hydroperoxides are unstable molecules. They may induce oxidation reactions and generate the formation of lipid radi-cals that may further support the oxidation process or may be converted to a variety of so-called secondary lipid oxidation products, including carbonyl compounds (aldehydes, ketones, oxo-fatty acids) alcohols, epoxides, dimers, polymers, among others (Belitz et al., 2009; Schaich, 2005). Typically, carbonyl compounds have been related to rancidity and used as markers for lipid oxidation (Ross and Smith, 2006; Panseri et al., 2011; Pignoli et al., 2009), although recently Mubiru et al. revealed the unexpected high content of epoxy fatty acids in a variety of fresh oils with a low hydroperoxide value suggesting that some of the lipid oxidation markers already used for decades are potentially not always reliable (Mubiru et al., 2013). A typical example of such an oxidation marker used to assess lipid oxidation in foods and other biological matrices is malondialdehyde (Requena et al., 1996; Del Rio et al., 2005).

Malondialdehyde (MDA) is generally recognized for its generation during the oxidation of  $\omega$ -3 and  $\omega$ -6 fatty acids. It is a three carbon dialdehyde mainly present as its enol in aqueous and organic solutions (George and Mansell, 1968; Xu et al., 2015). Though, in aqueous solutions, MDA can exist in various forms depending on the pH (as shown in Fig. 1). The pK<sub>a</sub> of the enolic OH group is 4.46. Therefore, under neutral and alkaline conditions the predominant form of MDA is the enolate anion, whereas at acidic pHs, MDA mainly exists in its undissociated enol in equilibrium with the dicarbonyl form (Esterbauer et al., 1991). Besides, the Z-isomer of the enol can stabilize itself via the formation of an intramolecular hydrogen bond resulting in a chelated form

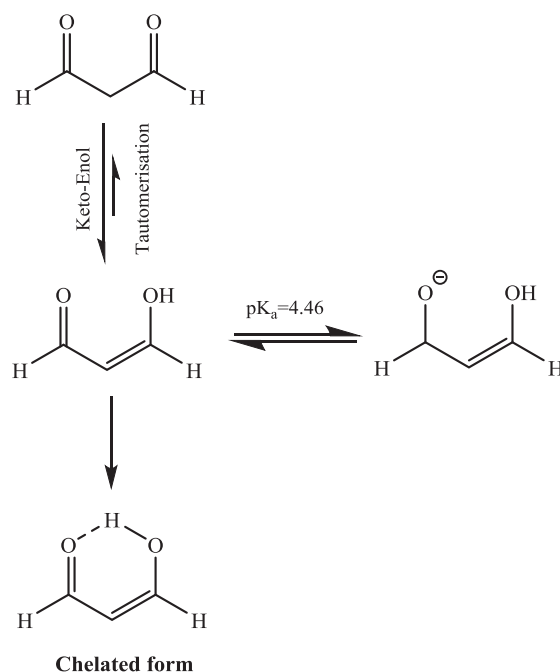


Figure 1 Behavior of MDA in aqueous solutions.



(Freitag et al., 2007). However, in aqueous solutions the relative stability of the intramolecular hydrogen bond diminishes because of competing hydrogen bond formation with the solution (Xu et al., 2015; Freitag et al., 2007). Hence, in aqueous solutions the reactivity of MDA depends on the pH, in contrast to oils in which MDA is produced. In oil-in-water (O/W) emulsions, however, MDA can partition between the oil and aqueous phase depending on the nature of its molecular environment (e.g. the used emulsifier type, droplet size, oil content, etc.) (Berton-Carabin et al., 2014; Osborn and Akoh, 2004; McClements and Decker, 2000; Lethuaut et al., 2002). Vandemoortele et al. showed that, after spiking MDA into the aqueous phase, 36% of the MDA was translocated to the oil and/or interface of the O/W emulsion (Vandemoortele and De Meulenaer, 2015; Vandemoortele et al., 2017).

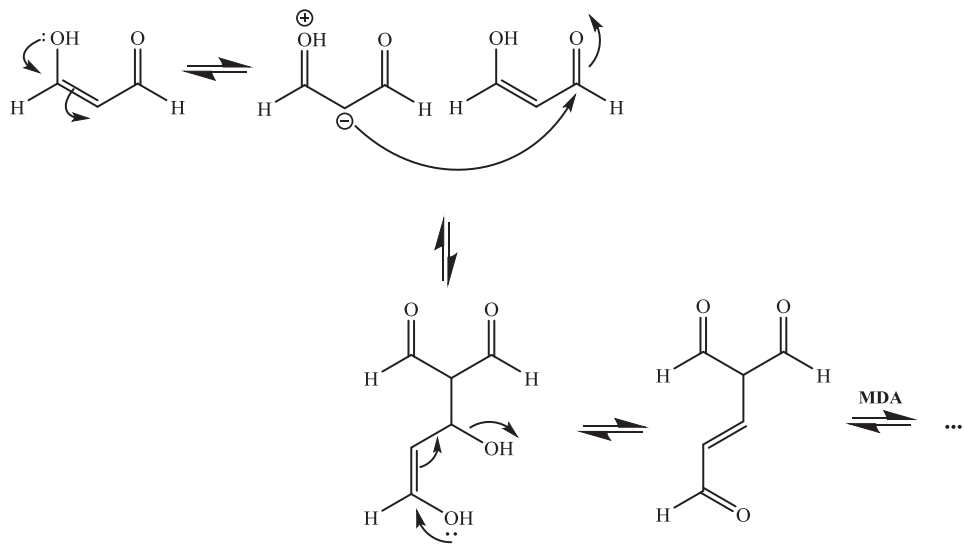
Several methods have been developed to assess the MDA content, including spectrophotometric or fluorimetric techniques, high performance liquid chromatography (HPLC), gas chromatography and immunological techniques (Del Rio et al., 2005). The most common method for MDA determination in foods is the spectrophotometric measurement of the pink-colored fluorescent MDA-thiobarbituric acid (TBA) complex (Botsoglou et al., 1994). However, the spectrophotometric TBA method has been criticized because of the lack of sensitivity, since TBA reacts not only with MDA but also with many other compounds (e.g. carbohydrates, amino acids, pyridines, other secondary lipid oxidation products, etc.), resulting in considerable overestimation of the MDA content (Mendes et al., 2009; Papastergiadis et al., 2012). Therefore, chromatographic techniques (e.g. HPLC-fluorescence detection) are preferred to quantify the MDA content due to their high separation power, specificity and accuracy (Grotto et al., 2009). The aim of this review is to evaluate critically the general applicability of MDA as marker for assessing lipid peroxidation in foods. Thereby, no summary will be made about the plurality of methods for analyzing the MDA content in foods, as it has been reported before (Del Rio et al., 2005; Grotto et al., 2009; Barriuso et al., 2013). Though, the focus will be on the stability of MDA in foods and the subsequent consequences for the MDA determination via HPLC-fluorescence detection of MDA-TBA adducts (the TBA-MDA assay).

## Reactivity of MDA

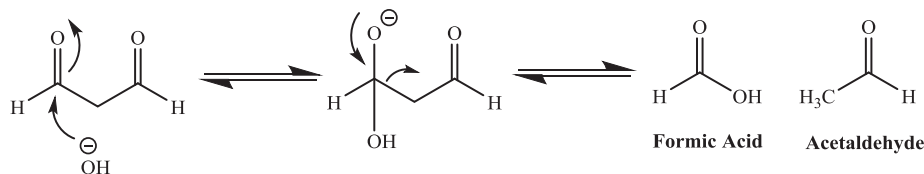
### Aldol Self-condensation and Hydrolytic Cleavage of MDA

MDA can undergo aldol self-condensation and hydrolytic cleavage under physiological conditions (aqueous medium, pH near neutrality, room temperature) (cf. Fig. 2). The aldol self-condensation of MDA leads to the formation of acid labile dimers and

#### A Aldol self-condensation of MDA



#### B Hydrolytic cleavage of MDA



**Figure 2** Reaction mechanism of (A) the aldol self-condensation and (B) hydrolytic cleavage of MDA.

oligomers and is restricted to pH 5–7 (Esterbauer et al., 1991; Gómez-Sánchez et al., 1990; Janero, 1990). MDA condensation products have been reported to be approximately equipotent to MDA with respect to their mutagenicity (Niedernhofer et al., 2003; Riggs and Marnett, 2001). Moreover, these condensation products of MDA are suspected to be involved in protein modifications (Esterbauer et al., 1991; Janero, 1990), complementary to MDA as will be discussed in detail below. In addition, hydrolytic cleavage of MDA can occur (pH 4–7), generating formic acid and acetaldehyde (Vandemoortele et al., 2017; Gómez-Sánchez et al., 1993). Multiple studies have shown that acetaldehyde is mutagenic and even carcinogenic (Seitz and Meier, 2007; Salaspuuro, 2009). Its interaction with a variety of proteins resulted in the formation of protein-acetaldehyde adducts that may interfere with the biological functions of the modified protein (Tuma et al., 1996; Thiele et al., 2001). Vandemoortele et al. reported that both the aldol self-condensation and hydrolytic cleavage of MDA can occur in a lipid medium. Moreover, the aldol self-condensation of MDA was very dependent on the storage and operating temperature of the oil and increased dramatically with increasing temperature. About 1.5% and 68% of the added MDA underwent aldol self-condensation at 4 and 40 °C, respectively, after 24 h. Though, no temperature effect was observed on the hydrolytic cleavage of MDA in oil (which accounted for 7% loss). Surprisingly, greater hydrolytic cleavage was found in a lipid medium than in an aqueous buffer (pH 6.8) (cf. about 3% of the added MDA). In aqueous buffer (pH 6.8), the main reaction route was the aldol self-condensation of MDA (cf. 5% of the added MDA at 4 and 40 °C) (Vandemoortele and De Meulenaer, 2015; Vandemoortele et al., 2017).

### Interaction Between MDA and Food Proteins

Lipid peroxidation and protein oxidation are often addressed together. Oxidative damage to protein may be induced indirectly by reaction with lipid oxidation products in general, and by lipoperoxidation-derived  $\alpha,\beta$ -unsaturated aldehydes in particular. These aldehydes are able to bind spontaneously to proteins, causing protein modification (e.g. cross-linking, aggregation) and generation of adducts (termed “advanced lipid peroxidation end products” or “ALE”) (Domingues et al., 2013).

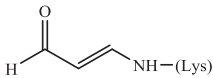
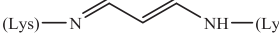
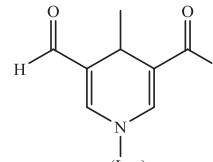
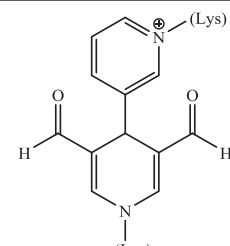
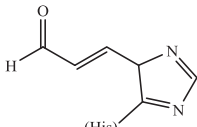
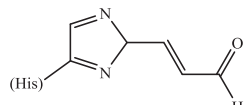
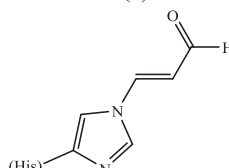
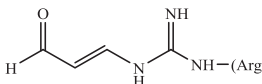
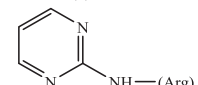
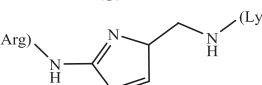
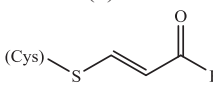
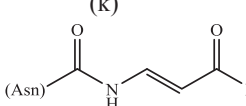
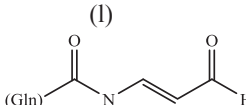
The interaction of different proteins with MDA under physiological conditions has been shown to generate potentially various toxic adducts and cause cross-linking of proteins (Domingues et al., 2013; Uchida et al., 1997; Refsgaard et al., 2000). Table 1 provides an overview of the different MDA-protein adducts.

The predominant derivatives resulted from the reaction of MDA with proteins have been identified as MDA-lysine adducts. The reaction of MDA with the free amino groups of lysine residues leads to the formation of imines, specifically N<sup>ε</sup>-(prop-2-enal)lysine derivatives or enaminals (a) (Uchida et al., 1997; Ishii et al., 2008). These adducts have been detected as the major forms in which endogenous MDA is excreted in rat and human urine (Draper et al., 1984; Draper et al., 1988). Because of the bifunctional nature of MDA, nucleophilic addition of a second free amino group of lysine can occur, generating a 3-amino-1-iminopropene cross-link (b) (Slatter et al., 2000). Formation of such iminopropene cross-links in an *in vitro* reaction has been reported for polylysine and human low-density lipoprotein (Requena et al., 1997). In the presence of alkanals, MDA addition can generate fluorescent adducts, 4-substituted 1,4-dihydropyridine-3,5-dicarbaldehyde derivatives (DHP-type adducts), with lysine. For example, MDA and acetaldehyde (derived from MDA cleavage *in vitro*) can react with each other in a concerted manner forming 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde (c) (Tuma et al., 1996; Mooradian et al., 2001). These DHP-type adducts can induce further cross-linking, leading to the formation of another fluorescent adduct containing dihydropyridine and pyridinium rings (pyridinium DHP (d)), although, these pyridinium DHP adducts were not detected on protein or peptide level (Itakura et al., 1996).

In addition to these adducts, MDA may provide many other derivatives upon reaction with the side-chain groups of histidine, arginine, cysteine, asparagine and glutamine as well as the N-termini of peptides. Modification of the imidazole group of histidine by MDA to various imine derivatives (e–g) has been reported (Slatter et al., 2004; Zhao et al., 2012). Besides, MDA imine formation at the side chain of arginine (h) has been identified on peptide level (Zhao et al., 2012). Also the conversion of the guanidine group of arginine to a 2-aminopyrimidine residue by MDA (i) under strong acidic conditions has been described in peptides and proteins (Foettinger et al., 2006). Moreover, Slatter et al. reported the feasibility of a MDA-derived cross-link formed between arginine and lysine, giving 2-ornithinyl-4-methyl(1<sup>ε</sup>-lysyl)1,3-imidazole (j) (Slatter et al., 2004). Buttkus demonstrated that MDA could react with the sulfhydryl groups of cysteine (k) (Buttkus, 1969). A decrease in the sulfhydryl groups of soy proteins was found upon the increase of the MDA level (ratio MDA/free sulfhydryl groups: 0.0025–2.5 mol mol<sup>−1</sup>) (Wu et al., 2009). The reactivity of asparagine and glutamine with aldehydes is expected to be low due to the reduced nucleophilicity of the amino group in the carboxamide residue. Nevertheless, MDA imine formation at the side chain of both amino acid residues (l, m) was detected (Zhao et al., 2012).

Interaction of MDA with food proteins during processing and storage can lead to a decrease in the nutritional quality of proteins (e.g. damage to amino acid residues) and a change in their physicochemical properties. Progressive conjugation or cross-linking by MDA can cause protein browning, protein denaturation, polymerization, aggregation, and loss in solubility (Wu et al., 2009; Traverso et al., 2004; Adams et al., 2008). Protein aggregation can lead to a decrease in proteolytic susceptibility and also affect the digestibility of proteins (Chen et al., 2013; Obando et al., 2018). Further, enzymes containing reactive amino acid groups can be inactivated (Tironi et al., 2002). Protein immunogenicity and allergenicity could also be reduced due to MDA-induced conformational changes (Song et al., 2015; Nikolić et al., 2017). Thus, MDA-protein interaction is of biological importance because of the induced profound alteration in protein biochemical properties and correlation to chronic diseases such as atherosclerosis (Duryee et al., 2010), Alzheimer's disease (Pizzimenti et al., 2013), diabetes (Slatter et al., 2000), etc.

**Table 1** Structures of MDA-modified amino acid residues identified in various proteins and model systems

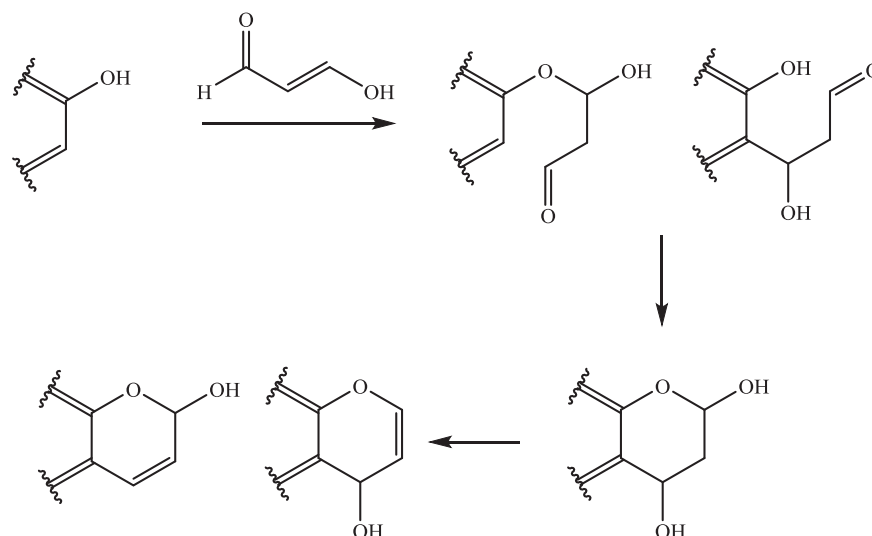
Lysine (Lys, K)	   
Histidine (His, H)	  
Arginine (Arg, R)	  
Cysteine (Cys, C)	
Asparagine (Asn, N)	
Glutamine (Gln, Q)	

Besides some immunological methods (Khan et al., 2002; Wang et al., 2012; Bevan et al., 2003), lacking sensitivity and accuracy, only the analytical method of Requena et al. (1997) revealed two MDA-lysine adducts (a, b), although its applicability for food matrices needs to be assessed.

### Interaction Between MDA and Other Food Constituents in Food

Analogous to amino compounds, MDA interacts with other food compounds such as phenolic compounds, ascorbic acid, nitrite, DNA and phospholipids.

Phenolic antioxidants are widely used to protect lipids from oxidation by acting as free radical scavengers and chelators (Yin et al., 2012; Racicot et al., 2012). In addition, these compounds are able to act as lipid-derived carbonyl scavengers. Carbonyl-phenol adducts are produced with phenolic compounds that have either aromatic carbons or hydroxyl groups with a high nucleophilicity. This reaction occurs mainly with *m*-diphenols such as resorcinol, phloroglucinol, epicatechin, theaflavin, hesperetin (Hidalgo and Zamora, 2014; Hidalgo et al., 2017). The interaction between phenolic compounds and MDA or other reactive carbonyl substances such as propanal, pentanal, hexanal, acrolein, pent-2-enal, oct-2-enal, and 4-hydroxy-non-2-enal (HNE) have been reported (Hidalgo et al., 2017; Zhu et al., 2009a,b). Zhu et al. (2009a) proposed that green/black tea polyphenols might directly trap MDA, a phenomenon similar to that occurring between black tea polyphenols and methyl glyoxal (Lo et al., 2006; Sang et al., 2007). However, at present, the structures of MDA-phenol adducts have not yet been proposed. Based on the reaction

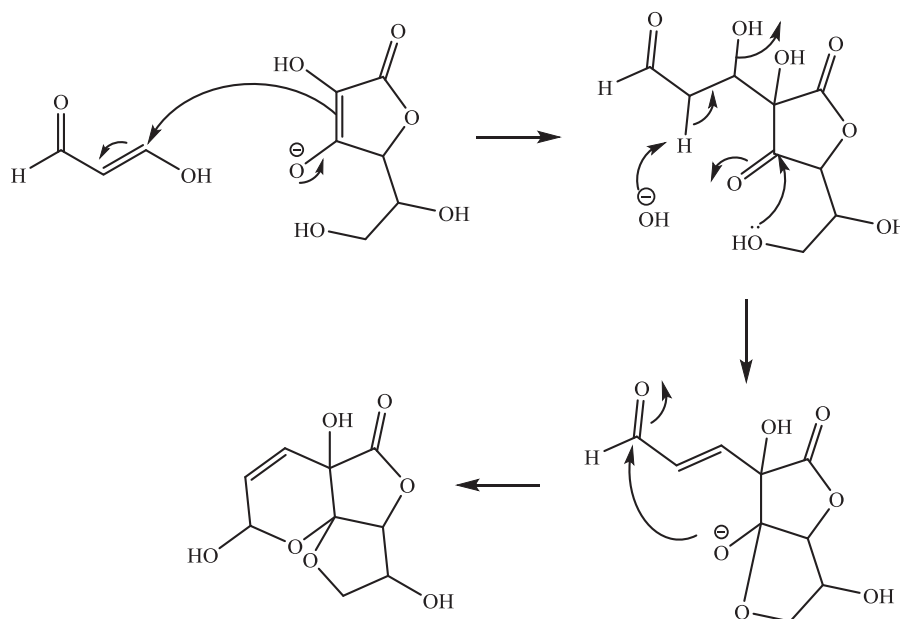


**Figure 3** Proposed structures of MDA-phenol adducts. Only the portion of the phenolic compound that takes part in the reaction has been shown. Adapted from Zamora, R., Hidalgo, F.J., 2018. Carbonyl-phenol adducts—an alternative sink for reactive and potentially toxic lipid oxidation products. *J. Agric. Food Chem.* 66, 1320–1324.

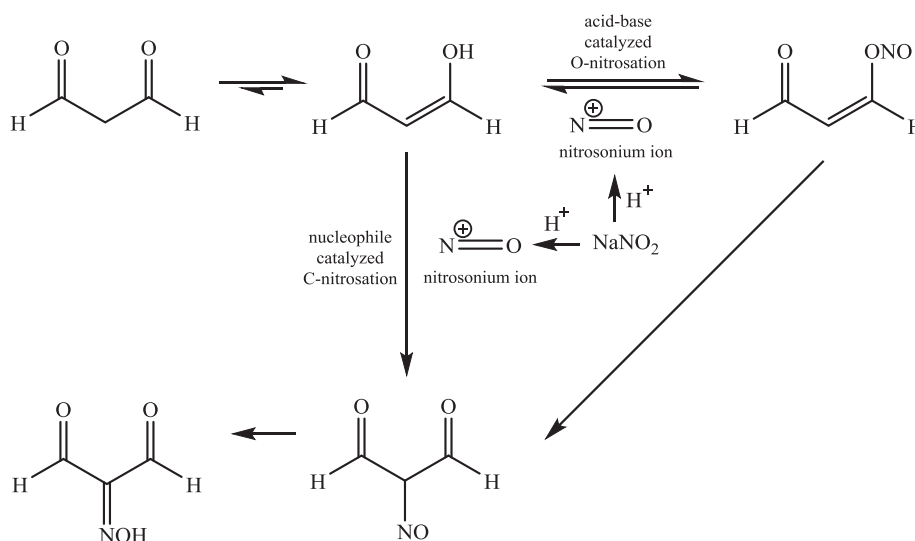
mechanism proposed for alk-2-enals and *m*-diphenols (Hidalgo and Zamora, 2014; Zamora and Hidalgo, 2016, 2018), these structures may be as those shown in Fig. 3.

Like phenolic compounds, ascorbic acid can also function as a nucleophile (which is less appreciated compared to its antioxidant action). The formation of Michael adducts between ascorbic acid and lipoxidation products such as HNE and acrolein has been demonstrated (Fodor et al., 1983; Sowell et al., 2004, 2005). Currently, no interaction between ascorbic acid and MDA has been reported. Though, a similar adduct as acrolein (Fodor et al., 1983) can be expected for MDA-ascorbic acid reaction (as shown in Fig. 4).

MDA can react with nitrite in an acidic medium (pH 1.3–6), even at room temperature. Nitrite is an important additive in cured meat due to the induced characteristic cured-meat color, antioxidant activity and antimicrobial effect (Kolodziejaska et al., 1990). However, the presence of residual nitrite in meat samples could lead to nitrosation of MDA (which cause limitations of the TBA-MDA method in cured meat) (Fernandez et al., 1997; Ulu, 2004). Nevertheless, the TBA-MDA assay has still been used for analyzing the MDA content in cured meat despite the potential nitrosation (Moawad et al., 2012; Sharma et al., 2012; Berardo



**Figure 4** Suggested reaction mechanism between ascorbic acid and malondialdehyde. Based on Fodor, G., Arnold, R., Mohacsi, T., Karle, I., Flippen-Anderson, J., 1983. A new role for L-ascorbic acid: Michael donor to  $\alpha$ ,  $\beta$ -unsaturated carbonyl compounds. *Tetrahedron* 39, 2137–2145.



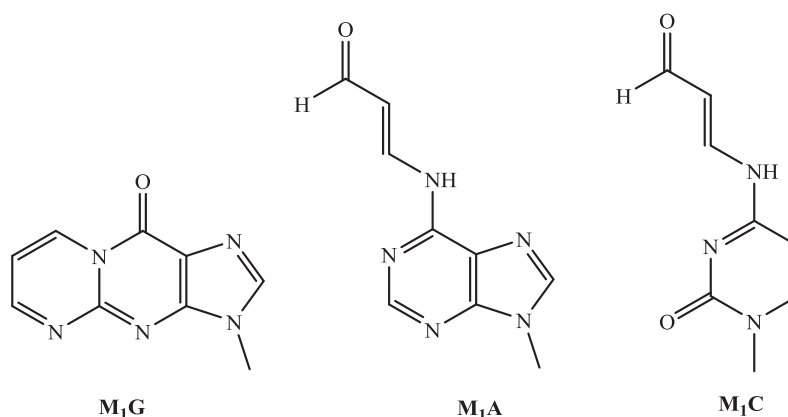
**Figure 5** Mechanism proposed for MDA nitrosation in aqueous solutions. Adapted from Garcia-Rio, L., Mejuto, J.C., Parajó, M., Pérez-Lorenzo, M., 2008. First kinetic discrimination between carbon and oxygen reactivity of enols. *J. Org. Chem.* 73, 8198–8205.

et al., 2016). In Fig. 5, the possible mechanism underlying MDA nitrosation in aqueous solution is presented, although this mechanism is still under discussion since no MDA-nitrite adducts have been isolated so far.

The mutagenicity of MDA has been linked to its reaction with DNA bases. Indeed, MDA can interact with nucleic acid bases to form multiple adducts (as shown in Fig. 6) (Niedernhofer et al., 2003; Marnett, 1999). Among the various MDA-DNA adducts produced *in vitro*, the pyrimido[1,2- $\alpha$ ]purin-10(3H)-one ( $\text{M}_1\text{G}$ ) is probably the major adduct followed by  $\text{N}^6$ -(3-oxopropenyl)deoxyadenosine ( $\text{M}_1\text{A}$ ) with  $\text{N}^4$ -(3-oxopropenyl)deoxycytidine ( $\text{M}_1\text{C}$ ) only found in trace amounts (Marnett, 1999; Guéraud et al., 2010). Further, the amine-containing head groups of phospholipids (e.g. phosphatidylserine or phosphatidylethanolamine) can also form adducts with MDA (Bhuyan et al., 1986). In the last decade, the latter interaction has not gained much attention in contrast to the action of phospholipids as antioxidants.

### Consequences of the Reactivity of MDA in View of Its Use as Lipid Oxidation Marker in Food

Since MDA represents a secondary lipid peroxidation product and sensitive analytical methods are available for its isolation and quantification, MDA has been considered as an excellent marker for lipid peroxidation of  $\omega$ -3 and  $\omega$ -6 fatty acids in foodstuffs. However, as discussed in detail in above paragraphs, MDA is a highly reactive substance towards not only various compounds in food but also itself. The commonly used MDA-TBA assay would measure only the free MDA content. It remains unknown whether or not the various MDA adducts and reaction products are stable under the harsh test conditions used in the TBA-MDA assay. MDA bound to proteins would definitely not be determined by this method because proteins (including those bound with MDA) are mostly excluded prior to TBA derivatization (to avoid interference). Attempts have been made to measure the



**Figure 6** Structure of MDA-DNA adducts.

MDA bound to proteins including the treatment via alkaline hydrolysis prior to TBA derivatization (Grintzalis et al., 2013; Tukozkan et al., 2006; Jung et al., 2016). During this treatment, MDA would mainly exist in its anionic form (which is much less reactive than its undissociated enol) (Chuaqui-Offermans and Chuaqui, 1983). However, the stability of MDA during such alkaline hydrolysis and the degree of protein hydrolysis are still concerns (Grintzalis et al., 2013; Tukozkan et al., 2006; Jung et al., 2016; Mendonça et al., 2017; Carboneau et al., 1991). The ignorance of the occurrence of free amino acids after this hydrolysis step and their interaction with MDA would lead to incorrect analysis results of MDA content, even in acidic medium (cf. arginine). In the study of Grintzalis et al., the proteins were first precipitated with trichloroacetic acid before the alkaline hydrolysis, although resolubilization of precipitated protein is often difficult (Nandakumar et al., 2003). As for MDA, little is known about the stability of the reaction products between MDA and co-existing food components during MDA analysis. Consequently, the MDA determination in a particular food using the conventional TBA-MDA assay is likely inaccurate and the results are greatly affected by the food ingredients and food physico-chemical properties (cf. pH and temperature).

Proteins and peptides can act as multifunctional antioxidants to inhibit lipid oxidation in foods by inactivating reactive oxygen species, scavenging free radicals, chelating prooxidative transition metals, reducing hydroperoxides and altering food physical properties (Elias et al., 2008). Several studies evaluated the antioxidant potential of proteins and peptides by the TBA-MDA assay (Pena-Ramos and Xiong, 2001; Peña-Ramos and Xiong, 2002, 2004; McCarthy et al., 2001a,b; Kęska et al., 2017). The resultant antioxidant activities of protein and peptides would likely be overestimated, because the MDA-protein interaction is not a reaction truly based on an antioxidant mechanism (it does not inhibit lipid oxidation but instead, masks the extent lipid oxidation). Similar conclusions can be made for ascorbic acid (Shalata and Neumann, 2001), phospholipids (Hidalgo et al., 2005) and phenolics (Ahn et al., 2002; Brettonnet et al., 2010). Hence, high reactive MDA as a general oxidation marker for lipid peroxidation in foods and as a measure for antioxidant activity is of concern. Further research is needed to examine the stability of MDA adducts during the TBA-MDA assay and amounts of the MDA oligomers and MDA-protein adducts.

### Consequences of the Reactivity of MDA With Respect to Food Safety

Modern diets contain significant amounts of oxidized lipids due to the inclusion of fried, cooked, and emulsified lipids, unsaturated fats and other lipid-containing foods, as well as the increasing proportion of prepared meals. Papastergiadis et al. studied the dietary exposure to MDA for the Belgian population by combining food consumption data with the determination of free MDA in foods specifically available in Belgium. On the basis of the threshold of toxicological concern, they indicated no risk to human health related to the consumption of such foods for the vast majority of analyzed consumers (Papastergiadis et al., 2014). The total dietary exposure to MDA for humans cannot be estimated based on the measured of free MDA content in foods, otherwise, the risk associated with the presence of potentially toxic MDA adducts along with the products derived from cleavage and aldol self-condensation would be neglected (Vandemoortele et al., 2017). Besides, MDA can be formed during gastric digestion (Steppeler et al., 2016; Larsson et al., 2016; Tullberg et al., 2016; Gorelik et al., 2008a), and in fact, its postprandial absorption and accumulation in the human plasma, most likely in the forms of MDA-lysine adducts, were reported (Gorelik et al., 2008b, 2013; Sirota et al., 2013; Giron-Calle et al., 2002). Giron-Calle et al. found that  $N^{\epsilon}$ -(prop-2-enal)lysine (**a**) can be absorbed from the gut and would then reach many organs, although this finding was based on the *in vivo* digestion of radioactive labeled MDA-lysine adduct rather than the MDA-modified food proteins or peptides. Moreover, MDA adducts can also be formed during the digestion process and then react further with co-existing food components (which may help to prevent the absorption of MDA during digestion). Actual consequences of the resultant MDA-containing species would be on a case-by-case basis, which represents another stream of future research.

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# Protein-Lipid Interactions and the Formation of Edible Films and Coatings

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## Introduction

The need to protect consumer health, as well as environmental concerns, led to exploitation of natural food grade materials in food packaging. Such biodegradable films extend shelf life by acting as selective barriers against moisture and oxygen. They also reduce lipid oxidation by controlling oxygen transmission and volatile compounds which are effective in producing undesired odor and flavour (Koushki et al., 2015). Polysaccharides, proteins, and lipids or a combination of these have been used to prepare biodegradable edible films. In general, the main film-forming materials are polysaccharides (i.e. starch, carrageenan, pectin, alginate, carboxymethylcellulose and chitosan), proteins (i.e. wheat gluten, whey protein isolate, caseinate and soy protein), and lipids (i.e. waxes and fatty acids) (Falguera et al., 2011). Among these materials the inherent properties of proteins make them excellent components for producing edible films. Edible films can be used for versatile food products to reduce loss of moisture, restrict absorption of oxygen, lessen migration of lipids, improve mechanical handling properties, provide physical protection, and/or offer an alternative to the commercial packaging materials (Enujiugha et al., 2013). Composites and mixtures of these films have shown more remarkable functionality in terms of barrier properties and food freshness preservation, when compared to individual biopolymer-based films. An example is the combination of proteins and lipids to form biodegradable edible films and coatings, whereby the low water vapour resistance of protein films is compensated for by the wiper-repelling properties of the incorporated lipids.

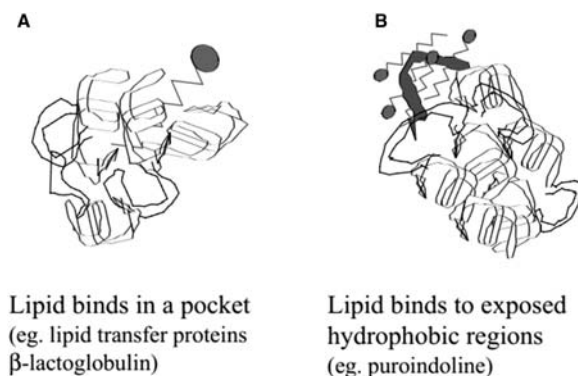
Protein-lipid films are formed as a result of endothermic polymerization of heat denatured proteins or lipoprotein monomers at the liquid surface promoted by surface dehydration (Enujiugha et al., 2013). Heat treatment leads to a change in the three-dimensional structure of proteins and results in exposing sulphhydryl groups and hydrophobic side chains. During film formation, lipid acts as a surfactant which moves to the air/water interface and interacts with proteins by hydrophobic interactions. The protein creates a continuous network structure wherein the lipids are dispersed as droplets. Covalent bonding of lipids to proteins through lipolization offers unique opportunities for film formation with improved properties. Lipid addition reduces significantly the water vapour permeability of protein-based films (Handa et al., 1999a,b; Enujiugha et al., 2013).

Foods are living tissues before and shortly after harvest with specific membrane functions at the cellular level. Within the cell, the mechanism by which a protein interacts with lipids has significant biological consequences, because it determines the specificity and affinity of membrane binding as well as possible effects of the protein on the morphology and dynamics of the membrane (Zhao and Lappalainen, 2012). Processing and preservation of food materials have obvious effects on the cellular activities, and at this level, the interaction of proteins with lipids deals more with the disturbance of the original structure by exposing of the hydrophobic core of the molecule. According to Bourtoom (2009), modifying properties of protein by chemical and enzymatic methods, combining with hydrophobic material or some polymers, or by using a physical method primarily focuses on improving the mechanical strength and moisture barrier properties. In this technical review, the underlying mechanisms of protein-lipid film formation are examined and the applications of the films in food systems x-rayed, with a view to enhancing packaging quality. The potential of edible films to control water transfer and to improve food quality and shelf life is key to the currently increasing attention given to their applications in food systems.

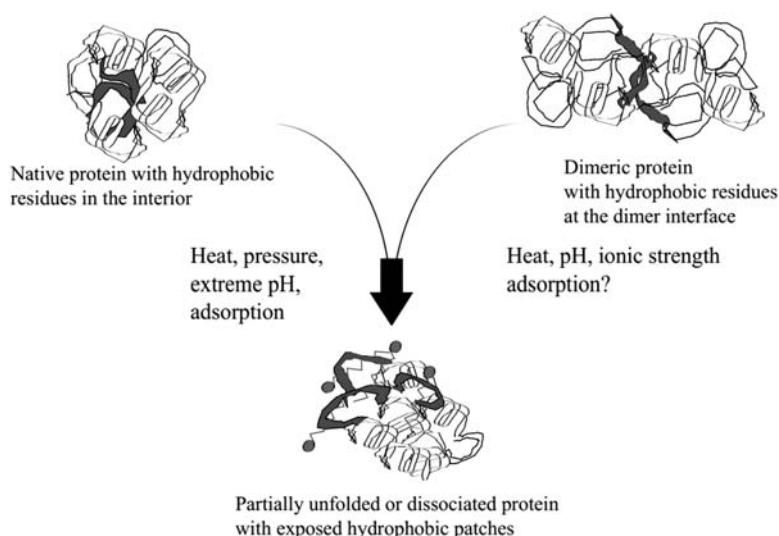
## The Mechanisms of Protein-Lipid Interactions

The hydrophobicity of a protein determines, to a large extent, the value of its interfacial tension, which usually changes as the number and density of hydrophobic residues increase. Proteins generally are amphiphilic molecules, that is, possessing both hydrophobic and hydrophilic regions within their structures. They are commonly surface active and form adsorbed layer at the boundary or interface between the hydrophilic and hydrophobic phases. It is therefore noteworthy that increased surface hydrophobicity of a protein has been associated with increased rates of adsorption. In a study of the physical, thermal and structural properties of amaranth flour films, Tapia-Blácido et al. (2007) observed that there was a strong association between the lipids and the proteins, and consequently established that the presence of both proteins and lipids in the flour films had an important effect on film solubility and also on the color and opacity of the films.

Fillery-Travis et al. (2000) stated that native proteins are able to bind lipid in two main ways, either in a cavity or binding, or through less well defined hydrophobic patches which lie close to the surface of the protein (Fig. 1). For example, the milk whey protein  $\beta$ -lactoglobulin binds a wide variety of aliphatic components in its binding site, including lipids, while, on the other hand, the cereal nonspecific lipid transfer proteins seem to have an important functional role at interfaces, and are known to bind lipids in a central pocket. Apart from the naturally occurring lipid-binding sites, new binding sites can be induced by processing using heat or pressure, or as a consequence of the pH and ionic strength of a food system (Fig. 2). Thus, the proteins may unfold to reveal the more hydrophobic sites normally only present in the centre of the protein, or the conditions may cause dimeric proteins to dissociate to reveal the hydrophobic faces normally buried in the dimer interface (Fillery-Travis et al., 2000). Heating of soymilk, for example, has been found to lead to a change in the three-dimensional structure of the soy proteins and results in



**Figure 1** Modes of lipid binding in native interfacially active food proteins. (A) proteins which bind lipids in a pocket. (B) proteins which bind lipid through exposed hydrophobic regions. From Fillery-Travis, A., Mills, E.N.C., Wilde, P., 2000. Protein-lipid interactions at interfaces. *Grasas Y Aceites* 51 (1–2), 50–55.



**Figure 2** Processes which introduce lipid-binding regions in proteins through dissociation and unfolding of native proteins. From Fillery-Travis, A., Mills, E.N.C., Wilde, P., 2000. Protein-lipid interactions at interfaces. *Grasas Y Aceites* 51 (1–2), 50–55.

exposing sulphhydryl groups and hydrophobic side chains. The protein creates a framework in the protein-lipid film structure, while lipids are dispersed in it as droplets within the protein network (Enujiughu et al., 2013).

Zhao and Lappalainen (2012) reported that the interactions between proteins and lipids can be studied using isolated and model membranes or food matrices in vitro. They observed that proteins can interact with lipids through an unspecific association, for example, by inserting an amphipathic  $\alpha$ -helix into the bilayer, and through electrostatic interactions between the protein and lipid head groups. There are many possible ways by which proteins interact with lipids in real food systems. For example, in the study of physical and molecular properties of egg-white lipid films, Handa et al. (1999a,b) observed that oleic acid increased tensile strength and elongation, and surface sulphhydryl group concentrations in egg white solutions. They suggested that reducing the water vapour permeability of hydrophilic egg white films would probably require cross-linking treatments (e.g., Maillard reaction) that promote covalent bonds other than S–S bonds.

The respective contents of proteins and lipids seem to exert an effect on the productivity of protein-lipid films (Enujiughu et al., 2013), with poor productivity occurring in systems with low protein-lipid ratio under 1.00. This is especially the case with traditional soybean food (yuba) which is a cream-yellow bland flavoured surface film of high nutritional value formed during the heating of soymilk. Furthermore, time and temperature of protein denaturation usually affect the solubility, tensile properties and oxygen permeability of resultant edible protein-lipid films. During the film-formation, lipids act as surfactants which move to the air/water interface and interact with proteins by hydrophobic interactions.

A composite film made of a protein and a lipid can be divided into laminates (in which the lipid is a distinct layer within or atop the biopolymeric films) and emulsions (in which the lipid is uniformly dispersed throughout the biopolymeric film). Both the laminate and emulsion films offer advantages (Enujiughu et al., 2013). The laminate films are easier to apply with regard to the temperature, due to the distinct natures of the support matrix and lipid. During the casting of the lipid onto the protein film,

the temperatures of the film and lipid can easily be controlled separately. When producing the emulsion films, the temperature of the emulsion must be above the lipid-melt temperature but below the temperature for solvent volatilization of the structural network. The main disadvantage of the laminated films, however, is that the preparation technique requires four stages; two casting and two drying stages. This is why the laminated films are less popular in the food industry despite their being good barriers against water vapour. The preparation of the emulsion films requires only one casting and one drying stage, but the finished films are still rather poor barriers against water vapour, since the water molecules still permeate through the non-lipid phase (Enujiughu et al., 2013). The reason for this is the nonhomogeneous distribution of lipids. However, they have the advantages of exhibiting good mechanical resistance, and to require a single step during the manufacture and application process, against one step per layer for multilayer films. It has been shown that for emulsion-based films the smaller the lipid globule size is, and the more homogeneously distributed they are, the lower the water vapour permeability.

## Properties of Protein-Lipid Films

The properties of protein-lipid films include the physical properties (color and opacity; solubility, as total soluble matter; film thickness; moisture content), the mechanical properties (tensile strength; Young's modulus; elongation at break), barrier properties (water vapour permeability; oxygen permeability) and molecular properties (surface and total sulphhydryl groups; electrophoretic patterns; protein content; concentration of saturated and unsaturated fatty acids) (see Table 1). Properties such as tensile properties, water vapour resistance and gas permeability are usually affected by film structure and the nature of protein-lipid interactions.

Water vapour permeability comprises sorption, diffusion and adsorption and is largely governed by the interactions between the polymer and the water molecules. Water permeation through a film usually occurs through the hydrophilic part of the film, thus the relation of the hydrophilic/hydrophobic portions influences water vapour permeability. Polymers with high hydrogen bonding produce films that are susceptible to moisture, while polymers with hydrophobic groups make excellent barriers to moisture (Souza et al., 2010). Water vapour permeability is also dependant on the pore size of the film, and tends to increase with polarity, degree of unsaturation and degree of ramification of the lipids. Gas permeabilities of edible films and coatings depend on several factors such as the integrity of the film, the ratio between crystalline and amorphous zones, the hydrophilic–hydrophobic ratio and the polymeric chain mobility. Oxygen is known to cause food oxidation, inducing several unwanted changes such as odor, color and flavor, as well as nutrients deterioration. Therefore, films providing a proper oxygen barrier can help improve food quality and extend shelf life. Carbon dioxide, on the other hand, is formed in some foods due to deterioration and respiration reactions, and therefore has to be removed from the package to avoid food deterioration and/or package destruction (Souza et al., 2010).

Tensile strength and elongation-at-break are frequently used to characterize the mechanical properties of films. Tensility is the maximum tension supported by the film until the moment it collapses, while elongation is a measure of the flexibility of the film and defines the ability of the film to deform in place before it collapses. Factors, such as film composition, temperature, relative humidity and storage time, affect tensile properties. Films containing low-molecular-weight proteins have higher chain mobility of the matrix. Film formation generally takes place through the development of a three-dimensional network of protein molecules by ionic, hydrogen, hydrophobic and disulphide bonds (Gounga et al., 2007). Young's modulus is also an important property, as it measures the stiffness of a solid material. Edible films show elastic deformation, whereby they revert to original shape after an exerted force is lifted.

**Table 1** Comparison of physical and mechanical properties of selected protein-lipid films

Properties	<i>Amaranth flour</i>	<i>Pistachio globular protein—stearic acid</i>	<i>Hake protein—thyme oil</i>	<i>Egg white—Oleic acid</i>
Thickness (mm)	—	—	0.023 ± 0.005	0.103 ± 0.005
Tensile Strength (MPa)	1.20 ± 0.10	7.80 ± 0.10	5.61 ± 1.56	5.71 ± 0.20
Elongation at break (%)	39.0 ± 1.1	62.0 ± 2.0	146.3 ± 45.3	89.2 ± 1.6
Young's Modulus (MPa)	14.8 ± 0.8			
Water vapour Permeability (gmmh <sup>-1</sup> m <sup>-2</sup> KPa)	0.4 ± 0.02	55.497 ± 6.09	4.0E <sup>-11</sup> ± 0.1	8.3 ± 0.2
Oxygen Permeability (cm <sup>3</sup> μmm <sup>-2</sup> d <sup>-1</sup> KPa)	33.7 ± 7.4			
Moisture content (%)	17.0 ± 0.6	34.76 ± 3.562		73.3 ± 0.6
Solubility (%)	39.9 ± 2.5	42.504 ± 3.251		45.7 ± 0.6
Opacity	20.3 ± 0.1	22.5 ± 0.1	14.9 ± 0.5	
L <sup>a</sup>	78.3 ± 1.0		93.65 ± 0.25	95.85 ± 0.14
a <sup>a</sup>	0.4 ± 0.1		-1.73 ± 0.14	-1.40 ± 0.12
b <sup>a</sup>	18.9 ± 0.2		5.90 ± 0.64	6.00 ± 0.34
ΔE	24.1 ± 1.2		4.74 ± 0.66	
References	Tapia-Blacido et al. (2007)	Zahedi et al. (2010)	Pires et al. (2011)	Handa et al. (1999a,b)



Fatty acids are characterized by their chain length and unsaturation degree and both have influence, as increasing chain length of fatty acids results in lower water vapour permeability of emulsion films. The longer the hydrophobic tail of these molecules, the lower the film water solubility, and as a consequence, the lower the moisture transfer rate. [Fernández et al. \(2007\)](#) examined the effect of the unsaturation degree and concentration of fatty acids on the properties of whey protein isolate-based edible films and found that surface tension was significantly decreased by adding unsaturated fatty acids (oleic and linoleic acid), whereas the greatest effect on water vapour permeability reduction was achieved with stearic acid. Unsaturated fatty acids have one or more double bonds, thereby causing a sharp bends in the carbonyl chain. Increasing unsaturation degree reduces the contact between acyl chains, which lowers the melting temperature. Stearic acid plays a key role in controlling the physical and mechanical properties of films and any increase in the ratio of this component could increase the consistency coefficient, shear thinning behavior, elasticity, rate of drying and flexibility of edible films ([Rezvani et al., 2013](#)).

Covalent disulfide (S–S) bonds are considered important for film formation with proteins containing cysteine and/or cystine amino acids, as they are involved in network formation. In the work of [Handa et al. \(1999a\)](#), concentration of surface sulfhydryl (SH) groups was increased ( $P < 0.05$ ) (3.81–19.45 mM/g protein) with both pH and heating, presumably due to protein denaturation and cleavage of disulfide (S–S) bonds. Concentration of surface SH groups correlated ( $P < 0.05$ ) with film tensile strength ( $r = 0.70$ ), elongation at break ( $r = 0.86$ ), and film total soluble matter ( $r = -0.94$ ). Most likely, surface SH groups formed S–S bonds through air oxidation and/or sulfhydryl/disulfide interchange, thus contributing to film formation.

Visual characteristics of biodegradable films such as gloss, color, and transparency can affect consumer acceptability and even food quality. In a study by [Zahedi et al. \(2010\)](#), the color of control pistachio globulin protein film was yellow and the color intensity depended on film thickness. The addition of fatty acids increased the film opacity, and the opacity increased significantly ( $P < 0.05$ ) as fatty acid level increased.

## Development of Edible Films and Coatings

Edible films and coatings potentially increase food shelf life, improve their quality. They provide the potential to control transport of moisture, oxygen, aroma, oil, and flavor compounds in food systems. Also, edible films and coatings can be used as carriers of food additives, such as antimicrobials, flavors, antioxidants, nutrients and colorants, to enhance preservation, physicochemical and organoleptic properties of food. Such films and coatings provide mechanical integrity and physical protection for food products which are susceptible to injury during transport. The contribution of charge is highlighted by the observation that surface viscoelasticity reaches a maximum when the pH is close to a proteins' isoelectric point, i.e., its net charge is zero. By increasing the overall charge on a dispersed phase (lipid) droplet through the charge of the adsorbed protein the dispersion can be stabilised by the electrostatic repulsion of the droplets hindering their close approach ([Enujiugha et al., 2013](#)).

The efficiency of an edible film against moisture transfer cannot not be simply improved by addition of hydrophobic lipid materials unless the formation of a homogenous and continuous lipid layer inside the hydrocolloid matrix is achieved ([Karbowski et al., 2007](#)). As a result, optimization of film composition and its rheological behavior is one of the most important steps in edible films production. The coating surface is influenced by rheological behavior of components, mechanism of application and changes in properties associated with transition from liquid to solid state. As most coating systems are pseudoplastic, viscoelastic and thixotropic, their rheological state is strongly dependent on the coating process which involves shear in different magnitude. The application of homogenization to the initial biopolymer-oil mixture is required, to obtain a uniform droplet distribution with small-sized droplets so as to increase the tortuosity factor and improve the water barrier performance of the film. In a study by [Matsakidou et al. \(2013\)](#), homogenization of the film-forming oil bodies dispersion led to size reduction and improved to some extent the surface related characteristics of the films although their moisture barrier and the tensile properties were not significantly affected.

Edible films are formed through extrusion, co-extrusion, spreading, casting, roll coating, drum coating, pan coating or laminating techniques. Edible coatings, on the other hand, are mainly applied using spraying, drum coating, spray fluidization, pan coating or falling film techniques. Film-forming mechanisms of biopolymers include intermolecular forces such as covalent bonds (e.g., disulphide bonds and cross-linking) and/or electrostatic, hydrophobic or ionic interactions ([Souza et al., 2010](#)). Physical modifications of edible films and coatings include lamination, formation of composites, addition of particles or emulsions, perforation, over-coating, annealing heat curing, orientation, radiation, ultrasound treatment and electric field treatments. For edible coatings, knowledge of the surface properties of the coating-forming solutions is crucial for the characterization of their performance.

The development of films with a uniform and compact layer can be an important achievement toward the improvement of various film properties, such as their permeability to gases ([Souza et al., 2010](#)). Scanning electron microscopy is frequently used to evaluate film homogeneity, layer structure, pores and cracks, surface smoothness and thickness, while X-ray diffraction is mainly used to evaluate the degree of crystallinity of the films. Coatings can be obtained either by dipping the product into, or by brushing or spraying it with solution containing film ingredients, so as to deposit the film directly on food surface, or by creating standalone film from solution or through thermoformation for subsequent covering of food surface. Thermoformation is rarely used to create edible films, because most edible components cannot be molded at elevated temperatures without causing irreversible structural changes to the material. Direct application of any lipid to a hydrophilic or wet surface results in weak adhesion at the film–food interface. Dual-coating is one possible solution to this problem, as it provides protection against more than one permeate through use of different laminate layers.

## Conclusion

Proteins and lipids have been shown to interact in many different ways to form effective edible films and coatings. Such composite protein-lipid films are capable of combining the effective structural and oxygen barrier properties of protein films with the high moisture barrier characteristics of lipids. However, the film-forming conditions and the composition and structural variations of the constituent lipids affect film performance and properties, with regard to aesthetics and keeping quality of packaged food systems. An understanding of the mechanisms of the interaction of proteins with lipids helps in elucidating the important properties of the resultant films and coatings.

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# Protein Ingredients in Low- and Intermediate-Moisture Systems<sup>☆</sup>

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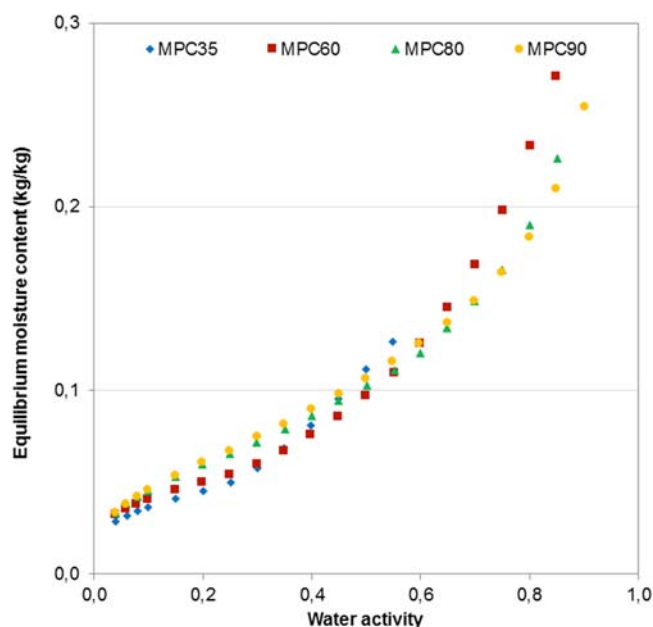
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## Introduction

Proteins play a crucial role from nutritional and physicochemical perspectives in a wide array of food products. In some cases, proteins are included as part of the materials in which they naturally occur, e.g., the use of milk to prepare cheese or yoghurt, but in many cases, proteins are included in products in the form of (partially) purified protein ingredients. While in some cases this can be done in the form of concentrated liquid protein ingredients, in most cases, dried protein ingredients will be used. Drying of protein ingredients can be beneficial from the perspective of extending shelf life and reducing volumes for transport and standardization. However, some undesirable effects are also observed during production and storage of these protein ingredients, most notably a loss of solubility and a concomitant loss of functionality, as well as a reduction in nutritional value, because of the Maillard reaction and other modifications of amino acids. Therefore, despite low moisture content and water activity ( $a_w$ ) in these low-moisture systems (LMSs), considerable changes will still occur in protein ingredients. Although the use of most protein ingredients typically involves reconstitution and rehydration of proteins in high-moisture systems, some protein ingredients are also used in intermediate-moisture systems (IMSs), most notably high-protein bars. As in the LMS, considerable interactions can still occur in the IMS even though proteins are not fully hydrated. In this article, the behavior of proteins and protein ingredients in the LMS and IMS will be described and discussed, with particular emphasis on milk protein ingredients.

## Water Relations in Protein Ingredients

When considering protein ingredients in the LMS and IMS, insights into how water is bound and distributed within the ingredients are necessary. Insights into such phenomena can be gained from moisture sorption isotherms, which describe the relationship between  $a_w$  and equilibrium moisture content (EMC). In general, sorption isotherms of proteins can be described well based on the sum of the main individual components, i.e., typically proteins and carbohydrates contribute heavily to moisture sorption, whereas fat does not (Foster et al., 2005). Moisture sorption isotherms for milk protein concentrate (MPC) samples containing 35%–90% protein in the dry matter are shown in Fig. 1.



**Figure 1** Moisture sorption isotherms at 40 °C for milk protein concentrate (MPC) samples containing 35%, 60%, 80%, and 90% protein in the dry matter.

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Moisture sorption by proteins can be separated into three zones (Kinsella et al., 1986): at low  $a_w$  values, i.e.,  $a_w < \sim 0.3$ , most water occurs as structural/hydrogen-bonded water and hydrophobic hydration water, which are strongly bound by the polar, charged, and hydrophilic residues of proteins. This water is typically considered to be nonfreezable and not available for chemical reactions and requires intense drying conditions for removal. At  $a_w$  values between 0.3 and 0.7, proteins can sorb the so-called multilayer water (which is less strongly bound than the monolayer water but is more firmly bound than free water) and some capillary water (which is physically held in capillaries, voids, clefts, cavities, or crevices in  $a_w$  range of 0.5–0.7). Multilayer water refers to layers of hydrogen-bonded water surrounding structural water, thus can be considered as the continuous transition from the monolayer to free water. At  $a_w > 0.7$ , free water is also found, primarily in the forms of capillary water in  $a_w$  range of 0.7–0.95 (which is held in large capillaries, voids, and crevices within structures) and hydrodynamic hydration water (which is loosely associated with the proteins at  $a_w > 0.99$ ). This free water behaves the same as bulk water.

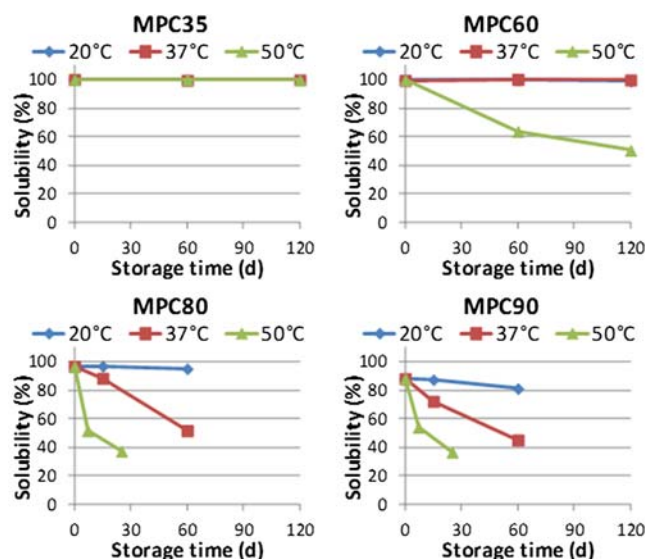
Because moisture sorption and desorption induce some structural changes in proteins and powder particles, hysteresis is sometimes observed in moisture sorption isotherms between the adsorption and desorption isotherm (especially when the desorption isotherm lies above the adsorption isotherm). This is particularly important when using moisture adsorption isotherms for predicting the drying behavior of products. In cases where a large hysteresis loop is observed between adsorption and desorption, notable variations in drying behavior (e.g., the degree of dryer fouling or powder stickiness) can occur when only adsorption isotherms are used as input parameters. Hysteresis can also influence the chemical and physical changes in stored foods, e.g., decrease the accessibility of the polar residues of proteins. Typically, EMC at a given  $a_w$  is higher in the desorption isotherm than in the adsorption isotherm (Al-Muhtaseb et al., 2002). Hence, moisture adsorption of a protein powder during storage does not restore the same situation at a given  $a_w$  that existed during desorption.

In addition to moisture sorption by proteins, sorption by carbohydrates is also important for protein-containing ingredients, particularly in ingredients where carbohydrates are present at high concentrations. For example, in milk powder containing 50%–55% carbohydrate in the form of lactose, the carbohydrate forms a continuous amorphous matrix in which the rest of the constituents are suspended. The extremely high viscosity of this amorphous (glassy) phase ensures that it behaves like a solid, but an increase in  $a_w$  or in temperature results in plasticization of the amorphous phase and the transition from a glass to a highly viscous phase that has been described as sticky and rubbery. The critical temperature above which this change occurs can be determined by differential scanning calorimetry and is referred to as the glass transition temperature ( $T_g$ ). In this viscous phase, crystallization of carbohydrates may occur. Furthermore, reducing carbohydrates are more prone to participate in reactions such as the Maillard reaction when temperatures are above the glass transition temperature.

## Changes During Storage of Protein Ingredients

As outlined in the introduction, a number of changes can occur during storage of protein ingredients, most notably the loss of solubility and (chemical) modification of amino acid residues. Solubility is a key functional property for any application of protein ingredients. Whereas ingredients containing a significant amount of carbohydrate typically have good solubility and maintain the solubility during storage, many high-protein ingredients are prone to loss of solubility during processing and storage. Among the milk protein ingredients, milk protein concentrates and micellar casein isolate are particularly well known to lose solubility easily (Anema et al., 2006; Havea, 2006; Gazi and Huppertz, 2015). Solubility of milk protein concentrates during storage is shown in Fig. 2. Both milk protein concentrates and micellar casein isolate are prepared by membrane filtration of milk and lead to powdered products containing a high concentration of casein in the micellar form. Although being remarkably heat-stable during heating of fresh milk, casein micelles tend to have poor stability in more concentrated systems, particularly when both temperature and  $\text{Ca}^{2+}$  activity are high but pH is low (Crowley et al., 2014; Gazi and Huppertz, 2015). This manifests itself in the aggregation of casein micelles during drying, in a manner similar to heat-induced coagulation; this aggregation leads to a network of casein micelles, which has poor solubility. Development of insolubility during drying can be reduced through careful selection of appropriate processing conditions and product properties (De Castro-Morel and Harper, 2003). Further aggregation and development of insolubility can occur during storage of milk protein concentrates (Anema et al., 2006; Havea, 2006; Gazi and Huppertz, 2015). Insolubility becomes more severe with increasing storage time and temperature as well as with elevated water activity. Protein analysis of the soluble and insoluble phases revealed that, like during processing, casein micelles could lose solubility during storage whereas the other constituents (lactose, salts, and whey proteins) remain soluble. The progressive insolubility of such ingredients results primarily from the noncovalent interactions between casein micelles; aggregation of casein micelles appears to be more extensive on the surface of the particles than in the core. Higher heat load on the surface of particles during drying along with depletion of lactose to a greater extent on the surface of particles compared with the core of the particles could certainly contribute (Kelly et al., 2015). It should further be considered that on drying, the environment of the particles changes considerably. The hydrophilic aqueous phase is replaced by a more hydrophobic air phase; as a result, protein interactions are promoted. Higher degree of insolubility development during storage and at a higher  $a_w$  and temperature can be related to the increased mobility, and thus propensity to aggregation, for the proteins. If the interactions are noncovalent, solubility can be reattained in most cases when sufficient shear and time (especially, hydration time) are applied. However, when the interactions are covalent, irreversible protein aggregation can occur.

Chemical modifications can occur during storage of protein ingredient powders. The Maillard reaction is probably the most studied chemical reaction, particularly for milk protein ingredients. The Maillard reaction requires the presence of a reducing carbohydrate along with accessible lysine residues. As part of the Maillard reaction, the so-called Amadori compounds (mainly



**Figure 2** Solubility as a function of storage time (unit: day) at 20, 37, and 50 °C for milk protein concentrate (MPC) samples containing 35%, 60%, 80%, and 90% protein in the dry matter. Data from Gazi, I., Huppertz, T., 2015. Influence of protein content and storage conditions on the solubility of caseins and whey proteins in milk protein concentrates. *Int. Dairy J.* 46, 22–30.

lactulosyl-lysine for most milk products) are formed, which can undergo further reactions including dehydration and deamination to form other intermediates, by-products, and advanced Maillard reaction products. These advanced Maillard reaction products include many heterocyclic compounds, and melanoidins are formed in the final stage via polymerization causing browning (Van Boekel, 1998). The rate of the Maillard reaction increases with increasing temperature and  $a_w$ , although at a high  $a_w$ , the rate of the Maillard reaction can decrease again (Morgan et al., 2005; Gonzales et al., 2010). Because of the browning effect and loss of the essential amino acid lysine, Maillard reactions in protein ingredients are generally considered undesirable, although controlled glycation of proteins can improve certain protein functionality. Studies have shown that glycation of proteins can improve emulsifying and foaming properties, solubility, and heat stability.

Another reaction involving lysine can occur during storage of protein powders, which is the formation of isopeptide bonds as a result of dephosphorylation of phosphoserine. A dehydroalanine side chain is then formed, which can cross-link with adjacent lysine residues to yield lysinoalanine (LAL). Histidine and cysteine may also form cross-links with the dehydroalanine side chain. The reaction is promoted by heat treatment (i.e. a greater reaction rate at a higher temperature). Owing to the requirement for phosphoserine, the formation of isopeptide bond is limited to the proteins containing phosphoserine residues, most notably caseins, and other products such as milk protein osteopontin and egg protein phosvitin. During storage of caseinates at temperatures above 30 °C, LAL formation rates can exceed 100 ppm/month. The LAL level is considerably lower in Ca-caseinate than in Na- or K-caseinate. The interaction of Ca with phosphoserine, which also occurs in milk powder, milk protein concentrate, and micellar casein, is likely the contributor to reduce LAL formation. Other modifications of amino acids in protein ingredients include the oxidation of methionine to methionine sulfoxide and the oxidation of tryptophan (Higgs and Boland, 2009).

## Application of Protein Ingredients in Intermediate-Moisture Systems

IMSs ( $a_w$  0.3–0.7) involving protein ingredients have also gained considerable interest since the early 2000's. This can be attributed primarily to the rapidly expanding market for high-protein bars. The high-protein bars usually contain milk and/or plant proteins, carbohydrate syrup, and fat as the main ingredients. Water activity of these products is typically in the range of 0.5–0.7. One of the main issues associated with these products is bar hardening, which occurs mostly during storage. The behavior of protein ingredients is the primary cause of bar hardening.

Insights into the behavior of protein ingredients in the IMS have been primarily gained from model systems mimicking consumer protein bars. Such systems contain protein ingredients with/without carbohydrate syrups and/or polyols. Bar hardening in these model systems was found to increase with storage time and temperature. Irrespective of what model system was used, elevated bar firmness was observed with increasing protein content. Considerable differences, however, are found between models involving different protein ingredients (single use or in combinations). Both protein type and powder properties can strongly affect bar hardening. With respect to protein type, proteins with reasonably high solubility such as whey proteins (particularly protein hydrolysates) tended to produce soft bars (Zhou et al., 2008; Hogan et al., 2012, 2016; Rao et al., 2016). Protein hydrolysates, however, may present sensory issues such as those associated with excessive Maillard-induced browning. As a result, the amount of protein hydrolysate used in protein bars is limited (Childs et al.,



2007). The firmest bars are likely attained when protein ingredients with low solubility are used. In terms of milk protein ingredients, bars produced with micellar casein, milk protein concentrate, or caseinate were found firmer than those with whey protein concentrate, isolate, or hydrolysate (Hogan et al., 2012). Remarkably firm bars are feasibly produced when insoluble protein ingredients such as acid or rennet casein are used. In addition to protein type, powder properties are also important factors that influence bar hardening. For example, powders with a high  $a_w$  tend to reduce hardening in bars (Hogan et al., 2012), whereas powders with a higher particle density and lower amount of occluded air likely yield softer bars. Agglomeration of powder particles would reduce bar hardening. Compared with the influence of proteins on bar hardening, the effect of the carbohydrate fraction in bar is limited.

Accordingly, on mixing the different ingredients of a protein bar, a notable gradient in  $a_w$  is established between the protein powder and carbohydrate syrup. As a result, sorption of the aqueous phase (i.e., the carbohydrate syrup) into the protein powder particles takes place. This results in an increase in the volume fraction of protein particles because part of the continuous phase becomes “entrapped” in the serum phase of the bar system. The volume fraction of powder particle can further increase if the powder particles swell on sorption of the aqueous phase. If the volume fraction of powder particle becomes sufficiently high, interactions between powder particles can occur, giving rise to bar hardness of protein bars (Hogan et al., 2012). Over time, fusion of interactive protein particles into a continuous mass, not unlike the fusion of *para*-casein micelles in cheese curd, can occur. To monitor protein bar hardness, controlling the volume fraction of protein powder and the interactions between powder particles is essential. The volume fraction of protein powder correlates negatively with particle density but positively with occluded air. Furthermore, the polydispersity of the particle size distribution can also affect bar hardness, as the maximum volume fraction attainable before the system is “full” would be higher. The same phenomenon was observed for bimodal particle size distributions. These findings partially explain why protein bars prepared with blends of protein ingredients often exhibit lower hardness than those prepared with a single type of protein ingredient. Blends of protein ingredients typically contain mixtures of protein ingredients with different water solubility. Protein hydrolysates and whey proteins typically exhibit higher solubility than casein-based or vegetable protein-based ingredients. (Partial) dissolution of proteins not only causes a reduced powder volume fraction but also leads to increased serum phase viscosity.

## Conclusion

As outlined above, understanding the behavior of proteins and protein-based ingredients in the LMS and IMS is crucial. It enables the production of soluble and functional protein ingredients and retention of their desirable properties during storage. Protein ingredients are the key ingredient in high-protein bars. In these food systems, the sorption behavior of protein powder particles and the interactions between the powder particles are the key to controlling bar hardness. In both LMS and IMS, it is crucial to ensure that proteins are not fully hydrated, and desirable sorption behavior and particle interactions occur in very different environments of LMS and IMS. Careful consideration of these environmental changes is required to control and optimize protein interactions in low- and intermediate-moisture food products.

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# Interactions Between Starch, Proteins and Lipids and the Formation of Ternary Complexes With Distinct Properties

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## Glossary

**Full width at half maximum** The width of a spectrum curve measured between those points on the y-axis which are half the maximum amplitude.

**Long-range molecular order** The long-range ordered array of double helices, often referred to as crystallinity, which is often determined by X-ray diffraction.

**Self-assembly** A process in which a disordered system of pre-existing components forms an organized structure or pattern as a consequence of specific, local interactions among the components themselves, without external direction.

**Short-range molecular order** The ordered array of double helices at a short-range scale, which is measured usually by FTIR and Raman spectroscopy.

**Starch gelatinization** A process of breaking down the intermolecular bonds of starch molecules in the presence of water and heat, allowing the hydrogen bonding sites (the hydroxyl hydrogen and oxygen) to engage more water.

**Starch retrogradation** A process in which disaggregated amylose and amylopectin chains in a gelatinized starch paste reassociate to form more ordered structures during cooling and storage.

**Thermodynamically incompatible** Two or more (incompatible) polymers cannot achieve uniform dispersion at the molecular level, and phase separation between them often occurs even in concentrated systems.

## Introduction

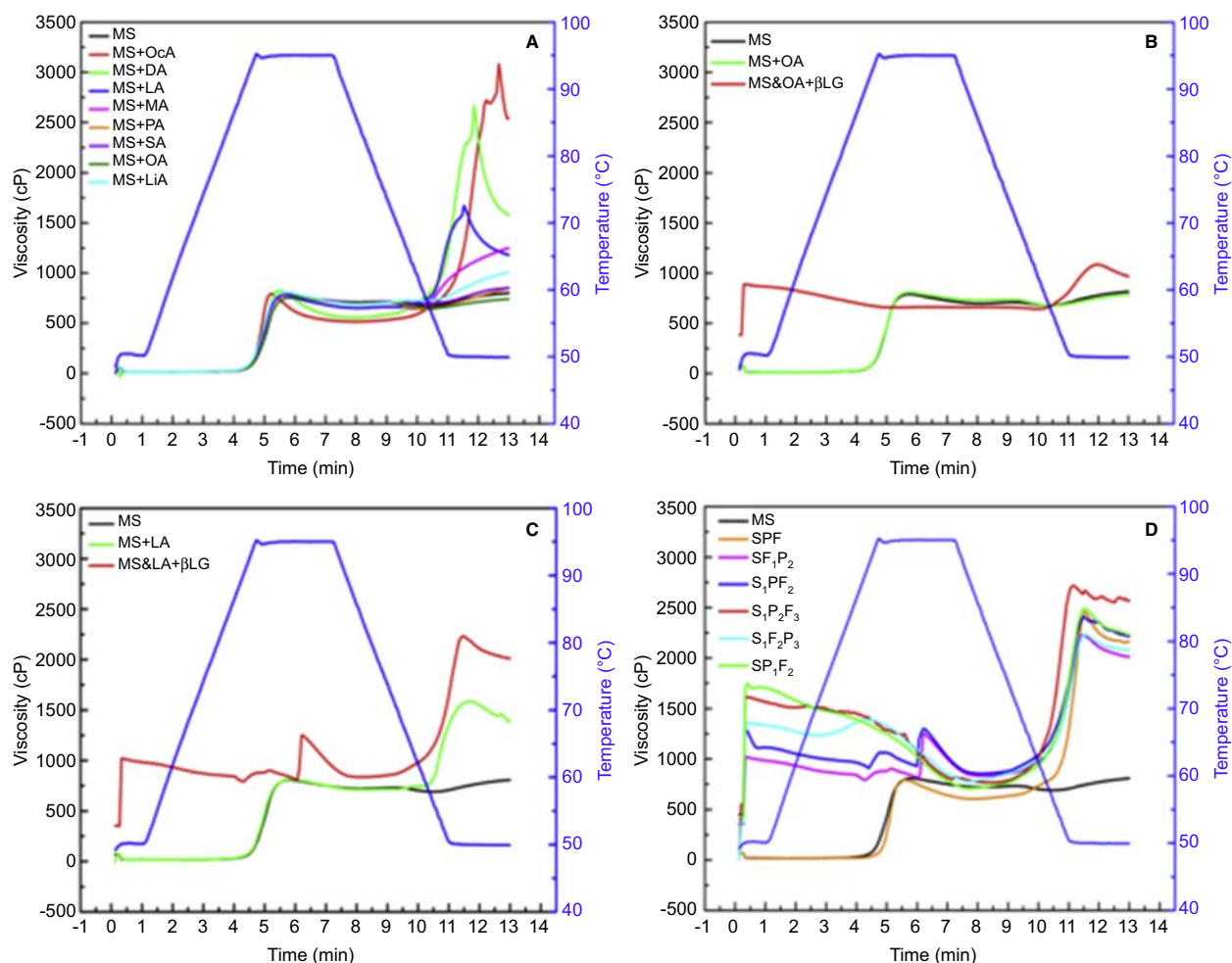
Starch, lipids and proteins are three major macronutrients in human diet that provide the human body with energy (calories). These three components often occur together in many food systems, especially the starchy foods. During food processing, each macronutrient undergoes a series of changes. Such processing-induced changes and associated interactions among these macronutrients have a direct impact on the quality of final food products such as flavor, mouth-feel, texture, shelf-life and nutritional value (Parada and Santos, 2015; Wang and Copeland, 2013). The interactions among starch, proteins and lipids are very complex including their binary interactions and ternary interactions. Understanding how these interactions occurred during food processing may help to optimize processing parameters to create food products with desired microstructure and functionality.

Binary interactions among the starch, lipids and proteins have been studied extensively, and their effects on the properties of starch and derived finished foods were found. Starch-lipid complexes naturally occur in native starch or are formed during food processing and storage (Ai et al., 2013; Tang and Copeland, 2007; Wang et al., 2016c), and the formation of such complexes reduces swelling power and starch solubility, retards the gelatinization and retrogradation of starch and slows the enzymic digestion rate (Copeland et al., 2009; Putseys et al., 2010; Wang and Copeland, 2013). Protein-lipid interactions occurring in real foods can act as emulsifiers at the interfaces (Rodríguez Patino et al., 2001), mainly via hydrophobic interactions, and electrostatic and/or polar interactions (Cornell and Patterson, 1989; Ioffe et al., 2007). Starch and protein are two thermodynamically incompatible macromolecules (Polyakov et al., 1997). The interactions between protein and starch such as those in wheat flour may afford desirable health benefits, for example, protein network-coated starch granule in white flour exhibit decreased glycemic response and digestibility (Jenkins et al., 1987).

To fully understand the functionality and digestibility of starch and other macronutrients in foods containing starch, lipids and proteins, not only binary interactions but also ternary interactions have been examined. A number of studies have confirmed the occurrence of a ternary starch-lipid-protein complex during food processing, which led to alterations in starch pasting and gelatinization, the digestion rate and extent of the three macronutrients, as well as the rheological properties of the finished foods (Parada and Santos, 2015; Zhang et al., 2003). More importantly, both starch-lipid complexes and starch-lipid-protein complexes were found to possess the capacity to carry sparingly soluble small molecules in the lumen of the amylose helices not occupied by the hydrophobic carbon chain of lipids. Such a capacity enables the potential of starch-lipid or starch-lipid-protein complexes in the delivery and release of hydrophobic bioactive substances contained in functional foods and health products (Bhopatkar et al., 2015).

## The Formation of the Ternary Starch-Lipid-Protein Complexes

The starch-lipid-protein complex formed between starch, proteins, and fatty acids (FAs) was discovered by Zhang et al. who studied the pasting properties of sorghum flour, which was believed to account for the viscosity peak for sorghum flour during rapid



**Figure 1** RVA profiles of starch with different fatty acids (octanoic acid (OAcA), decanoic acid (DA), lauric acid (LA), myristic acid (MA), palmitic acid (PA), stearic acid (SA), oleic acid (OA) and linoleic acid (LiA) and  $\beta$ -lactoglobulin). (A) maize starch (MS) with different fatty acids in one cycle; (B) MS, MS-OA (in one cycle) and MS-OA (in one cycle) +  $\beta$ LG (in the second cycle); (C) MS, MS-LA (in one cycle) and MS-LA (in one cycle) +  $\beta$ LG (in the second cycle); (D) MS- $\beta$ LG-LA in different mixing sequences. Starch control, SPF in one cycle,  $SF_1P_2$ ,  $S_1PF_2$ ,  $S_1P_2F_3$ ,  $S_1F_2P_3$ ,  $SP_1F_2$  (S, P, and F, are for starch, protein and lauric acid, respectively). Wang et al. (2017). Reproduced with permission from ACS publications.

viscosity analysis (RVA) cooling stage by self-assembling amylose molecules along with lipids and proteins. A model starch-rich system containing normal starch, soluble proteins and FAs showed a RVA pattern different from that of normal starch but similar to that of sorghum flour (Zhang and Hamaker, 2000). Addition of long chain FAs alone (e.g. palmitic, oleic and linoleic acids) to starch can only cause an increase in starch pasting viscosity at the cooling stage (Fig. 1A), whereas, the addition of  $\beta$ -lactoglobulin ( $\beta$ -LG) to these starch-FA pasting systems led to the occurrence of a viscosity peak during the cooling stage of the following RVA run (Fig. 1B). Interestingly, the addition of  $\beta$ -LG to the pasting systems containing starch and short chain FAs can further increase the viscosity peak during the second RVA run (Fig. 1C) (Wang et al., 2017). However, the addition of whey protein to the system containing starch alone caused no effect on starch pasting behavior. Accordingly, the formation of starch-protein-lipid complexes during RVA pasting has been believed to be a common phenomenon that might occur during thermal processing of starchy foods (Wang et al., 2017; Zhang and Hamaker, 2003).

The addition sequence of starch, proteins and lipids during RVA runs is critical for the formation of a ternary complex, and as a consequence, viscosity would change during the cooling stage of the RVA protocol (Fig. 1D). For example, the addition of lauric acid (LA) together with  $\beta$ -lactoglobulin ( $\beta$ -LG) after starch alone was pasted in the RVA (i.e.  $S_1PF_2$ ) or addition of LA after the pasting of starch along with  $\beta$ -LG ( $SP_1F_2$ ) would result in higher viscosity, compared with the addition of LA in the first RVA run ( $SF_1P_2$ ) or second RVA run ( $S_1P_2F_3$ ). Adding LA in the third run ( $S_1P_2F_3$ ) led to the highest viscosity peak. The systems with  $\beta$ -LG added in the second ( $SF_1P_2$ ) or third RVA run ( $S_1F_2P_3$ ) exhibited a smaller viscosity peak, compared with the system in which all three components were mixed together (SPF). The prior mixing of starch and  $\beta$ -LG could promote the formation of a ternary complex whilst the addition of LA in the RVA cycle before adding  $\beta$ -LG did not. The emulsifying action of  $\beta$ -LG likely contributed

to the complex formation among starch,  $\beta$ -LG and LA, but a prior interaction of starch with LA could make  $\beta$ -LG a less effective emulsifier causing weaker interactions between starch,  $\beta$ -LG and LA (Wang et al., 2017).

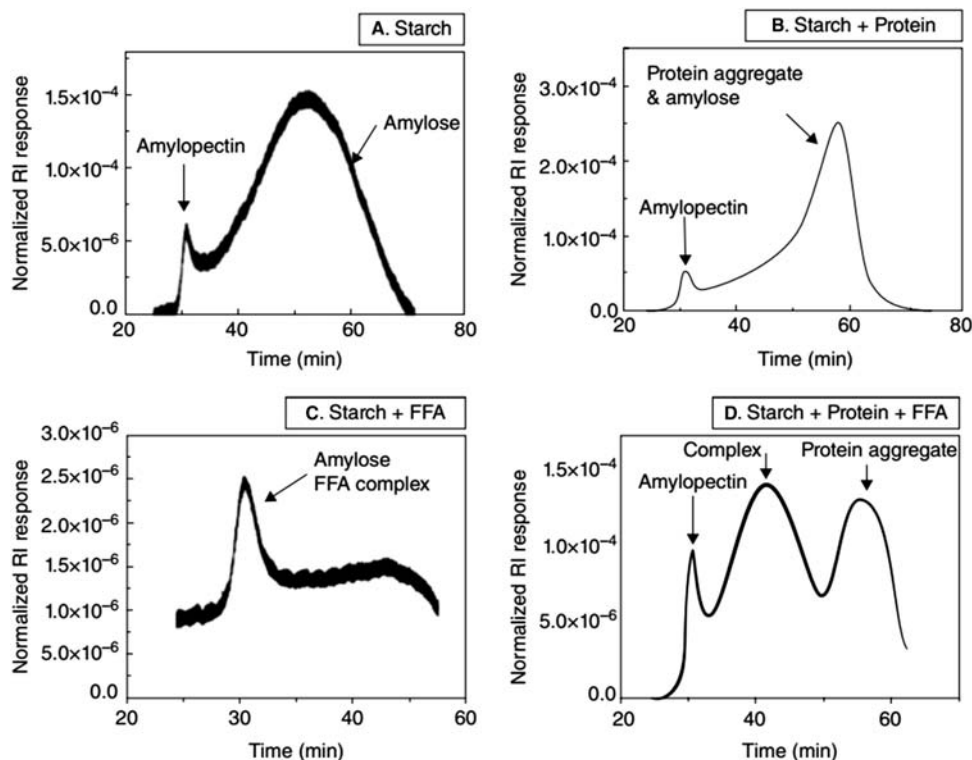
### Identification of the Ternary Complex of Starch, Proteins and Lipids

The starch-lipid-protein complex can be identified by high-pressure size exclusion chromatography (HPSEC) with multi angle laser scattering (MALLS)—detector (Shah et al., 2011; Shah, 2009; Zhang et al., 2010; Zhang et al., 2003) and FTIR spectroscopy (Wang et al., 2017). For pure starch, two HPSEC peaks are typically found (the smaller peak corresponding to amylopectin molecules while the larger one derives from amylose molecules) (Fig. 2A). In comparison, a very small amylopectin peak and a prominent amylose cum protein peak were found for the starch-protein mixture (Fig. 2B). SEC profiling revealed only a void volume peak for the starch-FA mixture (Fig. 2C, indicating the formation of an insoluble amylose-lipid complex), but three distinct peaks for the ternary system (Fig. 2D, corresponding to the amylopectin molecules, ternary complex, and protein aggregate or protein-lipid complex (Zhang et al., 2010, 2003). The molecular mass and the radius of gyration of the ternary complex were  $\sim 10^6$ – $10^7$  Da and 20–100 nm, respectively, as estimated by MALLS/HPSEC (Shah et al., 2011; Zhang et al., 2010).

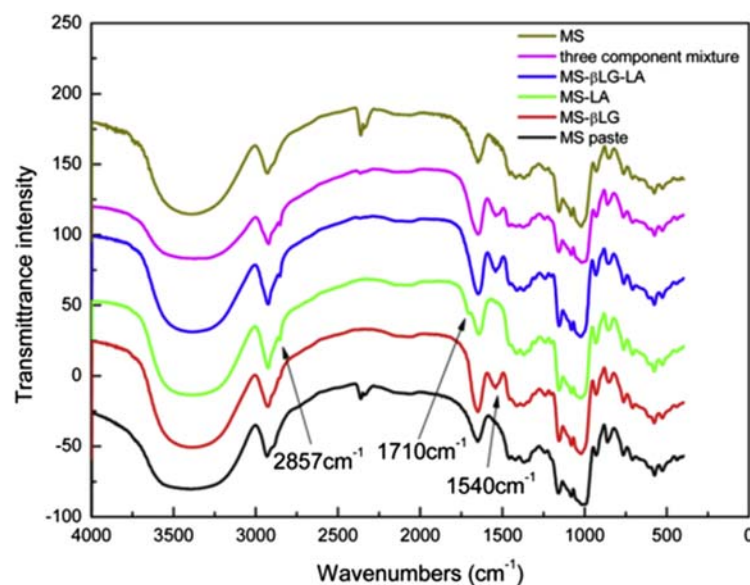
The identification of starch-lipid-protein complex by FTIR is demonstrated in Fig. 3 which contains spectra of native starch, the three-component mixture and samples obtained after RVA pasting. In comparison with maize starch (MS), two additional absorptions at  $1710\text{ cm}^{-1}$  and  $2846\text{ cm}^{-1}$  were found in the FTIR spectra of MS-LA complex (assigned to vibration of carbonyl groups and C–H asymmetric stretching vibration of FA's methylene groups, respectively), with a concomitant shift of the absorption peak around  $1705\text{ cm}^{-1}$  (corresponding to the carbonyl group of LA) to a higher value of  $1710\text{ cm}^{-1}$  (Guillen and Cabo, 1997; Koca et al., 2007; Safar et al., 1994). Moreover, the absorption band at  $1710\text{ cm}^{-1}$  was absent in the spectra when the MS- $\beta$ LG-LA complex was formed due to its attenuating effect on the IR absorbance of the carbonyl group of LA (Wang et al., 2017).

### Changes in Thermal Properties Caused by the Starch-Lipid-Protein Complex

Differential scanning calorimetry is used to analyze the thermal transition parameters of systems containing starch, lipid and proteins, by which the melting and formation of starch-lipid complex or starch-protein-lipid complex were examined. Upon heating of the MS- $\beta$ LG-LA mixture, three endothermic transitions were observed including melting of LA (around  $43^\circ\text{C}$ ), starch



**Figure 2** High pressure size exclusion chromatography (HPSEC) results of different materials: (A) starch control, (B) starch + protein, (C) starch + FFA, and (D) starch + protein + FFA. Shah et al. (2011). Reproduced with permission from Elsevier.

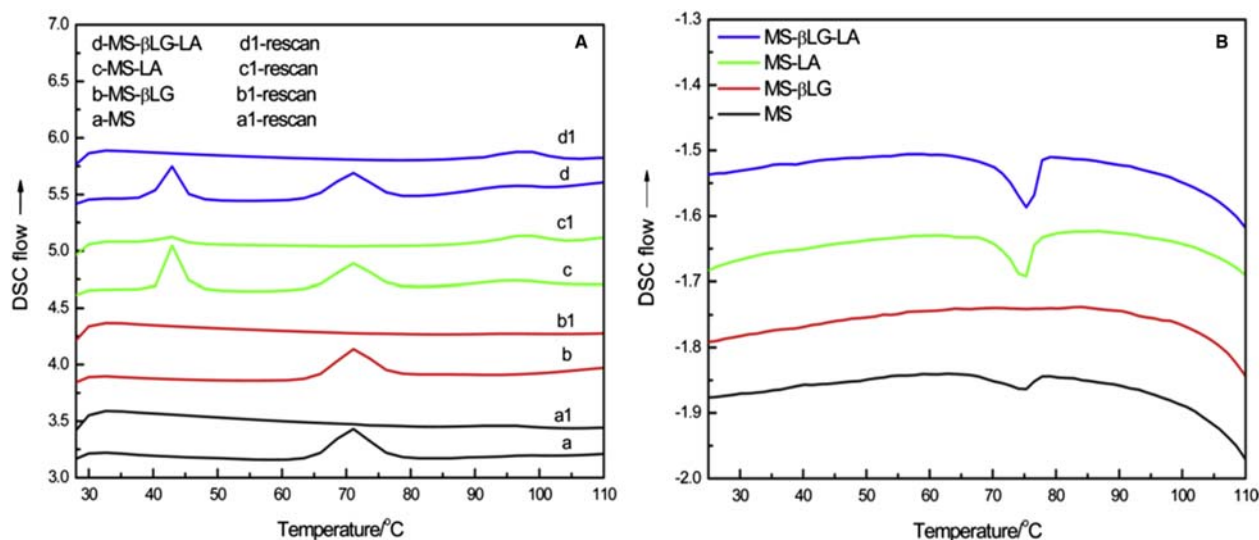


**Figure 3** FTIR spectra of MS, three-component mixture, binary and ternary complex samples. Wang et al. (2017). Reproduced with permission from ACS publications.

gelatinization (65–75 °C) and melting of starch-lipid complex (95–105 °C) (Fig. 4A). Compared with MS, a greater enthalpy change was found for both MS-LA and MS-βLG-LA samples indicating the formation of starch-lipid complex during DSC heating. During the DSC cooling stage, an exothermic transition was observed at 65–80 °C, with the enthalpy changes following the order of MS < MS-LA < MS-βLG-LA (Fig. 4B). These results indicated the formation of an MS-βLG-LA complex during cooling and a more ordered structure of the MS-βLG-LA complex compared with the MS-LA sample. On an immediate DSC reheating of the cooled samples, a small endothermic transition for MS, MS-LA and MS-βLG-LA samples occurred at 90–105 °C. The greatest enthalpy changes for MS-βLG-LA sample further confirmed the more ordered structure of MS-βLG-LA complex compared to the MS-LA complex (Wang et al., 2017).

### The Ordered Structure of the Ternary Starch Lipid-Protein Complex

The ordered structure of starch can be characterized by various analytical techniques, including X-ray diffraction, small-angle X-ray scattering, solid state NMR, FTIR and Raman spectroscopy. X-ray based methods are the only commonly used means to analyze quantitatively and qualitatively the long-range crystalline structure in starch. In contrast, there are a number of methods available



**Figure 4** DSC curves of MS, binary and ternary mixtures. (A) First heating and reheating process. (B) Cooling process. Wang et al. (2017). Reproduced with permission from ACS publications.



to measure the short-range double helical order in starch, for example, NMR, FTIR and Raman spectroscopy. During food processing, the long- and short-range ordered structures of native starch are disrupted, and the gelatinized starch chains regain an ordered structure during subsequent cooling and storage. Such changes vary with the quantity of water (excess or limited amount) as well as the nature of the heating and subsequent cooling processes (Wang and Copeland, 2013; Wang et al., 2015). The formation of a ternary starch-lipid-protein complex during food processing could affect these structural changes of native starch.

### Long-Range Ordered Structure

The long-range ordered structure of starch is often measured by X-ray diffraction technique by which four major types of diffraction patterns of starch, A-, B-, C- and V-type, can be characterized. Starch-lipid complexation can give rise to a typical V-type pattern with two major diffraction peaks at 13 and 20° (2 $\theta$ ). After pasting of starch-LA and starch- $\beta$ LG-LA mixtures in the RVA, four diffraction peaks at 2 $\theta$  values of 12.8 and 19.8° (assigned to V-type amylose-fatty acid crystallites), and 21.3 and 24.2° (assigned to fatty acid aggregates) can be clearly seen in the diffraction profiles of their freeze-dried samples (Fig. 5A). Further, the intensities of peaks at 12.8° and 19.8° were much stronger for the ternary complex than for MS-LA complex, suggesting the presence of V-type crystallites and more ordered structures in MS- $\beta$ LG-LA complex (Wang et al., 2017; Zhang and Hamaker, 2004).

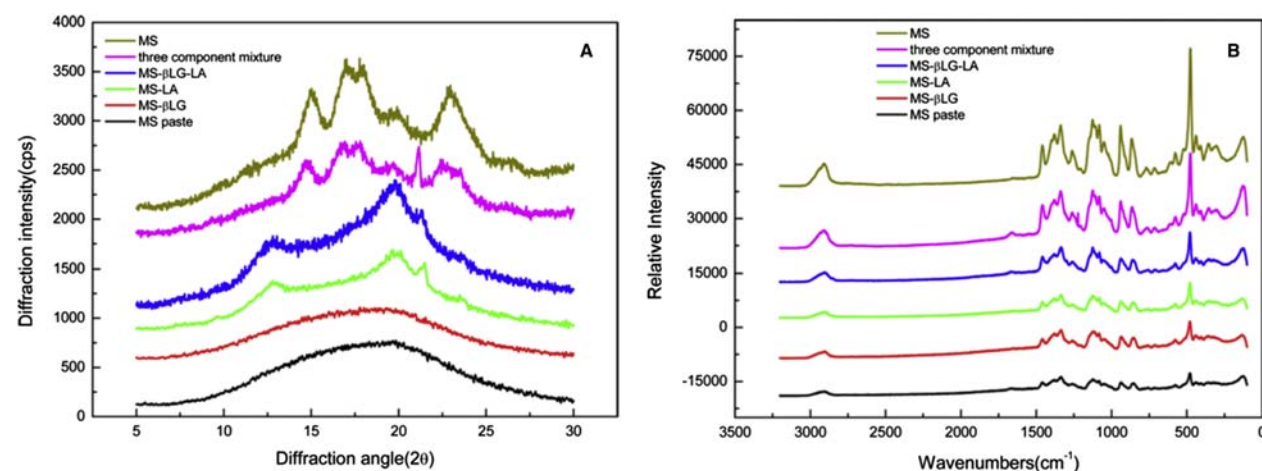
### Short-Range Ordered Structure

The short-range molecular order of double helices in starch can be characterized by Raman spectroscopy (Mutungi et al., 2012; Wang et al., 2016d), and measured based on the full width at half maximum (FWHM) of the band at 480 cm<sup>-1</sup> i.e. a smaller FWHM value indicates a greater degree of structural order (Wang et al., 2016b,d). Native MS sample typically exhibits very intense bands at 480, 865, 943, 1264 and 2900 cm<sup>-1</sup> whilst weaker bands are normally presented in the spectra of gelatinized starch and MS- $\beta$ LG-LA three-component mixture (Fig. 5B). Of the RVA samples, the Raman band intensity ranks in the order of MS- $\beta$ LG-LA complex > MS-LA complex > MS- $\beta$ LG sample. After RVA pasting, the FWHM value of gelatinized starch (22.31) was higher than those of MS-LA and MS- $\beta$ LG-LA samples (16.37 and 16.18, respectively). All these results confirmed the greater short-range ordered structure of the MS- $\beta$ LG-LA complex sample (Wang et al., 2017).

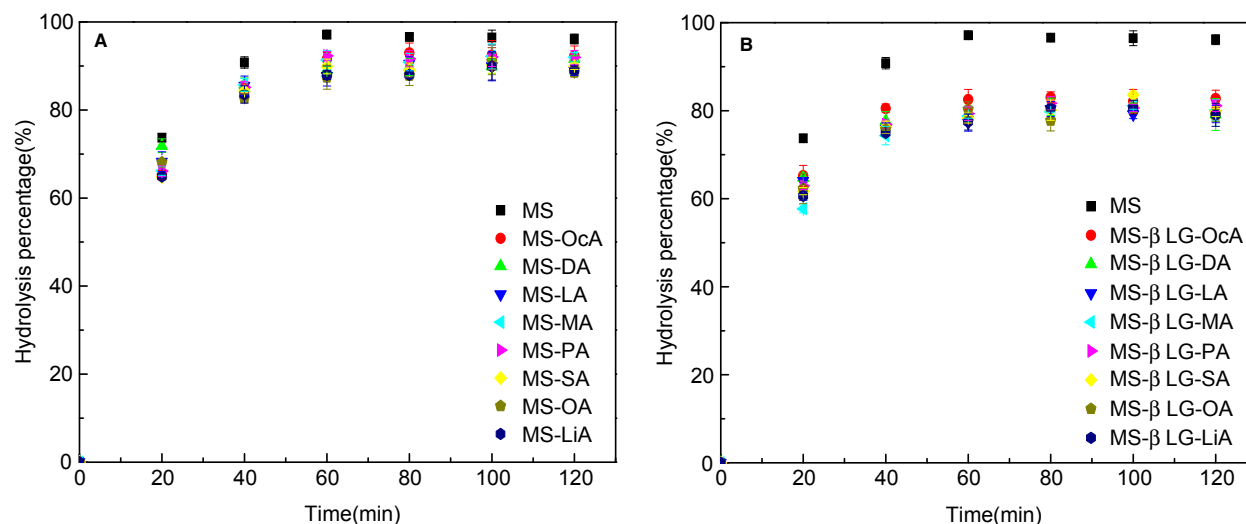
### Digestibility of the Ternary Complex by Amylolytic Enzymes

Carbohydrates including starch are the main energy source in human diet. Thus, the rate and extent of starch digestion to produce glucose for absorption into the bloodstream are of great interest. Native starch is attacked slowly by amylolytic enzymes due to its granular structure, but the susceptibility of the hydrothermally processed starch to enzymatic breakdown would be considerably increased. Treatments of starch such as retrogradation or modification of starch such as complexation with lipids were found to reduce the susceptibility to enzymatic hydrolysis (Copeland et al., 2009; Wang and Copeland, 2013; Wang et al., 2015). The processing-induced formation of ternary complex such as starch-protein-lipid complex could influence the *in vitro* digestibility of starch and *in vivo* glycemic response of starchy foods (Parada and Santos, 2015).

Native MS paste, and the MS-FA and MS- $\beta$ LG-FA complexes obtained after RVA pasting, behave differently during *in vitro* enzymatic digestion (Fig. 6): 90% of MS paste was digested by amylase and amyloglucosidase after a 40-min incubation. Compared with MS paste, the digestion extent of the binary MS-FA complexes at each time points was slightly lower with the digestion after 2 h close to 90% (Fig. 6A). When MS paste, MS-FA and MS- $\beta$ LG-FA complexes were compared, the ternary complex exhibited lower



**Figure 5** XRD patterns (A) and LCM-Raman spectra (B) of maize starch (MS), three-component mixture, and binary and ternary complex samples. Wang et al. (2017). Reproduced with permission from ACS publications.



**Figure 6** *In vitro* starch hydrolysis of pasted MS, MS-FA (A) and MS-βLG-FA (B) complex samples. (Unpublished data from our laboratory).

digestion percentages at each time point with the final digestion reaching about 80% (Fig. 6B). The fact that ternary complexes have a higher resistance to amylolysis than binary complexes is likely owing to the higher structural order and greater steric hindrance resulting from the presence of protein in the ternary complexes.

### The Use of Ternary Complex as Self-Assembled Nanocapsule to Carry Sparingly Soluble Functional Molecule(s)

In the starch-lipid-protein ternary complex, there are still sufficient spare spaces in the lumen of the amylose helices to accommodate the sparingly soluble small molecules (Liu et al., 2009). This characteristic enables the ternary complex as a high potential carrier for the delivery and release of hydrophobic bioactive substances. 1-Naphthol, an insoluble small molecule, has been successfully incorporated into the nanoparticle of ternary complex, and is located in the lumen of amylose helix along with FA molecules (Bhopatkar et al., 2015). Similarly, 5-fluorouracil (5-FU) used to treat cancer could also be incorporated into the hydrophobic lumen of the amylose helix of the ternary nanoparticle (Zhang et al., 2015).

### Conclusions

Mixing starch, FAs and proteins under heating and cooling conditions can induce complex interactions and result in confounding effects including desirable outcomes e.g. amylose-FA complexation, FA aggregation into micelles, melting/solidification of FA, starch retrogradation, protein-starch, protein-FA and starch-protein-FA interactions. The ternary complexes formed among starch, proteins and lipids would have a higher degree of ordered structure compared with the starch-lipid binary complex. The MS-βLG-FA complexes exhibit more resistance to enzymatic digestion than the MS-FA complexes. Further, the starch-lipid-protein complex can be used as carriers for the entrapment of hydrophobic bioactive substances to improve their delivery and release in the human body. Future studies should be directed towards the investigations on the structures and functionality of starch-lipid-protein complexes to obtain a better understanding of how the ternary complexes are formed during food processing and their effects on the quality and nutritional value of final food products.

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- <http://www.starch.dk/isi/starch/index.asp> – 'International Starch Institute'.
- <http://www.livestrong.com/article/488214-starch-and-digestion/> – 'Live Strong'.
- <http://www.nutrientsreview.com/carbs/polysaccharides-starch.html> – 'Nutrients Review'.
- <http://time.com/3754097/rice-calories-starch/> – 'TIME'.

## O/W Emulsions Stabilized by Interactions Between Proteins and Polysaccharides

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### Glossary

**Complex coacervation** is a liquid–liquid phase separation phenomena caused by the complexation between macroions of opposite charge in aqueous solution, by which the insoluble complex formed deposit onto an interface or sediment as a bottom phase.

**Electrophoretic mobility** is the ratio between the observed velocity of migration of a charged solute and the applied electric field strength in a given medium.

**Isoelectric point (pI)** the isoelectric point of a protein is the pH at which the net charge of the molecule is zero, i.e., the protein has a neutral charge.

**Maillard reaction** is a cascade of consecutive and parallel complex reactions initiated between an amino group on a protein, peptide, or amino acid and the  $\alpha$ -hydroxy carbonyl moiety on a reducing sugar; at its initial phases the Maillard reaction produces two intermediate products, the Amadori and Heyns products; in the final stage, melanoidins and brown pigments are formed.

**Steric stabilization** refers to the stabilization of colloidal particles involving non-ionic macromolecules that are attached to the particle surface. Each particle becomes surrounded by a layer of solvated polymeric molecules and, when approaching, two particles overlap and penetrate these polymer layers, in such a way that the increased polymer concentration in the overlapping region and the osmotic pressure favour the solvent transportation to this area, causing the particles be repelled away from each other again. At the same time, the polymer molecules have their conformation restricted in the overlapping area, which leads to entropy decreasing and, consequently, to a repulsion potential.

**Zeta potential** is the electric potential that develops between the surface of a solvated particle and the liquid solvating medium, measured at the shear plane, i.e., at the boundary between the edge of the solvated layer and the bulk liquid; it is also known as the electrokinetic potential and is measured in millivolts.

### Introduction

Oil-in-water (O/W) food emulsions are dispersed systems in which oil, generally a triglyceride oil, is dispersed in small droplets into a continuous aqueous phase. Common examples of O/W food emulsions include milk, cream, mayonnaise, salad dressings, sauces, and soups. In spite of their intrinsic thermodynamic instability, emulsions may be kinetically stabilized in the presence of surfactants that lower interfacial tension, help breaking up larger droplets into smaller ones, and prevent coalescence. Surfactants may be classified, regarding their molar mass, as small amphiphilic molecules - such as monoacylglycerols and phospholipids -, or as polymeric surfactants, mainly represented by proteins (Walstra, 2003).

Proteins, as well as polysaccharides to some extent, have significant advantages over monomeric surfactants because they present several adsorption sites that can anchor simultaneously onto the oil-water interface, forming viscoelastic films around the dispersed droplets with improved resistance to mechanical stresses, in addition to providing steric stabilization. These attributes turn them the preferred emulsifiers in many food systems. Even so, new and very suitable amphiphilic structures can be produced from interactions between proteins and polysaccharides, with the resulting complexes being able to replace the synthetic low molar mass emulsifiers. These association colloids do not require special legislation and may have textural and stability advantages. The interfacial functionality in emulsions is achieved not only from the direct adsorption on the oil–water interface during emulsification, but also from the established associative interactions with the stabilizing protein layer after the emulsion formation (Benichou, Aserin, and Garti, 2002; Dickinson, 2003). To better understand the role of protein-polysaccharide interactions in stabilizing O/W emulsions, it is useful to examine the mechanisms by which proteins alone exert their interfacial activity.

### Proteins as Interfacial Stabilizing Agents

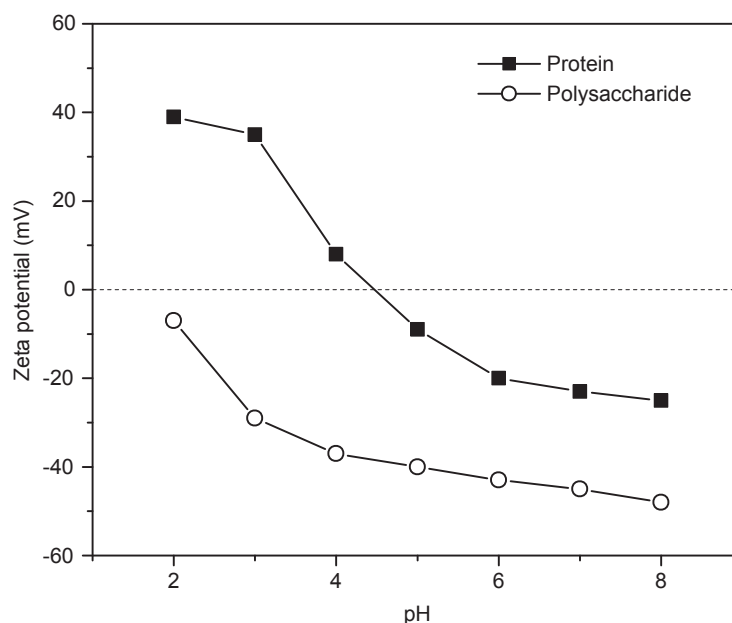
The ability of proteins to act as emulsifiers is related to their amphiphilic character, which in turn derives from the number of polar and nonpolar surface groups present in the polypeptide chain. The number of neutral, anionic, and cationic amino acids present in a protein molecule depends on the protein type and source, and on the extraction process, whereas the exposed hydrophilic and lipophilic groups may also be affected by the solvent properties (pH, ionic strength), temperature, and denaturation extent. A

greater number of hydrophilic sites increase the protein solubility, which positively affects the diffusion toward the oil-water interface during emulsion production. On the other hand, the presence of lipophilic groups is important to allow the protein adsorption on the oil side of the interface, which leads to formation of a viscoelastic interfacial film (Damodaran, 1996; Lam and Nickerson, 2013).

Proteins are polyelectrolytes, i.e., they contain electrically charged groups, including both positively (basic) and negatively (acidic) charged ones. The surface charge of polyelectrolytes may be described by the zeta potential, or electrokinetic potential, which is related to the electrophoretic mobility and gives a measure of the net charge on the surface of the macromolecule. Important properties of colloidal systems are determined by the electrical charge on the particle surface, as the electrical potential distribution determines the interaction energy between the particles and this, in many situations, determines if the particles will be stable in suspension or, otherwise, will undergo coagulation and subsequent destabilization (Hunter, 1981). In proteins, the zeta potential varies as a function of pH and changes from high positive charge at low pH, passing through neutrality at the isoelectric point (pI) of the protein, and attaining highly negative values at alkaline conditions (Fig. 1). Knowledge of the pH that corresponds to the protein pI, which may be affected by the ionic strength of the aqueous medium, is important to determine the conditions of stability/instability of protein stabilized O/W emulsions, since at  $\text{pH} \approx \text{pI}$  the protein molecules will present a net zero charge and, in the absence of a sufficiently thick protein layer or of a complementary stabilization mechanism, probable droplet aggregation will take place (McClements, Bai, and Chung, 2017).

At this point it is important to distinguish between the roles played by emulsifiers and stabilizers. The emulsifying agent is responsible for rapidly and substantially lowering the interfacial tension at the oil-water interface, permitting formation of large interfacial area due to a great number of small dispersed droplets, which requires adsorption of the amphiphilic molecules onto the droplet surface at a sufficient concentration. Once the fine emulsion has been produced, the aggregation/flocculation and coalescence of droplets must be prevented in order to attain long-term stability. This is possible by the action of stabilizers whose performance is based on steric and electrostatic phenomena; the short-range steric interactions are important for preventing coalescence, while the electrostatic interactions, which are short-to-long range in nature, work by hindering droplet flocculation. In order to provide steric stabilization it is desirable that, in addition to the hydrophobic groups that will keep permanently attached to the oil droplets' surface, the macromolecular structure at the oil-water interface presents a large fraction of hydrophilic chain segments that will protrude from the surface and increase the thickness of the stabilizing layer. At the same time, if this macromolecular structure contains charged groups that contribute to increasing the net repulsive electrostatic interactions, it would help to prevent aggregation of adjacent droplets caused by the attractive van der Waals forces (Dickinson, 2003; Ozturk and McClements, 2016).

Proteins alone may be unsuitable to fulfil all the requirements to providing extended stability to emulsions, as the interfacial coverage formed around oil droplets by protein molecules is often not thick enough. In addition, the functional properties of proteins may be lost due to aggregation and precipitation that occur under acidic conditions near their isoelectric point, at high concentrations of electrolytes (high ionic strength), at high temperatures, and in the presence of organic solvents. In order to overcome these adverse factors, promising alternatives to enhance the emulsion stabilizing properties of proteins are showing to be feasible by taking advantage from their potential interactions with charged polysaccharides (Semenova, 2017; Akhtar and Ding, 2017).



**Figure 1** Typical behavior of zeta potential of proteins and polysaccharides as a function of pH.

## Nature of Protein–Polysaccharide Interactions

Proteins and polysaccharides may interact forming supramolecular complexes by two possible mechanisms: electrostatic interactions or covalent bonding. Both types of supramolecular structures will be able to combine the surface activity of proteins, which will adsorb onto the oil–water interface of the dispersed droplets, with the ability of polysaccharides to interact with the proteins and to increase the thickness of the stabilizing layer. In addition, the polysaccharides possess hydrophilic chain segments that will protrude from the surface enhancing steric stabilization, as well as binding great amounts of water and thus contributing to emulsion stabilization by increasing the viscosity or even causing gelation of the continuous aqueous phase. Both types of protein–polysaccharide interactions will be discussed as follows.

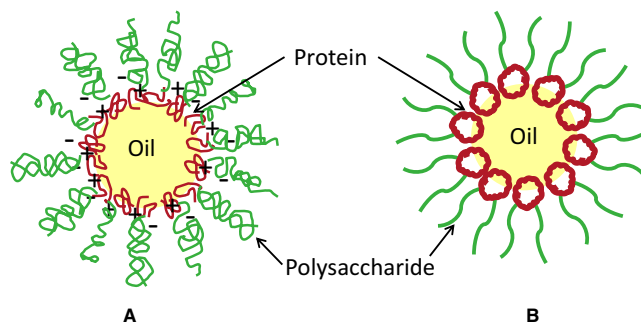
### Electrostatic Complexes Between Proteins and Polysaccharides

When mixed in a solution, protein and polysaccharides may interact with each other or remain freely dispersed, depending mainly on their surface charge and on the physical–chemical properties of the solvating medium, including pH and ionic strength. The biopolymer interactions can lead to two possible situations: segregative phase separation or associative phase separation. The segregative phase separation prevails when the biopolymers have similar electrical charges or they are neutral, and occurs when the biopolymer concentration exceeds a certain critical level. On the other hand, the biopolymers may associate with each other through relatively strong attractive electrostatic interactions. In turn, the resulting molecular complexes from these attractive interactions may be soluble, i.e. they remain dispersed in the solution with no macroscopic phase separation, or otherwise may be insoluble, leading to the formation of a sediment phase rich in biopolymers and an aqueous phase that is depleted in biopolymers (Moschakis and Biliaderis, 2017).

The insoluble supramolecular structures resulting from associative electrostatic interactions are known as complex coacervates and require two polymers with opposite charges - a polyanion and a polycation, whereas soluble complex formation may occur between an overall positively charged protein presenting a negative charge patch and a cationic polysaccharide (Kayitmazer, 2017). Even though, in most of the conventional situations, an anionic polysaccharide is used in combination with a protein bearing positive charge below its isoelectric point. The negative charge of polysaccharides is commonly due to the presence of carboxyl or sulfate groups. The only naturally occurring cationic polysaccharide is chitosan and in this case electrostatic interaction will take place with a protein at a pH above its pI. Regarding the ionic strength effect, in general, increasing salt concentration has a negative effect on polyelectrolyte complexation and above a critical salt concentration the attractive interaction is inhibited (Moschakis and Biliaderis, 2017; Evans, Ratcliffe, and Williams, 2013).

Both soluble electrostatic complexes, as well as the complex coacervates may be applied as emulsifiers/stabilizers in O/W food emulsions, although the insoluble complex coacervates have been mostly used to produce biopolymer-based particles, mainly to microencapsulation.

It has been recognized that even non-surface-active polysaccharides could contribute to interfacial stabilisation of an O/W emulsion if it can form an outer steric stabilising layer by electrostatic complexation with a protein adsorbed on the oil droplet interface (Fig. 2a) (Dickinson, 2013). Nevertheless, there are different approaches for producing protein–polysaccharide stabilized O/W emulsions and the resulting functionality of the complexes may vary depending on the production route adopted. Both biopolymers can be initially complexed in the absence of an oil phase, which will only be added at the end of the process then producing the so-called mixed emulsions. Alternatively, in the layering, bilayer, or double-layer approach, the protein forms the inner adsorbed layer on the primary emulsion droplets and a polysaccharide dispersion is subsequently added to the system to induce the formation of a complexed polysaccharide outer layer. The double-layer method is the most commonly used at industrial scale, despite it has been shown that the stability of a mixed emulsion prepared with soluble sodium caseinate – dextran sulfate complexes was greater than that of a bilayer emulsion prepared with the two-step method, in which bridging flocculation was observed (Jourdain, Leser, Schmitt, Michel, and Dickinson, 2008; Schmitt and Turgeon, 2011; Evans, Ratcliffe, and Williams, 2013).



**Figure 2** Schematic representation of oil droplets stabilized by (a) protein–polysaccharide electrostatic complexes and (b) protein–polysaccharide conjugates.

## Protein–Polysaccharide Conjugates

The most promising example of protein-polysaccharide complexes involving covalent bonding with good performance on emulsion stabilization is the Maillard-type conjugates, in which a polysaccharide is attached to a protein via the Maillard reaction and forms a permanently bonded conjugate. The Maillard reaction is a complex series of nonenzymatic reactions initiated between the amino groups of proteins and the reducing end carbonyl groups of polysaccharides (Troise, 2018; Oliveira, Coimbra, Oliveira, Zuñiga, and Rojas, 2016; Yaylayan, 1997). The protein-polysaccharide conjugates produced through the Maillard reaction have demonstrated ability of stabilizing O/W emulsions even under stressing conditions, such as high ionic strength, low pH, and along thermal and freeze-thaw processing. The improved emulsifying properties appear as a consequence of the enhanced steric stabilization provided by the bulky hydrophilic polysaccharide moiety attached to the protein (Fig. 2b). In fact, the parameter that most significantly improves emulsifying properties of protein-polysaccharide conjugates is the increasing molecular weight of the polysaccharide chain (Chen, Ji, Qiu *et al.*, 2018; Akhtar and Ding, 2017; Akhtar and Dickinson, 2007).

It is interesting to note the existence of naturally occurring protein-polysaccharide conjugates, including  $\kappa$ -casein that contains small side chains consisting of 3 or 4 sugar moieties that are thought to be crucial in stabilizing colloidal casein micelles in milk. Another typical example is gum Arabic that is largely applied as emulsifier/stabilizer in soft drinks and in beverage emulsions, and in which the proteinaceous fraction makes about 2% of the total polysaccharide. The relatively small fraction of proteins present in these few natural conjugates has contributed to the interest of generating alternative glycosylated proteins to induce the Maillard reaction (Ettelaie, Zengin, and Lishchuk, 2017).

The most used method for producing protein-polysaccharide conjugates is through dry thermal processing, although wet-heating has also been explored. In a typical process under dry heating conditions, the selected protein (mostly caseins, whey, albumin, soy or pea proteins) and polysaccharide (usually dextran, maltodextrin, or galactomannans) are mixed in a solution at the desired proportion, subjected to pH adjustment to slightly alkaline values, and freeze-dried. The powder is then heated at a controlled temperature and relative humidity for a given period. Heat energy input is essential for the Maillard reaction development and the rate reaction increases with temperature, which is typically set around 60 °C – 80 °C. Water activity has a major impact on the reaction rate that is maximal at water activity in the range of 0.3 to 0.7. The reaction time, of at least a few hours, must be carefully controlled in order to avoid the formation of the potentially harmful advanced glycation end-products that occurs at the advanced stages of the Maillard reaction (Ettelaie, Zengin, and Lishchuk, 2017; Oliveira, Coimbra, Oliveira, Zuñiga, and Rojas, 2016). Alternatively, wet-heating methods have been tested to produce protein-polysaccharide conjugates aiming to maximize the contact between protein and polysaccharide, as well as eliminating the freeze-drying step that is energy and time consuming. Recent approaches have included utilization of a spinning disc reactor (SRD) and microwave heating (Akhtar and Ding, 2017; Nasrollahzadeh, Varidi, Koocheki, and Hadizadeh, 2017).

## Conclusion

Independently of the mechanism by which proteins and polysaccharides interact forming supramolecular complexes, their complementary properties of emulsifying and stabilizing O/W emulsions have nourished plenty of research work aiming to clarify the various aspects of interest regarding their understanding and applications, which include thermodynamics, functional properties, processing approaches, and others. This will certainly enable the increasing tailored use of protein-polysaccharide pairs to improve food stabilization, enhancement of textural properties, and creation of reduced-fat food systems, microencapsulation of bioactive compounds, as well as other potential applications.

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## Changes in the Interactions Between Proteins and Other Macromolecules Induced by HPP

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### Glossary

**High pressure processing** This is a non-thermal technology for food preservation and food manufacture that found an early application in the food industry for extending the shelf life of foods.

**Storage ( $G'$ ) and loss ( $G''$ ) moduli** These are the indication of the hydrogel's ability to store deformation energy in an elastic and viscous manner and show solid-like property and liquid-like property respectively.

### Introduction

Proteins are a very important group of food component of the human diet, as they are essential to the maintenance of muscle mass, immune responses, cell signaling and repair of damaged cells (Lin et al., 2017). They also contribute to color and flavor developments in foods. However, the structures of single protein formed gels or films are always fragile. Proteins combined with other biopolymers, e.g., polysaccharides or proteins, to form functional complexes are widely used for improving the functionalities of proteins. In complex food systems, the interactions between proteins and other macromolecules will inevitably take place in a variety of ways. These interactions can potentially have great influences on the structures and properties of food products.

High pressure processing (HPP) is one of the promising non-thermal preservation techniques and has the advantage of satisfying the increasing consumer demand for food products having highly retained sensory and nutritional qualities during food processing in industrial settings (Knorr, 1996). HPP could extend the shelf life of food and improve the safety of food products (Knorr, 1996). In addition, HPP could also induce modification of macromolecular arrangements during protein denaturation, starch gelatinization, or other interactions between food ingredients (Devi et al., 2013a). The extent of macromolecular rearrangements caused by HPP is dependent on macromolecule's intrinsic properties, physicochemical environment, applied pressure level and temperature, duration of pressure treatment (Michel and Autio, 2002). This chapter discusses the changes in rheological properties, gel properties, surface/interfacial properties and structure owing to the interactions between proteins and other macromolecules (e.g., proteins, polysaccharides) induced by HPP.

### Changes in the Interactions Between Proteins and Proteins Induced by HPP

Applying HPP to protein molecules in solution can have a strongly disruptive effect on electrostatic and hydrophobic interactions causing denaturation and aggregation of proteins (Galazka et al., 2001). High pressure effects on proteins are mainly related to the rupture of non-covalent interactions between and/or within protein molecules, and the subsequent reformation of intra- and inter-molecular bonding. Many quaternary structures show complex behaviors, such as dissociation, followed by aggregation of subunits or precipitation upon a high pressure treatment (Apichartsrangkoon and Ledward, 2002). Changes in secondary structures occur at a very high pressure and likely lead to irreversible denaturation. Interactions of proteins at the oil-water or air-water interfaces can help maintain the stability of emulsions or foams, whilst the interactions between protein molecules in aqueous solutions are essential to the formation of protein gels and films (Lin et al., 2017). The protein-protein interactions induced by HPP can bring new functional properties of food products. Protein-protein interactions have been well investigated with the objectives of examining structure-function relationships, improving food quality, and developing new products. HPP is one of the useful technologies to enable changes in protein-protein interactions.

### Changes of Rheological Properties

Protein-protein interactions and the resulting changes of rheological properties (e.g., viscosity and flow behavior) depend greatly on the type of protein, protein concentration and applied pressure. In the study on the gluten and soy mixtures treated at 700 MPa by HPP (Apichartsrangkoon and Ledward, 2002), both high temperature and pressure were found to increase the strength of storage ( $G'$ ) and loss ( $G''$ ) moduli of all the gluten-soy mixtures, especially the mixtures having high gluten concentrations. The  $\tan \delta$  values, which are the ratios of loss ( $G''$ ) to storage moduli ( $G'$ ), were low for all the soy protein-rich samples, although the samples containing high amounts of gluten exhibited higher  $\tan \delta$  values at frequencies in the range 1–10 Hz. The HPP treatments affect

neither the flow behavior of gelatin alone, nor that of the skim milk-gelatin mixtures made with  $\sim 0.4\%$  weight/weight (w/w) gelatin. However, at a gelatin concentration  $\sim 0.4\%$  w/w, the mixtures treated with 300 and 450 MPa exhibited a peculiar flow behavior. The viscosity remarkably increased in the milk-gelatin mixtures prepared with a high milk solid concentration (15% and 20% w/w) following a HPP treatment at 300 MPa, whereas, HPP at 600 MPa caused a decline in viscosity (Hemar et al., 2010).

### Changes of Gel Properties

Gels are a kind of special decentralized systems in which molecules are connected to each other to form a network structure under certain conditions. Gaps in the networks may be filled with liquid or gas as a dispersed phase (Lin et al., 2017). Mixing different proteins to form gels is a good way to improve the sensory and nutritional properties of foods. The concentration of one protein in protein-protein mixtures should be high enough to act as a filler to fill the gaps in the networks formed by the other protein. In addition, pressure and temperature may influence the protein-protein gel properties. Mixed systems subjected to HPP in aqueous solutions had higher  $G'$  values than the mixed systems (e.g. whey protein and gelatin) subjected to HPP in the form of gels (Devi et al., 2014). The cooling profile of  $G'$  in mixed solutions was similar to that of the gelatin solution treated by HPP, which indicates that HPP could lead to a high degree of gelatin continuity (Devi et al., 2014). Confocal imaging analyses confirmed that the gelatin was in a continuous phase whilst whey protein aggregated in discontinuous inclusions within the mixed systems treated by HPP (Devi et al., 2014). High pressure applications at temperatures below the thermal denaturation of whey protein (i.e.,  $<65^\circ\text{C}$  at pH 6.8) permitted gelatin to maintain its continuity within the mixed systems (Devi et al., 2014). The microstructure of the mixed gel (whey protein and gelatin) at 600 MPa for 30 min was different from the single gel, possibly due to an associative phase separation (Devi et al., 2014). The  $G'$  value of mixed gel was still higher than that for the pure whey protein gels (Devi et al., 2014).

### Changes of Surface/Interfacial Properties

The surface/interfacial properties include the emulsification and foamability. Vegetable proteins (e.g., soybean protein, pea protein and gluten) and dairy proteins (e.g., casein and whey) are widely used as emulsifiers. There is a growing interest in mixing vegetable proteins with animal proteins or utilizing vegetable proteins instead of animal proteins for emulsification (Karaca et al., 2011). The thermal stability of mixed protein-stabilized emulsions can be increased due to protein-protein interactions. However, emulsions stabilized by mixed proteins are still sensitive to certain extreme conditions (e.g., some HPP treatments) (Lin et al., 2017).

High pressure-low temperature (HPLT) and HP (high pressure) treatments led to a decreased emulsion stability for emulsions derived from whey protein (WPI) solutions independent from a pH treatment, while the foaming stability was increased for these samples (Baier et al., 2015). The large flocs, which were induced in samples containing a high amount of micellar casein (MC), influenced greatly the rheological behaviors of these samples, while exerting no specific effect on foam and emulsion stability (Baier et al., 2015). HPLT treatments of MC-rich samples always led to the formation of some very large flocs which had a major influence on the functionalities. The treatments on mixtures could lead to varied results in comparison to pure dispersions which indicate the high influence of interactions between MC and WPI (Baier et al., 2015). The emulsifying capacity decreased after a HPP treatment at 200 MPa on myofibrillar proteins-egg white mixtures (Barrios-Peralta et al., 2012).

### Changes of Structure

Protein-protein interactions in protein solutions may lead to phase separation, synergistic interaction and aggregation (Firoozmand and Rousseau, 2015). In most cases, a mixture of two proteins will lead to phase separation, e.g., coagulation and segregation. When phase separation occurs, two proteins form independent phase-separated networks thus preventing the assembly of a uniform network structure (Sarbon et al., 2015). A mixture of two oppositely charged proteins can result in aggregation induced by electrostatic attraction. Synergistic interactions can lead to more desirable products with a uniform structure than those formed by each individual protein alone (Lin et al., 2017). Very complicated changes in structure owing to protein-protein interaction can be observed during HPP (Devi et al., 2013b).

The type of protein has an enormous effect on protein-protein interactions in solutions treated by HPP. The structures of the gluten-soy mixtures were very firm at 700 MPa and  $60^\circ\text{C}$ , exhibiting solid-like behaviors and a highly permanent cross-link density (Apichartsrangkoon and Ledward, 2002). The detected variations in structural modification of the gluten-soy mixtures were mainly due to the gluten rather than soy protein. Disulphide bonds contributed to the final structure of gluten-soy mixtures treated at a high temperature; however, other non-covalent bonds may also be involved. In the presence of gelatin, the state of the dissociated casein micelles is different to that of in the presence of skim milk alone. This was due to ruptured aggregates and phase separation as confirmed by confocal laser scanning microscopy (Devi et al., 2013b). Application of an intermediate to high pressure (300–600 MPa) results in changes in microstructure of the mixture depending on the milk solid content (Devi et al., 2013b). It appeared that milk solids occurring at levels of industrial interest ( $<20\%$  w/w) would lead to enhanced gelatin structure formation at refrigeration temperature during HPP (Devi et al., 2013b).

Pressure is also one of the most important factors that can significantly influence the protein-protein interactions in solutions treated by HPP. If the pressure applied is 150 MPa, extensive dissociation of the casein micelle does not occur and only the phase separation was observed in the skim milk-gelatin mixtures (Hemar et al., 2010). When the gelatin concentration is not lower than the critical concentration at which a gelatin gel is formed, the casein submicelle network would be entrapped in the gelatin network

(Hemar et al., 2010). If the gelatin concentration is lower than the critical concentration, then the system behaves as in good solvent conditions and the submicelle clusters can rearrange upon decompression. When the hydrostatic pressure applied is higher than 450 MPa, the submicelles do rearrange into more compact submicelle aggregates (Hemar et al., 2010).

## Changes in the Interactions Between Proteins and Polysaccharides Induced by HPP

Two main types of food macromolecules, proteins and polysaccharides, are often present together in a wide range of food products. Proteins and polysaccharides have a profound effect on the shelf life and texture of food products. Proteins are commonly recognized for their emulsifying and foaming properties whereas polysaccharides are known for their water-holding characteristics. Polysaccharides are used in admixture to proteins mainly to enhance stability of dispersed systems. Most high molecular weight polysaccharides are hydrophilic in nature and do not adsorb to the air–water interface. However, they can strongly enhance the stability of protein foams. The changes in protein-polysaccharide interactions under various conditions can be examined by measuring the rheological properties of resultant food fluids. Proteins and polysaccharides can form fine complexes via covalent bond(s) and/or non-covalent bond(s) (Ji et al., 2015). The covalent bonding, in this case, takes place during the Maillard reaction (Liu et al., 2012). The non-covalent bonds include hydrogen bond and electrostatic attraction. Generally, uncharged polysaccharides can form complexes with proteins mainly via hydrophobic interactions, whereas for ionic polysaccharides, complexes mainly are formed via electrostatic interactions (Lin et al., 2017). HPP can also influence the interaction between proteins and polysaccharides; hence, increasing interests have been attracted to the effect of HPP on systems containing mixed proteins and polysaccharides.

### Changes of Rheological Properties

HPP can cause changes in the rheological properties of protein-polysaccharide mixtures and the changes depend on the type, concentration, ratio of polysaccharide and protein, applied pressure and time. HPP treatment of sweet potato protein (SPP) in the presence of guar gum (GG) showed improvement in viscosity (Khan et al., 2015). The effects of HPP (800 MPa and 20 °C for 20 min) on the rheology of LMP (low methoxy pectin)/MC (micellar casein) mixtures depend on their concentrations and ratio (Abbasi and Dickinson, 2002a). The mixed gels subjected to high pressure displayed increasing  $G'$  and  $G''$  throughout the MC concentration range (0.5%–12% w/w) (Abbasi and Dickinson, 2002a). The enhancement of  $G'$  was likely due to either the intensified interaction between the positively charged parts of casein micelles and the negatively charged polygalacturonan chains, or the attachment of  $\text{Ca}^{2+}$  into the gap between the pectin backbones under pressure. However, the  $G'$  recovered at a high percentage of MC (12%–14% w/w), possibly because a network of caseins was developed upon pressure release (Abbasi and Dickinson, 2002a). An enhancement of  $G'$  was also found for skim milk powder (SMP) dispersions supplemented by LMP following HPP, especially when LMP (0.5% w/w) was mixed with SMP dispersions with MC ranging in 2%–8% and at 800 MPa (at 20 °C for 20 min) (Abbasi and Dickinson, 2002b). High pressure homogenization reduced the viscosity of hydroxypropyl cellulose (HPC)-casein complexes regardless of the molecular weight of HPC (Ye and Harte, 2014).

### Changes of Gel Properties

Proteins and polysaccharides are gelling agents mainly responsible for gelation, and for this reason, they play important roles in the food industry. Gels formed by mixed polymers usually have better mechanical properties than those formed by a single polymer due to the interactions between different polymers (Hou et al., 2015). The properties and concentration of polysaccharides would have great influences on the structures and properties of protein-polysaccharide gels. In addition, pressure, time and temperature can also influence the properties of a protein-polysaccharide gel.

High-pressure homogenization (HPH) delayed gelling of some mixed systems (e.g. peanut protein isolate (PPI)-chitosan, PPI-xanthan gum and PPI-guar gum mixtures), through the rupture of the molecular chains of PPI and polysaccharides (Jiao et al., 2018). A stronger gel network was formed for the PPI-xanthan gum mixed system as compared to that of PPI-guar gum mixtures, and the solid-like structure of the former system was unaffected by heating (Jiao et al., 2018).

The exudation of pressure-induced  $\beta$ -lactoglobulin gels (12% w/w and pH 7) could be prevented by incorporating 0.1% w/w of certain polysaccharides, i.e., LMP (low methoxy pectin), HMP (high methoxyl pectin), or Na-alginate (Dumay et al., 1999). When the concentration of polysaccharide is too high (i.e., 1% w/w), either no gel was formed (for  $\beta$ -lactoglobulin + Na-alginate), or a gel with poor water retention was obtained (for  $\beta$ -lactoglobulin + LMP), following 30 min treatments at 450 MPa and 25 °C.

The gelifying effect was studied through pressure treatments, at room temperature or 50 °C, on mixed systems of whey protein/polysaccharide ( $\kappa$ -carrageenan, xanthan gum and HMP) (Fernandes and Raemy, 1996). At room temperature,  $\kappa$ -carrageenan was the most efficient gelifying agent in whey protein/polysaccharides systems; however, it was no longer the case at 50 °C. A higher pressure did not always lead to a stronger gel. Xanthan gum and HMP were as efficient as  $\kappa$ -carrageenan at 50 °C. Large protein lumps enclosed by a pectin-rich phase were observed following an intense HPP treatment at 800 MPa. Due to the phase separation of protein and pectin, the concentrated pectin phase was probably the cause for the rise in viscosity of pressure treated mixtures whilst the protein network was developed during protein gelation (Fernandes and Raemy, 1996).

### Changes of Surface/Interfacial Properties

Emulsions stabilized by proteins combined with polysaccharides usually show better heat stability than those stabilized only by proteins. Generally, polysaccharides cannot adsorb onto the surface of oil droplets and accordingly cannot stabilize emulsions. However, they can improve the stability of emulsions in association with proteins. Among vegetable proteins, soy protein isolate (SPI) is the most frequently used protein as a foaming stabilizer due to its favorable foaming ability and potential health benefits (Lin et al., 2017). Interactions between proteins and polysaccharides at interfaces can enhance the foamability of proteins adsorbed onto interfaces (Baeza et al., 2005). HPP can also cause changes in the interfacial properties of protein-polysaccharide mixtures (Galazka et al., 2001).

The effects of dynamic high-pressure homogenization (HPH) on the interfacial and foaming properties of SPI and Methocel (E4M and E15) with different molecular weights were studied (Martínez et al., 2011). The obtained results showed that the interactions between SPI and polysaccharides apparently favored the foam overrun of untreated mixed systems. This interactive effect was promoted using HPH particularly in the case of E15 at 300 MPa. The effect of SPI-E4M was less pronounced than that observed for E15. Thus, the molecular weight of PS was a very important factor for the interaction with SPI under these high pressure conditions.

High pressure treatment of 11S globulin (11S) and  $\iota$ -carrageenan ( $\iota$ -CAR) or  $\kappa$ -carrageenan ( $\kappa$ -CAR) mixtures was found causing a remarkable improvement in emulsifying efficiency and emulsion stability (Galazka et al., 1999b). When  $\iota$ -CAR was added to 11S, the mixture gave a higher surface tension value ( $\gamma$ ) than did the untreated 11S alone. High pressure treatment of the 11S +  $\iota$ -CAR mixture led to lower  $\gamma$  values than native 11S, but a higher  $\gamma$  than pressure processed 11S alone. The tension data shown for the 11S +  $\iota$ -CAR mixtures are indicative of complexation between the protein and polysaccharide in the bulk solution, which perturbs the dynamic equilibrium of protein between bulk and interface in favor of the bulk. High-pressure treatment (200 MPa for 20 min) induces the formation of a more viscous steric stabilizing layer in a strongly time-dependent manner. The presence of  $\iota$ -CAR added to native 11S globulin at a low ionic strength was found to give emulsions with smaller droplets (whose size decreased with increasing polysaccharide concentration and degree of pressure treatment) (Galazka et al., 2001).

HHP treatment of sweet potato protein (SPP) in the presence of guar gum (GG) increased the stability of emulsions and decreased the creaming rate as compared to the control (Barrios-Peralta et al., 2012). Protein-starch interaction could increase the emulsifying capacity at pressures over 350 MPa for 3–5 min and the emulsifying capacity also increased in samples with agar (Barrios-Peralta et al., 2012).

The influence of high pressure (200–600 MPa) on surface tensions and surface shear viscosities of protein layers adsorbed at pH 6.5 or 8.0 from the mixtures of sulfated polysaccharides with  $\beta$ -lactoglobulin, bovine serum albumin (BSA) or ovalbumin (OVA) was studied (Galazka et al., 2001). The experimental interfacial tension for  $\beta$ -lactoglobulin + dextran sulphate (DS) indicated weak electrostatic complexation, whereas mixtures of BSA or OVA with DS were likely associated with strong protein-polysaccharide complex formation in bulk solutions. While the interactions of proteins with the polysaccharides might have only a small influence on the surface viscosities of the adsorbed proteins, they behaved like the strongest interaction for the most sulfated polysaccharide (DS). The pressure processing induces the dissociation of reversible electrostatic protein-polysaccharide complexes, and during the pressure treatment the protein was partly denatured and thus causing more charged groups exposed. When pressure is released, electrostatic protein-polysaccharide interactions could be strongly restored, as a result, re-complexation of the unfolded protein with polysaccharide is possible (Galazka et al., 2001).

### Changes of Structure

Thermodynamic incompatibility between proteins and polysaccharides often leads to separation, but two separate network structures formed by segregation can still form a rigid structure by physical or chemical force-driven intertwining. Properties of proteins and polysaccharides (e.g., charge density, molecular weight and branched chain) and their concentrations or ratio have a big influence on the protein-polysaccharide network structures (Lin et al., 2017). Pressure, time and temperature used during HPP can also influence the structure of protein-polysaccharide.

$\beta$ -Lactoglobulin at pH 7 in the presence or absence of dextran sulphate (DS) was subjected to a combined pressure and temperature treatment (pressures up to 280 MPa, temperature up to 60 °C and time up to 30 min) (Galazka et al., 1996). The treatments with combinations of moderate pressures and temperatures can result in distinct structures. Even under moderate processing conditions, it is still possible to optimize the processing parameters to obtain significant structural changes that potentially lead to functional advantages. The presence of DS could apparently reduce the degree of BSA unfolding induced by pressure but not for  $\beta$ -lactoglobulin, suggesting that DS either protected the protein against pressure-induced unfolding or enabled the pressure-denatured protein to regain some secondary structures.

Addition of a polysaccharide (DS or  $\iota$ -carrageenan) to the native protein at a low ionic strength leads to no change in fluorescence intensity (Galazka et al., 1999a). The presence of DS in the thermally processed samples appeared to inhibit the formation of aggregates. Under pressurization ovalbumin could form electrostatic complexes with DS or  $\iota$ -CAR at a low ionic strength and pH  $\leq$  7.0. As with BSA, the strength of protein-polysaccharide complex appeared to be dependent on the charge density of sulphate group on the polysaccharide (DS,  $\iota$ -CAR), and the extent of the interaction between ovalbumin and DS or  $\iota$ -CAR was enhanced during the pressure treatment at pH 6.5 (Galazka et al., 1999a). It seemed that protein-polysaccharide complexation would have protected the protein against pressure-induced aggregation (Table 1).

**Table 1** Selected examples of changes in the interactions between proteins and other macromolecules induced by HPP

Group	Mixtures	Change in the interactions	Main influence factors	References
Protein–Protein	Gluten-Soy	Rheological properties, Structure	Protein concentration, Protein sources, Temperature	Apichartsrangkoon and Ledward, 2002
	Skim milk-Gelatin	Rheological properties, Flow behavior, Structure	Pressure, Protein concentration	Hemar et al., 2010
	Milk-Gelatin	Rheological properties, Structure	Pressure, Protein concentration	Devi et al., 2013b
	WPI-MC	Rheological behavior, Emulsification properties, Foaming properties	Protein concentration, pH, Temperature	Baier et al., 2015
	WP-Gelatin	Rheological properties, Protein structure, Gel properties	Protein concentration, Temperature, Pressure	Devi et al., 2014; Walkenstörn and Hermansson, 1997
	Myofibrillar proteins-Egg white	Emulsifying capacity	Pressure, Time	Barrios-Peralta et al., 2012
Protein–Polysaccharide	SPI-Methocel	Foaming properties, Rheological properties, Interfacial properties	Pressure, Molecular weight of polysaccharide	Martínez et al., 2011
	PPI-chitosan, PPI-guar gum, PPI-xanthan gum	Rheological properties, Gel strength	Protein concentration, Polysaccharide sources	Jiao et al., 2018
	11S globulin+ $\iota$ -carrageenan, or $\kappa$ -carrageenan	Surface hydrophobicity, Emulsion properties and stability,	Pressure, Polysaccharide sources, Temperature	Galazka et al., 1999b
	SPP-GG	Emulsifying activity and stability, Viscosity	Polysaccharide concentration, Pressure, Time	Khan et al., 2015
	$\beta$ -Lactoglobulin + DS	Thermograms	Pressure, Temperature, Time	Aouzelleg and Bull, 2004
	$\beta$ -Lactoglobulin + DS, BSA-DS	Surface hydrophobicity, Structure, Thermograms	Pressure, Protein sources	Galazka et al., 1996
	$\beta$ -lactoglobulin + LMP, HMP, or Na-alginate	Gel properties	Pressure, Temperature, Polysaccharide sources	Dumay et al., 1999
	Ovalbumin-DS, Ovalbumin- $\iota$ -carrageenan	Formation of aggregates, Electrostatic interactions	pH, Ionic strength, Temperature, Pressure	Galazka et al., 1999a
	$\beta$ -lactoglobulin + DS, BSA-DS, OVA-DS	Surface tension, Surface shear viscosity	Polysaccharide sources, Pressure, pH	Galazka et al., 2001
	Myofibrillar proteins-potato starch, or agar	emulsifying capacity	Pressure, Time, Polysaccharide sources	Barrios-Peralta et al., 2012
	Casein- HPC	Particle size, Viscosity, Stability, Turbidity	Pressure, Time, Molecular weight of polysaccharide	Ye and Harte, 2014
	WP- $\kappa$ -carrageenan, xanthan gum, or HMP	Rheological properties	Pressure, Polysaccharide sources, Temperature	Fernandes and Raemy, 1996
	WP-pectin	Rheological properties	Pressure, Temperature	Michel et al., 2001
	MC-LMP	Rheological properties	Polysaccharide and protein of concentration, Pressure	Abbasi and Dickinson, 2002a
	SMP-LMP	Gel properties	Pressure, Temperature	Abbasi and Dickinson, 2002b

HPP, WP(), MC, SPP, PPI, GG, LMP, HLP, HPC, BSA, OVA, DS and SMP represent high pressure processing, whey protein(isolate), micellar casein, sweet potato protein, peanut protein isolate, guar gum, low methoxy pectin, high methoxy pectin, hydroxypropyl cellulose, bovine serum albumin, ovalbumin, dextran sulphate and skim milk powder respectively.

## Conclusion

The desired characteristics of food products such as structure, stability and textural attributes can be achieved through mixing polysaccharides and/or proteins, and monitoring the strength and nature of the interactions between the mixed biopolymers. Interesting effects may take place when HPP is applied to mixtures of macromolecules (polysaccharides and/or proteins) owing to their interactions under pressure and during decompression. HPP can induce modification of macromolecular structures and interactions of food biopolymers, causing changes that can potentially have great influences on the properties of food products. Thus, HPP can be used as a processing approach for manipulating properties of mixtures of proteins–proteins and proteins-polysaccharides. HPP may produce new ingredients and products (e.g., emulsifiers, stabilizers, foams or gels) containing protein–protein and protein-polysaccharide complexes for food industry.



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# Different Catalytic Activities of Microbial L-Glutaminases Against Bitter Amino Acid Phenylalanine in the Production of Kokumi $\gamma$ -Glutamyl Peptides

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## Glossary

**Gamma-glutamyl transpeptidation** The transfer of  $\gamma$ -glutamyl moieties from molecules such as glutathione (GSH), glutamine (Gln) or other  $\gamma$ -glutamyl compounds to an acceptor such as an amino acid, a peptide or water to form  $\gamma$ -glutamyl peptides or glutamate.

**Gamma-glutamyl-peptides** These are peptides in which an amide linkage occurs between the  $\gamma$ -carboxyl group of glutamic acid and the amino group of another amino acid.

**Kokumi** A Japanese expression ("rich taste") to describe a complicated sensory sensation of food that is detected through calcium channels on the tongue involving calcium-sensing receptor (CaSR) but not perceived as an independent taste quality, encompassing continuity, mouthfulness, thickness, taste-enhancing and persistent/long-lasting characteristics.

**L-Glutaminase** An amidohydrolase that mainly catalyzes the hydrolysis of  $\gamma$ -amido bond of L-glutamine (Gln) to produce L-glutamic acid, with some also catalyzing the transfer of  $\gamma$ -glutamyl moieties from Gln to other amino acids or peptides to form  $\gamma$ -glutamyl peptides.

## Introduction

The sensation of flavor plays an important role in food choice, dietary behaviors and even human development. Quite a lot of valuable nutrients and bioactives are naturally bitter or have unpalatable tastes including aromatic, basic or branched-chain amino acids and some short peptides such as aspartic acid, glutamic acid, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan and valine. While masking their unpleasant tastes through encapsulation is used in some cases, nonencapsulation approaches especially those based on food component interactions are often more desirable (Sun-Waterhouse and Wadhwa, 2013). Using bitter taste blockers for food formulation is a popular nonencapsulation approach but presents limitations related to the lack of universal bitter blocker. Thus, in situ taste improvement that can be included as part of the manufacturing process is preferred e.g. Gamma-glutamyl transpeptidation to improve the taste of bitter or bad-tasting peptides and amino acids generated in aged, fermented and hydrolyzed foods (Yang et al., 2017).

$\gamma$ -Glutamyl peptides including  $\gamma$ -glutamyl dipeptides or tripeptides such as  $\gamma$ -Glu-Val,  $\gamma$ -Glu-Met,  $\gamma$ -Glu-Glu,  $\gamma$ -Glu-Gln,  $\gamma$ -Glu-Gly,  $\gamma$ -Glu-Leu,  $\gamma$ -Glu-His,  $\gamma$ -Glu-Cys-Gly and  $\gamma$ -Glu-Val-Gly, are usually found in edible beans (Dunkel et al., 2007), cheese (Toelstede et al., 2009; Toelstede and Hofmann, 2009; Sgarbi et al., 2013; Roudot-Algaron et al., 1994) and soy sauces (Frerott and Chen, 2013).  $\gamma$ -Glutamyl peptides may possess antinociceptive, anticancer, antiplatelet, antiatherosclerotic, detoxifying and lipid-lowering effects (Kim et al., 2017; Keusgen, 2002; Ichinose et al., 1987; Main et al., 2012). These peptides are useful antioxidants and pro-therapeutics for Parkinson's disease, as they are quite stable in the body as their  $\gamma$ -glutamyl linkage cannot be cleaved by normal peptidases until at the location where specific enzymes such as  $\gamma$ -glutamyltranspeptidases are expressed. Besides the biological effects,  $\gamma$ -glutamyl peptides play important roles in sensory properties of foods and exhibit kokumi-imparting properties upon addition of a small amount. Kokumi is often listed together with the five basic tastes (sweet, salty, sour, bitter and umami), and describes a complicated taste sensation of food associated with continuity, mouthfulness, thickness, taste-enhancing and long-lasting characteristics. Moreover,  $\gamma$ -glutamyl peptides can be produced on a large scale via an enzymatic  $\gamma$ -glutamyl transpeptidation process, and  $\gamma$ -glutamyltransferases (EC 2.3.2.2) can catalyze the  $\gamma$ -glutamyl transfer from a  $\gamma$ -glutamyl donor (e.g. glutathione (GSH), glutamine (Gln) or other  $\gamma$ -glutamyl compounds) to a  $\gamma$ -glutamyl acceptor (e.g. other amino acids or peptides) to form  $\gamma$ -glutamyl peptides (Chen et al., 2015; Speranza and Morelli, 2012; Suzuki et al., 2002a,b,c, 2004, 2007). These two types of enzymes often possess several catalytic activities for the following reactions: 1) only hydrolysis (in which the  $\gamma$ -glutamyl moiety is transferred to a water molecule yielding glutamic acid), 2) only transfer reaction (in which the  $\gamma$ -glutamyl moiety is transferred to the acceptor is an  $\text{NH}_2$ -group containing compound), 3) hydrolysis of glutamine prior to a transfer reaction with some acceptors, 4) transfer reaction prior to hydrolysis (hydrolysis is largely suppressed by suitable acceptor in favor of a transfer reaction), and 5) autotranspeptidation (when the free  $\text{NH}_2$ - group of a donor acts as an acceptor itself). For example, the glutaminases from *Pseudomonas nitroreducens* (GPN) (Itoh et al., 2012) and *Aspergillus oryzae* (GAO) (Tomita et al., 1989) have transpeptidase activity and can catalyze the synthesis of L-theanine and a large number of  $\gamma$ -glutamyl peptides, respectively. GPN can catalyze the hydrolysis of glutathione prior to  $\gamma$ -glutamyltransfer to glycylglycine and also the  $\gamma$ -glutamyl transfer to ethylamine or methylamine with suppressed hydrolysis of glutamine. Thus, a series of  $\gamma$ -glutamyl peptides are normally produced during the enzymatic synthesis e.g.  $\gamma$ -Glu- $\gamma$ -Glu-Tau was found as a by-product of the  $\gamma$ -Glu-Tau synthesis catalyzed by  $\gamma$ -glutamyl transpeptidase (GGT) (Suzuki

et al., 2002c);  $\gamma$ -glutamyl-glutamine and other poly-glutamylated species were identified during the GGT-catalyzed synthesis of  $\gamma$ -glutamyl-peptides (Morelli et al., 2014).

### Biochemical Characteristics and Catalytic Activities of Microbial L-Glutaminases

L-Glutaminase has been in the spotlight since the discovery of its antitumour properties. Decades of research have been directed toward searching its sources, characterizing, utilizing and improving its catalytic properties. The enzyme naturally occurs as a family of species with varying enzymatic activities and biochemical characteristics and can be found in the whole biological world including human beings and microorganisms (bacteria, yeast and fungi). Most microbial species produce L-glutaminase at a neutral or slightly alkaline pH under mild conditions (25–37 °C). For the microbial L-glutaminases, L-glutamine is the main substrate, with D-glutamine, L-asparagine or D-asparagine at a lower concentration also as a substrate in some cases. In food industry, efforts have been made toward the exploration of their novel properties in specific food matrices and under food manufacturing conditions (e.g. desirable temperature, pH and salt tolerance). In particular, the catalytic activity of L-glutaminase for the synthesis of glutamic acid has been optimized in a range of food applications including soy sauce fermentation.

### Biochemical Characteristics and Transpeptidase Activity of Glutaminase

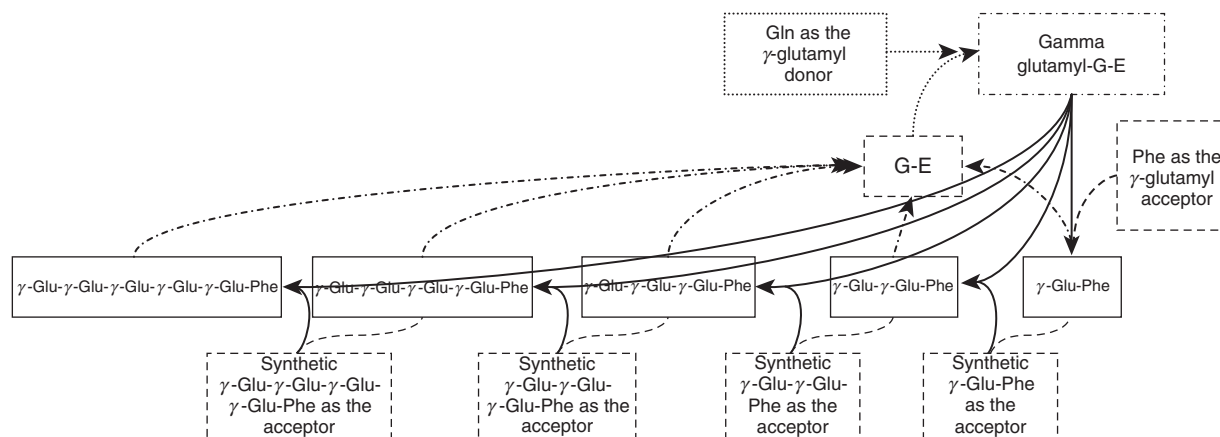
Glutaminases sourced from different microorganisms differ in molecular weight, optimal pH for stability and catalysis, optimal temperature for stability and catalysis, and substrate specificity (Tachiki et al., 1996; Yano et al., 1988; Koibuchi et al., 2000; Ye et al., 2013). Most L-glutaminases are monomers with some bacterial L-glutaminases as dimers and tetramers, and have molecular weight in the range of 40–148 kDa and high stability at 40–50 °C. The glutaminases from *Bacillus amyloliquefaciens* (GBA), GAO and GPN have a molecular mass of about 35 kDa, 82–113 kDa, and 40 kDa, respectively. The optimum pH and temperature for catalysis are 6.5 and 60 °C for GBA, 9.0 and 37–45 °C for GAO, and 9.0 and 30 °C for GPN. Different glutaminases exhibit varied salt-tolerance (which is an important aspect especially for food applications involving high salt conditions). For example, GAO can be significantly inhibited by high salt concentrations (e.g. 17%–18% salt concentration during soy sauce fermentation), whilst GBA still exhibits high activity (68% of the original activity) in the presence of 20% NaCl.

In terms of transpeptidase activity, GBA and GAO were found to possess transpeptidase activity i.e. 100 and 10 U/g, respectively, at the optimal pH of 10.0 and optimal temperature of 37 °C (Yang et al., 2017). The two enzymes can catalyze a  $\gamma$ -glutamyl transfer reaction to yield Phe-containing  $\gamma$ -glutamyl peptides in the presence of Gln and Phe i.e.  $\gamma$ -Glu-Phe and  $\gamma$ -Glu- $\gamma$ -Glu-Phe (by GAO), or  $\gamma$ -Glu-Phe,  $\gamma$ -Glu- $\gamma$ -Glu-Phe,  $\gamma$ -Glu- $\gamma$ -Glu- $\gamma$ -Glu-Phe,  $\gamma$ -Glu- $\gamma$ -Glu- $\gamma$ -Glu- $\gamma$ -Glu-Phe and  $\gamma$ -Glu- $\gamma$ -Glu- $\gamma$ -Glu- $\gamma$ -Glu- $\gamma$ -Glu-Phe (by GBA). Like any other enzyme, the specific activity of glutaminase is closely related to its degree of purity (which greatly depends on the purification method). GBA was found to have a final specific activity of 1.92, 25.70, 93.70, and 196.20 U/mg that corresponded to each purification step of a progressive purification process: supernatant after centrifugation at 12 000  $\times$  g for 10 min (to remove microorganisms), fraction coming out from the DEAE-Sepharose FF anionic exchange chromatography column, fraction from the hydroxyapatite column, concentrate after sephacryl S-200 (Ye et al., 2013). However, the glutaminases from *Escherichia coli* and *Pseudomonas aeruginosa* seemed to have no activity toward the transfer of  $\gamma$ -L-glutamyl-*p*-nitroanilide to Gly-Gly.

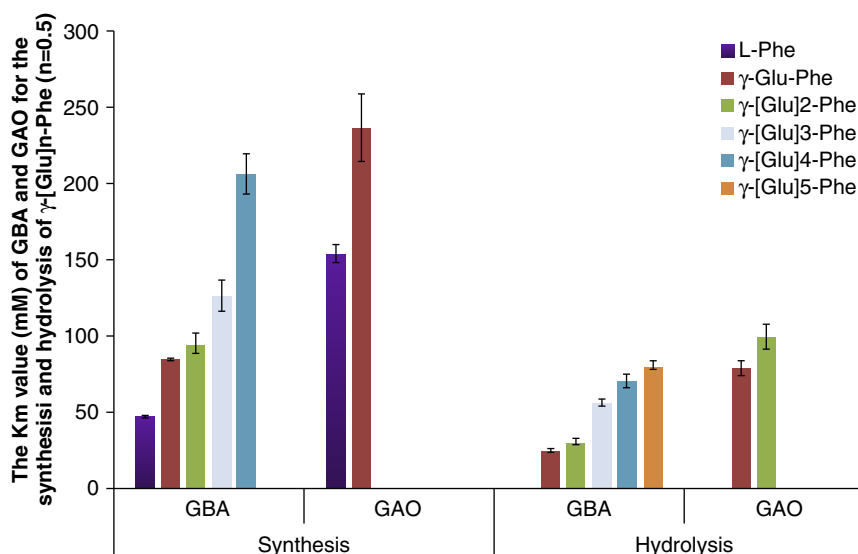
### Synthesis of $\gamma$ -Glutamyl Peptides in the Presence of Phe by GBA or GAO

Glutaminases of different microbial sources under the same enzymatic synthesis conditions (including type and concentration of substrates, pH, temperature, enzymatic activity, reaction time and method for inactivation of enzyme), can lead to different profiles of  $\gamma$ -glutamyl peptides. When GBA or GAO at 0.05 U/mL, the synthesis reaction was run at 37 °C for 2 h with substrate water solutions containing Gln and Phe (both at 200 mM, pH 10.0), yielding various Phe-containing  $\gamma$ -glutamyl peptides. The concentrations of  $\gamma$ -[Glu] $_n$ -Phe products differed between the two cases of GBA and GAO i.e.  $\gamma$ -Glu-Phe,  $\gamma$ -[Glu] $_2$ -Phe,  $\gamma$ -[Glu] $_3$ -Phe,  $\gamma$ -[Glu] $_4$ -Phe and  $\gamma$ -[Glu] $_5$  were 61.59, 27.64, 14.09, 1.50 and 0.13 mM, respectively, whereas, for GBA, only  $\gamma$ -Glu-Phe and  $\gamma$ -[Glu] $_2$ -Phe (20.31 and 3.10 mM, respectively) were detected for GAO (Yang et al., 2017). In both cases, a lower yield was likely achieved for the  $\gamma$ -[Glu] $_n$ -Phe product with a higher molecular weight. Such differences in the yield and type of  $\gamma$ -[Glu] $_n$ -Phe products indicated that GBA appeared to be more efficient than GAO in catalyzing the transfer of a  $\gamma$ -glutamyl group to a tripeptide or a higher molecular weight peptide. The synthesis of Phe-containing  $\gamma$ -glutamyl peptides is summarized in Fig. 1 (Yang et al., 2017).

A “Ping-Pong” kinetic mechanism was possible, causing the formation of a covalently bonded  $\gamma$ -glutamyl acyl-enzyme intermediate involving hydrogen bonding and charge interactions, then the intermediate reacted with Phe or existing peptides as acyl-acceptor substrates to yield a  $\gamma$ -glutamyl peptide (Itoh et al., 2012). GBA and GAO were found to exhibit a difference in affinity for acceptors such as Phe and  $\gamma$ -Glu-Phe, with GAO exhibiting a lower affinity than GBA for the acceptors (Fig. 2). The superiority of GBA and or GAO in the production of Phe-containing  $\gamma$ -glutamyl peptides can be evaluated through measuring the Michaelis-Menten constants ( $K_m$ ) for the synthesis of  $\gamma$ -[Glu] $_n$ -Phe and hydrolysis of  $\gamma$ -[Glu] $_n$ -Phe, respectively. For the  $K_m$  of the synthesis reaction, GBA or GAO, substrate (Phe or  $\gamma$ -[Glu] $_{(n<5)}$ -Phe), and donor substrate (Gln) were at 0.0025 U/mL, 5–50 mM, and 50 mM, respectively, with the reaction pH, temperature and time being 10.0, 37 °C and 30 min. For the  $K_m$  of the hydrolysis reaction, the same reaction pH, temperature, time and GBA or GAO (0.0025 U/mL) were used (Yang et al., 2017). For the transpeptidation, the  $K_m$  values generally increased with an elevated number of  $\gamma$ -glutamyl residue within the acceptor, and were 47.88 and 153.92 mM with Phe as the acceptor, 84.89 and 236.47 mM with  $\gamma$ -Glu-Phe as the acceptor, for GBA and GAO, respectively. Unlike



**Figure 1** A scheme for the synthesis of Phe-containing  $\gamma$ -glutamyl peptides. G-E refers to glutaminase.



**Figure 2** The  $K_m$  value (mM) of GBA and GAO for the synthesis and hydrolysis of  $\gamma$ -[Glu]<sub>n</sub>-Phe ( $n = 0-5$ ). GBA and GAO refer to L-glutaminases from *Bacillus amyloliquefaciens* or *Aspergillus oryzae*.

GBA, GAO could not catalyze the transfer of  $\gamma$ -glutamyl moiety to the tripeptide ( $\gamma$ -[Glu]<sub>2</sub>-Phe) or peptides with the number of  $\gamma$ -glutamyl residues higher than 2. Moreover, the  $K_m$  values (4.81–40.75 mM) for the hydrolysis catalyzed by GBA were lower than that for the synthesis catalyzed by GBA. All these findings suggest that GBA had a greater affinity than GAO for Phe and  $\gamma$ -Glu-Phe in the transpeptidation reaction. GBA was superior to GAO in the synthesis of Phe-containing  $\gamma$ -glutamyl peptides, and the transfer of  $\gamma$ -glutamyl moiety to a  $\gamma$ -[Glu]<sub>n</sub>-Phe became more difficult with increasing number of  $\gamma$ -glutamyl residues in the molecule (Yang et al., 2017).

### Sensory Characteristics of $\gamma$ -Glutamyl Peptides

$\gamma$ -Glutamyl peptides possess very interesting sensory properties. They can modulate the calcium sensing receptor (CaSR) in humans (which is an extracellular seven-transmembrane-spanning, G protein-coupled receptor (GPCR)).  $\gamma$ -Glutamyl peptides have a minimal flavor in water and exhibit only an unspecific, slightly astringent taste at high taste threshold concentrations. But as other kokumi substances, they modify the five basic tastes (especially sweet, salty and umami tastes), in particular, when added in a small amount to an umami solution or foods containing savory compounds such as sodium chloride and L-glutamic acid, they can substantially enhance the thickness, continuity, complexity, mouthfulness and palate length of the food. The important structural elements for  $\gamma$ -glutamyl peptides with a high CaSR activity have been proposed i.e. the presence of an N-terminal  $\gamma$ -L-glutamyl

residue, a moderately sized, aliphatic, neutral substituent of L-configuration at the second residue, and a C-terminal carboxylic acid, preferably with glycine occurring as the third constituent (Amino et al., 2016).

$\gamma$ -[Glu]<sub>(1 ≤ n ≤ 5)</sub>-Phe peptides were reported to exhibit astringency in water but kokumi properties in commercial soy sauce and model chicken broth (Yang et al., 2017). In detail,  $\gamma$ -[Glu]<sub>n</sub>-Phe in water (5 mM, pH 6.5) was astringent, with the threshold concentrations corresponding to n = 1, 2, 3, 4 and 5 as 2.5, 3.34, 3.58, 3.69 and 3.92 mM, respectively. However, when these  $\gamma$ -[Glu]<sub>n</sub>-Phe products were added at 2 mM separately to commercial soy sauce, enhanced continuity and umami taste were detected (p < 0.05), and all these fortified commercial soy sauce systems had a long-lasting taste and mouthfeel similar to that of an umami solution involving the peptide-associated Maillard reaction (Liu et al., 2015; Ogasawara et al., 2006). The kokumi-imparting properties of  $\gamma$ -[Glu]<sub>(1 ≤ n ≤ 5)</sub>-Phe peptides were also found in a model chicken broth (at 2 mM). The threshold concentrations in the commercial soy sauce and model chicken broth were all in the range of 0.78–1.42 mM. Interestingly,  $\gamma$ -[Glu]<sub>(n > 1)</sub>-Phe compounds (n > 1) had similar sensory characteristics to that of  $\gamma$ -Glu-Phe (n = 1), even though the taste-enhancing scores of  $\gamma$ -[Glu]<sub>(n > 1)</sub>-Phe were slightly lower than that of  $\gamma$ -Glu-Phe. The taste score in water and the taste-enhancing scores in commercial soy sauce and model chicken broth all decreased progressively with an increasing number of  $\gamma$ -glutamyl residues. The taste effect of the  $\gamma$ -[Glu]<sub>n</sub>-Phe would become weaker with an increased molecular weight. Similar findings were found with other  $\gamma$ -glutamyl peptides such as  $\gamma$ -Glu-Val-Gly and  $\gamma$ -Glu-Cys-Gly,  $\gamma$ -Glu-Cys- $\beta$ -Ala,  $\gamma$ -Glu-Leu, and  $\gamma$ -Glu-Val (Ueda et al., 1997; Ohsu et al., 2010; Miyaki et al., 2015).

## Conclusion

Microbial L-glutaminases hold the promise of catalyzing the production of  $\gamma$ -glutamyl peptides as bioactive flavor enhancers. Their potential as the biocatalyst for in situ taste improvement of foods with processing-induced bitter peptides or amino acids would enhance their applications in the food industry. Further, different species of glutaminases exhibit their distinct catalytic behaviors and lead to different profiles of  $\gamma$ -glutamyl peptides. For a specific type of  $\gamma$ -glutamyl peptide targets, one glutaminase may be superior to the other(s) owing to the intrinsic properties of the enzyme.

GBA, GAO and GPN all possess transpeptidase activity and thus can be used to yield specific  $\gamma$ -glutamyl peptides by altering substrates and optimizing reaction conditions. For the synthesis of Phe-containing  $\gamma$ -glutamyl peptides through transpeptidation using the same Gln and Phe as substrates, GBA was more suitable than GAO based on their K<sub>m</sub> values for the transpeptidation reaction. Under the same reaction conditions, the use of GAO or GBA would lead to different species of  $\gamma$ -[Glu]<sub>n</sub>-Phe at varied concentrations:  $\gamma$ -Glu-Phe and  $\gamma$ -Glu- $\gamma$ -Glu-Phe for GAO, and  $\gamma$ -Glu-Phe,  $\gamma$ -Glu- $\gamma$ -Glu-Phe,  $\gamma$ -Glu- $\gamma$ -Glu- $\gamma$ -Glu-Phe,  $\gamma$ -Glu- $\gamma$ -Glu- $\gamma$ -Glu-Phe,  $\gamma$ -Glu- $\gamma$ -Glu- $\gamma$ -Glu- $\gamma$ -Glu-Phe for GBA. These exciting findings encourage future research toward the discovery and development of novel glutaminases including those salt- and thermo-tolerant species for a broad spectrum of food applications.

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# Effect of the Structure of Tannins on Their Binding Site on a Human Salivary Proline-Rich Protein

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## Introduction

Tannins are plant secondary metabolites, and believed to play a role in plant defense against bacteria, fungi, pests and herbivores. There are two main classes of tannins, hydrolysable tannins and non-hydrolysable tannins (condensed tannins). Condensed tannins are also called proanthocyanidins, and are oligomers and polymers of flavan-3-ols. They have a wide variety of structure arising from the presence of several constitutive units and linkage positions, substitution, especially with galloyl groups, and varying degrees of polymerization. Proanthocyanidins are the major group of tannins in foods and beverages, and are particularly abundant in fruits such as blackthorn chokecherry, bird cherry, quince and persimmon fruits and in beverages such as tea and wine. Tannins play an important role in the perception of plant-based foods and beverages. In those foods, tannins induce a complex sensation, called astringency.

## The Proposed Molecular Mechanisms for the Origin of Astringency

Astringency is a trigeminal sensation, described as a drying-out, roughening, and puckering sensation felt in the mouth. It is generally described as a tactile sensation. Tannins have also the ability to bind to and precipitate proteins. Therefore, astringency is thought to result from the interaction between tannins and salivary proteins. Astringency and the affinity of tannins for proteins depend on the structural features of tannins and increase with chain length and degree of galloylation (Canon et al., 2010; Poncet-Legrand et al., 2006; Sarni-Manchado et al., 2002).

However, the molecular mechanisms for the origin of astringency are still not fully understood. Two main hypotheses have been proposed to explain the mechanical origin of astringency. One postulates that the precipitation of salivary proteins, in particular of PRPs, by tannins reduces the lubricating properties of saliva, leading to the perception of an increase of oral friction. The second hypothesis proposes that tannins reduce the lubricating properties of the mucosal pellicle, a thin layer of salivary proteins covering the oral epithelium, leading to an increase of the friction forces at the surface of the oral mucosa. In this hypothesis, PRPs play a role in the protection of the mucosal pellicle *via* their ability to bind tannins (Gibbins and Carpenter, 2013). It is supported by the increase of perceived astringency of tea after removal of saliva (Nayak and Carpenter, 2008). Moreover, recent results, obtained in a model of oral mucosa, have shown that tannins can aggregate the mucosal pellicle. The size of the aggregates depended on the structure of the tannin, while their formation was precluded by the presence the basic PRP (*bPRP*), IB5 (Ployon et al., 2018). In parallel, measurements on the surface of the oral mucosa have revealed an increase of the friction force after the aggregation of the mucosal pellicle (Ployon et al., 2018). A two-stage mechanism, involving adsorption of tannins on the mouth epithelium as the salivary proteins become depleted, has been proposed (Guinard et al., 1986). In these two hypotheses, PRPs appear as major actors in the astringency sensation. Therefore, interactions between PRPs and tannins are of particular interest to understand the molecular bases of astringency sensation.

## The Interactions Between PRPs and Tannins

### Three Different Stages of PRP-Tannin Interactions

The secretion of PRP, and especially of basic PRPs (*bPRPs*), whose known function is to bind tannins, is likely the first line of defense for herbivores against dietary tannins. For instance, in rats and mice, a tannin-rich diet induces the secretion of PRPs in their saliva (Mehansho and Carlson, 1983; Mehansho et al., 1985), allowing their survival. Moreover, PRPs are particularly prone to the interaction with tannins (Hagerman and Butler, 1981). The high affinity of PRPs for tannins is attributed to their particular structure. PRPs do not fold into a well-defined three-dimensional structure but show extended random coil conformations (Boze et al., 2010), except for small polyproline helix segments. They belong to the family of intrinsically disordered proteins (IDPs), which defy the classical structure-function paradigm (Tompa, 2006). The most widely accepted model of PRP-tannin interactions is composed of three different stages as the tannin concentration increases: (i) tannins, which are multidentate ligands, bind to several sites on the free protein, (ii) the stoichiometries of the complexes increase and tannins cross-link different protein molecules, (iii) the resulting multimeric aggregates grow until precipitation (Canon et al., 2013b).



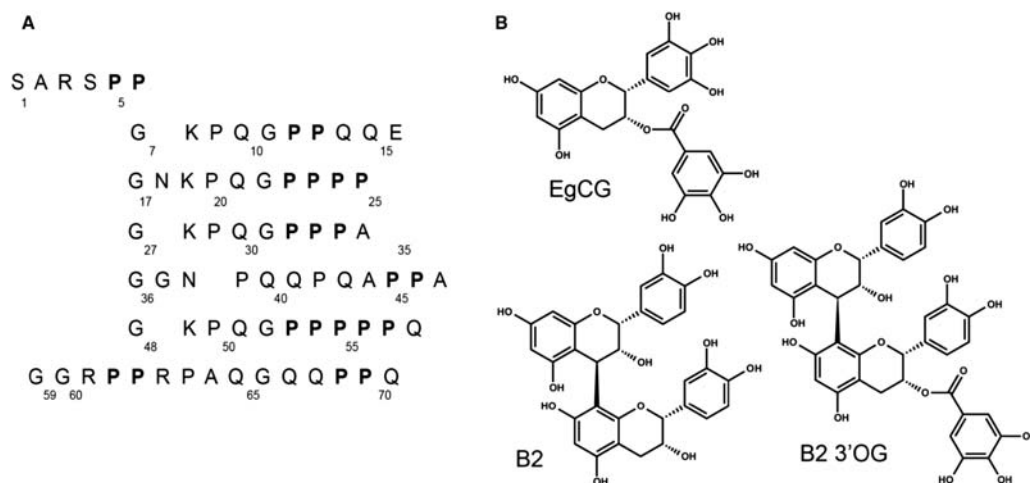
### Interactions of Human Salivary PRPs, IB5 or IB9<sub>37</sub>, With Three Different Tannins

A human salivary PRP, named IB5, and its interactions with tannins have been particularly studied. IB5 is a small bPRP of 73 residues. Its sequence contains 8 clusters of 2–5 proline residues and presents 5 tandem repeats of KPQGPP(P) (Fig. 1A). Small angle X-ray scattering (SAXS) (Boze et al., 2010), circular dichroism (CD) (Canon et al., 2011), nuclear magnetic resonance (NMR) (Pascal et al., 2009), and mass spectrometry (MS) (Canon et al., 2009) experiments have shown that IB5 has a random coil structure and undergoes transition from disorder to order upon the interaction with epigallocatechin gallate (EGCG) (Canon et al., 2011). The study of a smaller truncated PRP, IB9<sub>37</sub>, has also shown that structural rearrangements of the peptide chain occur during its binding to tannins. Charlton et al. (1996) were the first to propose that the peptide chain of PRPs may wrap around the tannin, in order to explain that IB9<sub>37</sub> and IB5 have a higher affinity for tannin than a peptide composed of a single proline-rich repeat (Charlton et al., 1996).

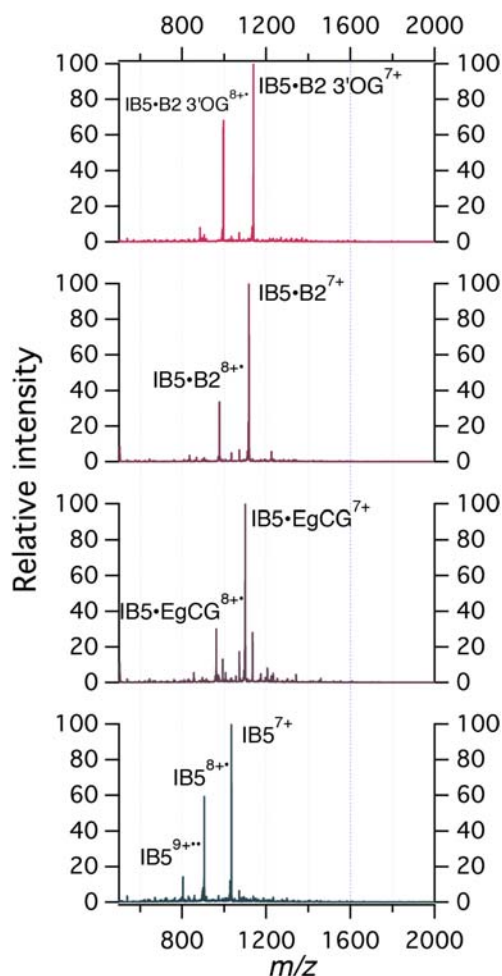
MS experiments have also revealed that the interaction between IB5 and EGCG could lead to the appearance of soluble non-covalent complexes with several stoichiometries (Canon et al., 2009). A multitechnique approach based on SAXS and MS demonstrated that at least three molecules of EGCG per protein are required to form PRP-EGCG aggregates (Canon et al., 2013b), a result in agreement with previous results obtained by NMR (Charlton et al., 2002). Comparison of different tannins showed that the stability of the non-covalent complexes depends on the number of tannin hydroxyl groups. This observation suggests that PRP-tannin interactions involve hydrogen bonds. Specific hydrophilic interactions between two proline-rich peptides (IB7<sub>14</sub> and IB9<sub>37</sub>) and tannins were also observed using NMR (Cala et al., 2012; Cala et al., 2010b). Fragmentation and titration experiments suggested the presence of 8 binding sites on IB5, corresponding to the number of proline clusters. NMR confirmed the involvement of proline residues in the interactions between IB5 or proline-rich peptides and tannins. However, the precise binding site of tannins could not be determined. The absence of a well-defined structure, the numerous repeated sequences and the coexistence in solution of numerous supramolecular edifices with different stoichiometries, make that the structural study of PRP-tannin interactions represents a challenge for classical techniques of structural biology, such as crystallography or NMR. In order to determine the binding site of tannins, we introduce a new technique of ion fragmentation based on the coupling of MS and synchrotron radiation. We applied this technique to the study of the interaction between IB5 and B2 3'-OG (epicatechin-(4b-8)-epicatechin-3-O-gallate). It generates numerous fragments of the protein, while preserving the non-covalent interaction between IB5 fragments and B2 3'-OG. The comparison of the two patterns of fragmentation (i.e. IB5 alone Vs IB5·B2 3'-OG), allowed to identify the fragments of sequence binding to B2 3'-OG and thus to determine its binding site onto IB5. The segment 'KPQGPPPPQGG' of the IB5 sequence constitutes the binding site. It contains a larger cluster of proline present into IB5 structure. In the cases of IB7<sub>14</sub> and IB9<sub>37</sub>, all tannin dimers and trimers tested bound to the same three binding sites, corresponding to PP (Pro-Pro) and GG (Gly-Gly) clusters, but with different strengths. Tannins having extended conformation and longer chain length showed a higher binding force.

Regarding these different observations, it seems important to know if, as for shorter IB9<sub>37</sub>, tannins with different structures bind onto the same site on IB5. Therefore, we recently apply the new methods of MS fragmentation using vacuum-ultraviolet (VUV) to localize the binding site of two others tannins: a monomer, EGCG, and another dimer, B2 ((epicatechin-4b-8)-epicatechin) (Canon et al., 2015). Fig. 1B presents the structure of the different tannins.

MS spectra of the mixtures containing IB5 and one of the three tannins, show ions with several charge states corresponding to the free IB5 and to the IB5·*n*Tannins non-covalent complexes with different stoichiometries. Ions corresponding to [IB5·1Tannin+7H]<sup>7+</sup> were then selected and activated by synchrotron radiation at 16 eV.



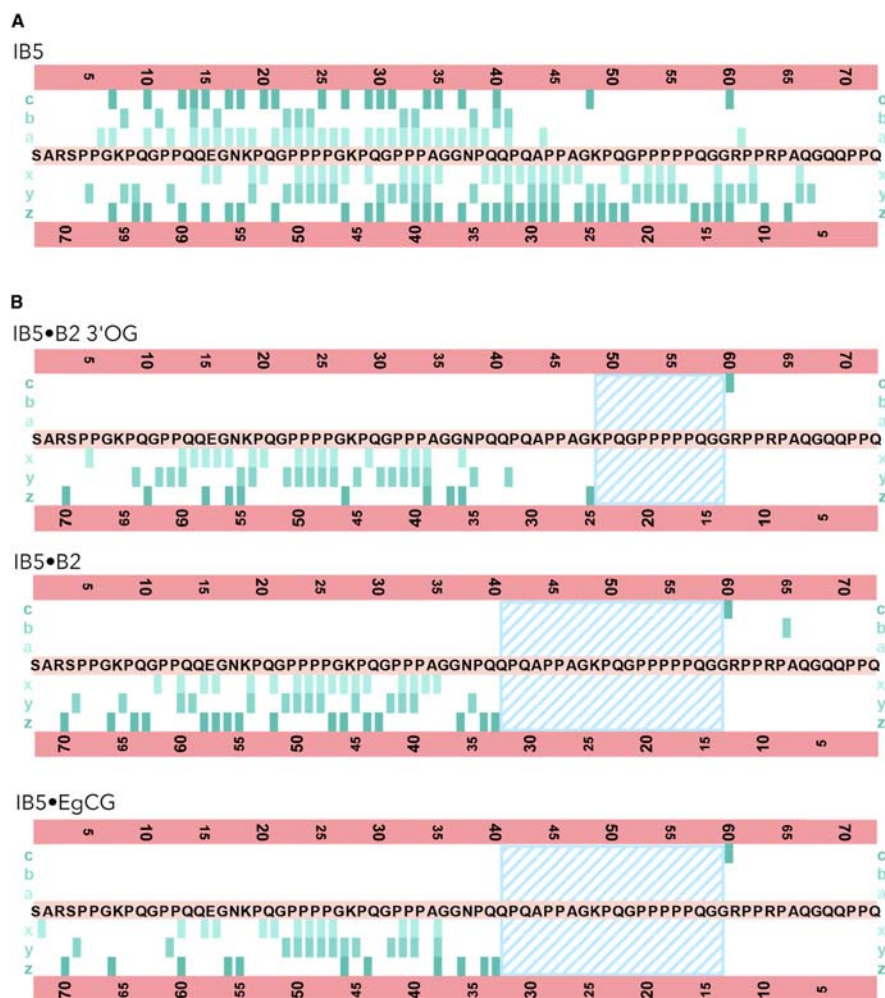
**Figure 1** (A) Sequence of the IB5 human salivary protein (B) Molecular structures of the epigallocatechin gallate (EGCG) (epicatechin-4b-8)-epicatechin (B2) and epicatechin-(4b-8)-epicatechin-3-O-gallate (B2 3'OG).



**Figure 2** (B) Dissociative photoionization (DPI) tandem mass spectra at 16 eV of the selected precursors  $[(\text{IB5} \cdot \text{tannin}) + 7\text{H}]^{7+}$ .

Fig. 2 presents the resulted mass spectra, in which are present the precursor ions, the  $[\text{M} + 7\text{H}]^{8+ \bullet}$  radical cation and fragment ions. The radical cation was produced upon the photoionization of the precursor ions. Activation at photon energies above the ionization threshold generated fragments through dissociative photoionization (DPI), with abundant formation of sequence ions of various natures (Giuliani et al., 2014). This regime of fragmentation has been previously reported to provide a higher sequence coverage than other techniques of activation, such as collision induced dissociation, electron capture dissociation or photodissociation (Canon et al., 2013a). Comparison of the 16 eV DPI MS/MS spectra of  $[\text{IB5} \cdot \text{1Tannin} + 7\text{H}]^{7+}$  with that of the bare protein ( $[\text{IB5} + 7\text{H}]^{7+}$ ) revealed the appearance of new fragments while others were not present anymore. The new ions correspond to the fragments of the peptide chain linked to the tannins, while ions of the corresponding bare fragments were not present anymore. Fig. 3 presents the fragments identified for the fragmentation of the bare proteins and of those linked to tannins for the fragmentation of the different non-covalent complexes. Analysis of all fragments linked to the tannins allowed to establish a map of the protein sequence containing the binding site of each tannin. Comparison of the different maps revealed that all tannins were bound to the following sequence 'QPQAPPAGKPQGPPPPQGG'.

This indicates that, as for the interaction with proline-rich peptides, tannins bound onto the same binding site. This sequence contains two clusters of proline and one of them is the longest cluster of proline into IB5 sequence. This part of the protein probably folds into a PPI (Pro–Pro I) or a PPII (Pro–Pro II) helix in solution (Simon et al., 2003). It probably provides a rigid section that facilitates the initiation of the binding. Indeed, it has been proposed that preformed structural element could facilitate the binding of intrinsically unstructured proteins (IUPs; also called intrinsically disordered proteins, IDPs) with their partners (Fuxreiter et al., 2004). This segmented is located between two clusters of glycine and alanine residues, which form flexible hinges allowing the establishment of additional hydrogen bonds (Chatterjee et al., 2005). These observations allows to propose a model of the interaction between PRP and tannins, in which clusters of proline form rigid regions, providing anchoring points to initiate the binding. Moreover, the carbonyl functional group in tertiary amides of proline residues is a more effective hydrogen bond acceptor than that in primary and secondary amides (Haslam, 1993). Flexible regions allowing the reeling of the peptide chain and the establishment of additional contact points for linking to these anchoring sections. This model is in agreement with the results obtained based on



**Figure 3** Fragmentation patterns of IB5 alone (A) and in association with B2 3'OG, B2 and EGCG (B). The binding sites identified for the tannins on the backbone are indicated by a box.

ion mobility indicating that a transition from extended to compact structures may occur (Canon et al., 2011), which also agrees with earlier findings obtained by NMR and circular dichroism (Pascal et al., 2009). Furthermore, molecular modeling of the interaction between IB9<sub>39</sub> and tannins indicates structural rearrangements of the peptide backbone (Cala et al., 2012). Such structural rearrangements do not seem to occur to shorter proline-rich peptides (Cala et al., 2010a; Simon et al., 2003). The model and associated observations may explain that the full PRP, IB5, has a higher affinity for tannins than a proline-rich peptide (Charlton et al., 1996). Interestingly, this model of the interaction involving preformed structural elements that initiate the binding and are linked by flexible hinges, which allow the establishment of additional interaction points, is rather common in the binding of IDP with their partners (Fuxreiter et al., 2004). Such a mode of interaction allows IDPs to bind to different partners and seems appropriate to the tannin-scavenging function of bPRPs, which have to adapt to a large variety of tannin structures.

## Conclusions

bPRPs are believed to be the first line of defense of mammals toward dietary tannins, as they are only present in the saliva of mammals for consuming tannins and their only known function is to bind tannins. To fulfill this function, PRPs have to adapt to a wide variety of structures exhibited by tannins. The present results indicate that two dimers (B2 and B2 3'OG) and one monomer (EGCG) bind to the preformed polyproline helix that forms a rigid contact point for initiating the binding. At the same time, the surrounding flexible hinges allow the structural transition and the establishment of additional hydrogen bonds. Thus, PRPs are likely to wrap around tannins, explaining that their interaction with tannins is stronger than the one of small proline rich-peptides. It should allow PRP to scavenge tannins more efficiently in order to protect the organism against their antinutritional effect, as well as the mucosal from aggregation. As astringency is thought to be triggered by the aggregation of the mucosal pellicle, it is likely that the presence of bPRPs in saliva precludes this sensation when tannin concentration is low. When tannin concentration is high and

that at least three tannins per protein are bound, aggregates are formed and grow in size with tannin concentration up to their precipitation. After PRP depletion, the mucosal pellicle is not anymore protected toward tannins leading to its aggregation, and subsequent increase of the friction and perception of astringency.

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# Interactions Between Polyphenols and Macromolecules: Effect of Tannin Structure

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## Introduction

In living plants, polyphenols, secondary metabolites, and macromolecules such as proteins and polysaccharides are usually compartmentally separated. During food and beverage processing, the plant cells de-compartmentalize under shearing and thermal forces. Polyphenols are released from vacuoles and bind to proteins and polysaccharides, notably those of the cell walls, and can also be oxidized by polyphenoloxidase (PPO). Various phenomena occur such as adsorption, oxidation, solubilization, and migration, leading to the emergence of new structures and new biological functions during processing, resulting from the establishment of physical and chemical associations. They will have an impact on technological, organoleptic and nutritional qualities of foods.

The polyphenols most involved in interactions with macromolecules are the tannins. The term 'tannin' has been employed classically to designate the molecule of plant origin capable of transforming fresh hide into leather. Tannins are widely widespread in plant and plant-based foods, in particular in fruits and vegetables, and derived beverages such as wine, tea, cocoa, beer and cider (Scalbert and Williamson, 2000). The term tannin includes two families of compounds: hydrolysable tannins and condensed tannins. Hydrolysable tannins consist of simple phenolic acids such as gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins) esterified to polyols, typically glucose (Fig. 1A and B).

Condensed tannins, also known as proanthocyanidins, are polymers and oligomers of flavan-3-ol units. Proanthocyanidins may differ by the nature of the constitutive units, by their mean degree of polymerization (mDP) which is the number average of constitutive units (varying between 2 and more than 100), and by the type of linkage (Cheynier, 2005; Fig. 2). They are divided into several classes based on hydroxylation of the constitutive units and the nature of the linkages. Their most common constitutive units are (epi)-catechins and (epi)-gallocatechins, leading to procyanidin and prodelphinidin structures, respectively. Flavan-3-ol subunits are most frequently linked through B-type bonds, that is, C4–C8 or C4–C6 interflavanic linkages. A-type proanthocyanidins are linked by an additional C2–O–C7 or C2–O–C7 ether bond producing doubly bonded proanthocyanidins.

Tannins are widely studied due to their health benefits that are ascribed to their ability to act as antioxidants (Hagerman et al., 1998). Tannins may exert their positive health effects either due to their antioxidant activity within the gastrointestinal tract producing local effects or due to bioactivity of their absorbable metabolites from colonic fermentation that may produce systemic effects in various organs (Serrano et al., 2009). Tannins contribute also to the organoleptic properties of plant-based foods, especially by their astringency, bitter taste, color, and by their participation to haze formation of various food products (Le Bourvellec and Renard, 2012).

Tannins interact with macromolecules either by non-covalent bonds, primarily driven by hydrogen bonding and hydrophobic interactions, or by covalent binding after polyphenol activation either by oxidation, i.e. as quinones, or as carbocations, resulting from proanthocyanidin cleavage under hot acidic conditions (Beart et al., 1985). Tannin macromolecule interactions has been the subject of many reviews in recent years (Zhu, 2017; Renard et al., 2017; Jakobek, 2015; Bordenave et al., 2014; Le Bourvellec and Renard, 2012; Hanlin et al., 2010). These reviews conclude that these interactions are dependent on both the structural characteristics of tannins and the physical properties and chemical composition of macromolecules, as well as the experimental and environmental conditions. In this context, we aim to synthesize the state of the science on the impact of tannin structures, i.e. molecular weight, constitutive units, substitution especially with galloyl groups, interflavanic bonds, conformation and flexibility, hydrophobicity on their interactions with macromolecules.

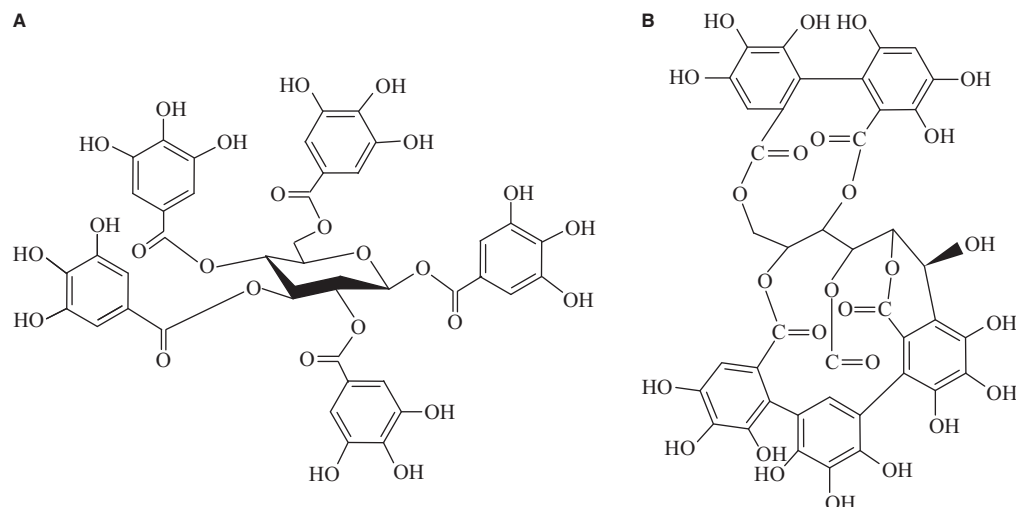
## Proanthocyanidins

### Molecular Weight

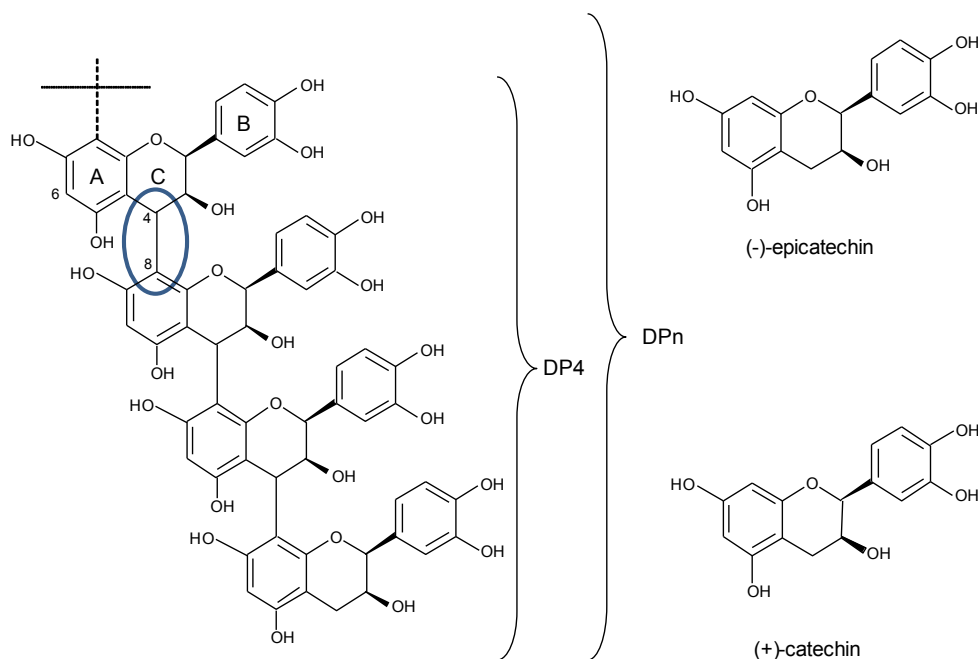
In general, increasing the molecular weight of proanthocyanidins increases the binding between macromolecules, i.e. proteins and polysaccharides, and proanthocyanidins (Girard et al., 2018; Ropiak et al., 2017; Ma et al., 2016; Barros et al., 2014; White et al., 2011; Cala et al., 2010a; Bindon et al., 2010; Bautista-Ortin et al., 2014; Le Bourvellec et al., 2004b, 2005; de Freitas and Mateus, 2001; Renard et al., 2001).

The NMR data and molecular modeling of wine proanthocyanidins binding further show that proanthocyanidin trimer (C2) exhibits a greater affinity to salivary peptide IB7 than four proanthocyanidin dimers (B1-4) (Cala et al., 2010a). Nephelometric and turbidimetric studies on glutenins, gliadins, BSA and gelatin also show that aggregation increases with increasing degree of polymerization (Girard et al., 2018; Ropiak et al., 2017). By Isothermal Titration Calorimetry (ITC) Kilmister et al. (2016) show that the calculated change in enthalpy ( $\Delta H$ ) and association constant ( $K_a$ ) between BSA and proanthocyanidin oligomers increase as the oligomer size increase. A positive relationship between the degree of polymerization of proanthocyanidins and salivary protein affinity is also revealed by Ma et al. (2016).





**Figure 1** Structure of the  $\beta$ -penta-O-galloyl-D-glucose (A) and of vascalagin (B).



**Figure 2** Structure of B-type.

Proanthocyanidins with higher degree of polymerization are also selectively adsorbed by cell-wall from various origins (Renard et al., 2001; Le Bourvellec et al., 2004b; Bindon et al., 2010; Bautista-Ortin et al., 2015), and by cell wall polysaccharides such as pectins (White et al., 2011), xyloglucans (Le Bourvellec et al., 2005) or cellulose (Le Bourvellec et al., 2005), and starch (Barros et al., 2014; Le Bourvellec et al., 2005), with the proanthocyanidins remaining in solution being low molecular weight polymers. Molecular weight of the proanthocyanidins also directly affect their binding to starch: the higher the molecular weight, the stronger the binding to amylose and the higher the resistant starch formation (Barros et al., 2014). An exception to the grape cell wall selective binding of large-size proanthocyanidins has been found for very large molecular mass proanthocyanidins (>15,000 g/mol) from skin of red grapes, which appear to be excluded from bindings when cell-wall porosity is low (Bindon et al., 2010).

Associations between proanthocyanidins and macromolecules, i.e. proteins and polysaccharides can be related to a tanning effect. This tanning effect is a function of both the presence of functional groups, i.e. hydroxyl groups and aromatic rings, able to form hydrogen bonds and hydrophobic interactions, and of the molecular weights, i.e. the size of the tannin (McManus et al., 1985). Therefore, larger proanthocyanidin molecules have more binding sites for the associations as they act as multidentate ligands able to bind simultaneously to more than one site of the macromolecule.



## Constitutive Units

### B-Ring Hydroxylation Pattern and Stereochemistry of the Proanthocyanidin Subunits

With regards to the B-ring substitution pattern proanthocyanidins could be divided in two groups: procyanidins, formed via the condensation of the flavan-3-ols catechin and epicatechin, are dihydroxylated, while prodelphinidins, formed via the condensation of the flavan-3-ols gallocatechin and epigallocatechin, are trihydroxylated. Catechin and gallocatechin are *cis*-flavan-3-ols whereas epicatechin and epigallocatechin are *trans*-flavan-3-ols.

Increasing the hydroxylation pattern of proanthocyanidins, i.e. from procyanidins to prodelphinidins, increases their ability to bind proteins (Helsper et al., 1993; Aerts et al., 1999). The dimer gallocatechin-C4-C8-epigallocatechin inhibits trypsin activity three times as much as the dimer catechin-C4-C8-epicatechin (Helsper et al., 1993). Moreover, increasing the prodelphinidins/procyanidins ratio, with concomitant increase of B the ring hydroxylation and of the molecular weight increases their capacity to complex with proteins (Aerts et al., 1999). Prodelphinidins have one more hydroxyl group than procyanidins and form more hydrogen bonds with proteins, increasing the strength of the interactions.

Trypsin inhibition activity of procyanidin dimers ranks in the order B2 > B4 > B3 (Helsper et al., 1993). NMR binding studies of four different procyanidin 4–8 dimers and the human saliva protein fragment IB7 also demonstrate that affinities are ranked: B2 > B4 > B1 > B3. The tendency to procyanidins diastereoisomers to adopt the extended versus the compact rotamer determines their relative binding affinities to IB7 in aqueous solution (Cala et al., 2010a,b). The extended conformation must facilitate the approach or/and the binding of the proanthocyanidin to the peptide: the more the extended conformation is preferred, the higher the affinity towards the peptide is. However, Bohin et al. (2012) and de Freitas and Mateus (2001) show that procyanidin B1 displays a better affinity for  $\beta$ -casein and salivary proline-rich proteins than procyanidin B2. Moreover, using a diverse panel of proanthocyanidins Ropiak et al. (2017) find no significant correlation between the procyanidin/prodelphinidin and *cis/trans*-flavan-3-ol ratios and BSA aggregation determined by turbidimetry. Besides, BSA tryptophan fluorescence quenching is positively correlated with procyanidin and *cis*-flavan-3-ols whereas BSA secondary structure is positively correlated with prodelphinidins (Ropiak et al., 2017). Hence, the effect of procyanidin monomer composition and conformation on their binding affinity for proteins might also be protein-dependent and method-dependent.

Cell wall material from grape flesh binds up to 47% and 57% w/w of total seed and skin proanthocyanidins respectively (Bindon et al., 2010). Both prodelphinidins and procyanidins are present in grape skins, whereas only procyanidins are present in seed. However, due to the complexity of skin and seed extracts (skin proanthocyanidins have a mDP of 17 and a percentage of trihydroxylation of 25 whereas seed procyanidins have a mDP of 7 and a percentage of galloylation of 17) it is not possible to conclude on the effect of the hydroxylation pattern (Bindon et al., 2010).

The stereochemistry of flavan-3-ol units also influence proanthocyanidin interactions with cell wall polysaccharides. Thus, proanthocyanidins comprising catechin as extension units have higher affinity to cell wall polysaccharides than proanthocyanidins composed mainly of epicatechin. A polymer composed of catechin forms a right-hand helix, whereas epicatechin forms a left-hand helix (Fletcher et al., 1976). The presence of catechin within the proanthocyanidin molecule may form a kink in the polymer increasing the number of reactive sites that are exposed on the outer side a helical conformation. Interactions with cellwalls are modulated by flavan-3-ol subunit composition: the presence of catechin leads to changes in proanthocyanidins conformation, with a more open and flexible conformation that may facilitate the formation of hydrogen bonds and hydrophobic interactions (Le Bourvellec et al., 2004b).

### Galloylation

The addition of ester-bonded gallic acid to proanthocyanidins plays a significant role in their macromolecules binding capacity (Soares et al., 2018; Bindon et al., 2010; Soares et al., 2007; Poncet-Legrand et al., 2006; Le Bourvellec et al., 2004b; de Freitas and Mateus, 2001; Sarni-Manchado et al., 1999; Ricardo da Silva et al., 1991).

Increasing the degree of galloylation of proanthocyanidins increases their ability to precipitate proteins (Soares et al., 2007, 2018; Poncet-Legrand et al., 2006; de Freitas and Mateus, 2001; Sarni-Manchado et al., 1999; Ricardo da Silva et al., 1991). The results of ITC and saturation transfer difference NMR indicate that the association constant ( $K_a$ ) between salivary proline-rich protein is higher for procyanidin B2 3'-O-gallate than for procyanidin B2 (Soares et al., 2018). Affinity of galloylated condensed dimers and trimers for proline-rich proteins is greater than that of ungalloylated analogues (Ricardo da Silva et al., 1991).

Proanthocyanidins with higher degree of galloylation are selectively adsorbed by apple and grape cell walls (Bindon et al., 2010; Le Bourvellec et al., 2004b). However, for grape cell walls, gel permeation chromatography has shown that the role of proanthocyanidins galloylation can be explained by the variation in size of galloylated material, as opposed to specific selectivity of cell wall galloylated proanthocyanidins (Bindon et al., 2010).

Three factors may explained the increased affinity observed for galloylated proanthocyanidins:

- 1) increased number of sites to form hydrogen bonds and hydrophobic interactions between proanthocyanidins and macromolecules;
- 2) increased hydrophobic character of proanthocyanidins as demonstrated by measuring the partition coefficients of monomeric and oligomeric flavan-3-ols between *n*-butanol and water (Plumb et al., 1998) which favors binding to macromolecules (Le Bourvellec and Renard, 2012);
- 3) greater structural flexibility to proanthocyanidins facilitating their binding to macromolecules.

## Interflavanic Bonds

### C4–C8 vs C4–C6

B-type proanthocyanidin subunits are mainly linked by two kinds of interflavanic bond, C4–C8 or C4–C6.

Procyanidin dimers linked through a C4–C8 interflavanic bond, such as procyanidin B3, have greater tannin specific activity for proline-rich proteins than their counterpart with a C4–C6 interflavanic bond, procyanidin B5 (de Freitas and Mateus, 2001), suggesting that the C4–C8 linkage promotes a more open configuration. However, Ricardo da Silva et al. (1991) find the opposite, i.e. proanthocyanidin dimers linked through a C4–C6 linkage bind more strongly with proteins than dimeric proanthocyanidins with a C4–C8 linkage. Santos Silva et al. (2017) confirm this result showing that procyanidin B6 (C4–C6 linkage) has a greater affinity for salivary proteins than procyanidin B3 (C4–C8 linkage). Discrepancies observed could be due to proanthocyanidin configuration and/or proteins used. However, further investigation concerning to interflavanic linkage is required.

To our knowledge no data are available in the literature concerning the impact of proanthocyanidins C4–C8 or C4–C6 interflavanic linkage on their binding with cell walls or polysaccharides.

### B-type vs A-type

Proanthocyanidins can be differentiated into B-type or A-type depending on their interflavanic linkages, the latter differing from the former by at least one double linkage consisting of a C–C bond and an additional ether bond (Fig. 2A and B).

Proanthocyanidin A2 has a higher trypsin inhibitor activity than proanthocyanidin B2 (Helsper et al., 1993). Proanthocyanidin A1 displays a higher affinity for  $\alpha$ -casein than proanthocyanidin B1 (Bohin et al., 2012). Cranberry proanthocyanidins, which are A-type, inhibit  $\alpha$ -amylase more strongly than grape proanthocyanidins which are B-type (Barret et al., 2013). The effect of the additional ether bond could be attributed to their higher hydrophobicity (McRae et al., 2010). However, cranberry proanthocyanidins have also a higher degree of polymerization than grape proanthocyanidins. A synergy between size and structural complexity could explain the results obtained.

Only indirect evidence supports the existence of interactions between A-type proanthocyanidins and the cell walls (White et al., 2011). To our knowledge, no study of the impact of the structure of A-type proanthocyanidins on their interaction with cell walls is available in the literature.

## Proanthocyanidin Oxidation

Proanthocyanidins are not substrates of PPO (Le Bourvellec et al., 2004a; Goodenough and Lea, 1979), but they can be oxidized by a coupled oxidation/reduction reaction involving primary *o*-quinones formed by PPO catalysis (Cheynier and Ricardo da Silva, 1991). Proanthocyanidins oxidation leads to both intra and intermolecular reactions. Intramolecular reaction results in the formation of new covalent bonds such as A-type linkages, reducing the proportion of acid-labile interflavanic bonds, thus limiting the configurational freedom of the macromolecules. Intermolecular reaction creates large macromolecules as evidenced by increased weight average degree of polymerization (Mouls and Fulcrand, 2012; Poncet-Legrand et al., 2010).

Oxidation of proanthocyanidins doubles their capacity to inhibit polyphenol oxidase (Le Bourvellec et al., 2004a). This effect could be due to the higher degree of polymerization of oxidized proanthocyanidins and to the presence A-type interflavanic bond inducing a higher hydrophobicity. However, ITC investigation of the interactions between poly-L-proline and grape or aged wine tannins, i.e. oxidized tannins, reveals that aged wine tannins have a lower protein affinity compared to grape tannins. The compact conformation of aged wine tannins may have limited their capacity to bind the poly-L-proline and the overall proanthocyanidin structure may be more important than hydrophobicity (McRae et al., 2010). Further investigation concerning oxidation effect is required by using well defined proanthocyanidins, because degrees of polymerisation, A-type linkages, and degree of molecular flexibility could explain the discrepancies observed. Moreover, initial concentration and degree of polymerization influence behaviour of proanthocyanidins upon oxidation: branched versus linear polymers are obtained, which have differences in their flexibility and hydrophobicity (Vernhet et al., 2014).

Oxidation of proanthocyanidins also increases their binding with cell walls (Le Bourvellec et al., 2009; Bautista-Ortin et al., 2014). The oxidation effect could be related to the increase of the degree of polymerization of proanthocyanidins as larger proanthocyanidins have more potential binding sites for the interactions.

## Hydrolyzable Tannins

### Tannin Molecular Weight

Like proanthocyanidins, it is generally accepted that hydrolyzable tannins with a higher molecular weight bind more strongly to macromolecules (Shimozu et al., 2017; Dobrev et al., 2014; Tang et al., 2003; Beart et al., 1985; McManus et al., 1985).

The size exclusion chromatography and polyacrylamide gel electrophoresis data show that isorugosin ellagitannin dimers exhibit a greater affinity for BSA than the monomer (Shimozu et al., 2017). ITC analysis confirms that for each ellagitannin monomer-dimer pair tested (vascalagin vs roburin A; pedunculagin vs oenothien B and pentagalloyl glucose vs gemin A) the dimer binds more strongly than the monomer (Dobrev et al., 2014).

Using thin layer chromatography [Tang et al. \(2003\)](#) show that the affinity of ellagitannins and gallotannins for cellulose is positively correlated with their molecular mass.

### Galloylation

Increasing the degree of galloylation of hydrolyzable tannin also increases their ability to bind macromolecules ([Tang et al., 2003](#); [Beart et al., 1985](#); [McManus et al., 1985](#)).

In the simple galloyl-D-glucose series, increasing the number of galloyl ester groups increases the protein binding capacity ([Tang et al., 2003](#); [Beart et al., 1985](#); [McManus et al., 1985](#)). This is attributed to an increase both of the number of hydroxyl group and aromatic ring able to interact with the protein.

[Tang et al. \(2003\)](#) also show that the affinity of ellagitannin and gallotannins for cellulose is positively correlated with the number of galloyl groups.

### Conformational Mobility and Flexibility

The flexibility of hydrolyzable tannin molecule is an important factor as well. Structurally flexible hydrolysable tannins bind more strongly to macromolecules than the more rigid molecules ([Karonen et al., 2015](#); [Deaville et al., 2007](#); [Hofmann et al., 2006](#); [Richard et al., 2006](#); [Frazier et al., 2003](#); [Tang et al., 2003](#)).

Vascalagin and castalagin bind much less effectively to proteins than their structural analogue  $\beta$ -penta-O-galloyl-D-glucose ([Beart et al., 1985](#); [McManus et al., 1985](#); [Hofmann et al., 2006](#); [Richard et al., 2006](#); [Tang et al., 2003](#)). Moreover, [Frazier et al. \(2003\)](#) show that tara gallotannins have a stronger affinity for BSA than myrabolan gallo- and ellagitannins. [Deaville et al. \(2007\)](#) and [Tang et al. \(2003\)](#) also observe that gallotannins have a stronger affinity for BSA and collagen than ellagitannins. Gallotannins are flexible and can easily change their conformation by intramolecular rotations whereas introduction of intergalloyl covalent linkages in the ellagitannins limits their capacity for conformational change, thus lowering their protein affinity. Using a series of oligomeric ellagitannins, [Karonen et al. \(2015\)](#) show by ITC that the binding constant of the monomer ( $K_a = 1.8 \times 10^4 \text{ M}^{-1}$ ) was higher than those for the dimer ( $K_a = 5.7 \times 10^3 \text{ M}^{-1}$ ) and trimer ( $K_a = 7.6 \times 10^3 \text{ M}^{-1}$ ) and similar to those for hexameric and heptameric ellagitannins ( $K_a = 1.7 \times 10^4 \text{ M}^{-1}$ ), thus the monomer to the trimer deviated from the overall trends. The differences observed are due first to the presence of an additional free galloyl group in the monomer and to the loss in the dimer structure of conformational flexibility and freedom, which affects its ability to bind to protein. Thus, hydrolysable tannins galloylation and flexibility may act in synergy in this case.

Ellagitannins show also much weaker interactions with cellulose than gallotannins having similar molecular mass, the same number of galloyl groups, and the same number of hydroxyl groups ([Tang et al., 2003](#)). Thus, interactions between hydrolyzable tannin and cellulose are also strongly dependent on the flexibility of galloyl groups, and the conformational restriction of ellagitannins limits their interactions with cellulose.

### Hydrophobicity

An inverse relationship is found between the strength of association with macromolecules and the solubility in water of hydrolyzable tannins ([Santos Silva et al., 2017](#); [Dobreva et al., 2014](#); [Tang et al., 2003](#)).

Vascalagin which is more hydrophilic than its stereoisomer castalagin has a lower affinity to salivary proteins ([Santos Silva et al., 2017](#)). [Dobreva et al. \(2014\)](#) also show using ITC that the more hydrophobic ellagitannins bind more strongly to BSA. Moreover, by evaluating the hydrothermal stability of sheepskin collagen treated with hydrolyzable tannins [Tang et al. \(2003\)](#) have shown that the hydrothermal stability of collagen is increases after polyphenol treatment and such effects are also positively correlated with the hydrophobicity of hydrolyzable tannins.

The strength of interaction between ellagitannins and gallotannins and cellulose is also positively correlated with hydrolysable tannin hydrophobicity ([Tang et al., 2003](#)).

### Conclusions

Some key structural characteristics namely molecular weight, the presence of galloyl groups, the conformation and the hydrophobicity that influence the ability of both proanthocyanidins and hydrolyzable tannins to interact with macromolecules are commune. However, some of structural characteristics are specific to each phenolic class:

- for proanthocyanidins: B vs A-type linkages, C4–C8 vs C4–C6;
- for hydrolysable tannin: gallotannins vs ellagitannins.

Some conflicting results appear in the literature: the polyphenol-macromolecule interactions also depend on the type of macromolecules as well as the experimental conditions and methods used.

In comparison to tannin-protein interactions, less is known regarding the interaction between tannin and polysaccharides and especially hydrolysable tannins and polysaccharides whereas they are both present simultaneously in many plant and food ([Okuda](#)

and Ito, 2011). Besides, polysaccharides play a role in regulating selective extraction of polyphenols from fruit to juice (Renard et al., 2017) and a role of “polyphenol carrier” by non-digestible polysaccharides has been described as an “essential physiological function” of polysaccharides (Saura-Calixto, 2011).

Because of a knowledge gap, mainly due to difficulties in tannin analysis, an in-depth study of the impact of certain structural characteristics of proanthocyanidins and hydrolysable tannins on their ability to interact with polysaccharides is still needed:

- for proanthocyanidins: impact of procyanidin/prodelphinidin ratio, C4–C8 vs C4–C6 and A-type vs B-type linkages.
- for hydrolysable tannins: interactions with cell walls and the different cell wall polysaccharides, i.e. pectins and xyloglucans, and starch.

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# A Molecular Thermodynamics Approach to Capture Non-specific Flavour–Macromolecule Interactions

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## Introduction

This short article provides an accessible practical guide to a new method for quantifying, from experimental measurements (Damodaran and Kinsella, 1981b; Hansen, 1997; Kuhn et al., 2006; Kuhn et al., 2008; Wang and Arntfield, 2016), how flavour molecules interact with food macromolecules and their assemblies. A serious problem of the traditional approach based on binding models (Wyman and Gill, 1990), is that it involves many unrealistic assumptions (Shimizu et al., 2017a; Shimizu et al., 2017b), such as the stoichiometric binding of flavour on binding sites distributed on macromolecular surfaces. The major shortcoming of such an assumption is the inability to grasp the non-specific and dynamic nature of the interactions. In contrast, the novel theory, which is rigorous, free of assumptions and surprisingly easy to handle, enables us to achieve this goal (Shimizu, 2004; Shimizu et al., 2017b). The fact that it works at the intuitive molecular level, rather than in terms of abstract thermodynamics, is a further advantage.

The need is to work out flavour-macromolecule interactions at the molecular level from typical experimental measurements, such as (a) dialysis equilibria using semi-permeable membranes (Arora and Damodaran, 2010; Damodaran and Kinsella, 1981a, 1981b; Guichard and Langourieux, 2000; Muresan, van der Bent and De Wolf, 2001; O'Neill, 1996; O'Neill and Kinsella, 1987b), (b) headspace vapour pressure measurements (Andriot et al., 1999, 2000; Fabre et al., 2002; Jung and Ebeler, 2003; Kuhn et al., 2006; O'Neill and Kinsella, 1987a; van Ruth, 2001; van Ruth and Villeneuve, 2002) and (c) denaturation temperature changes induced by flavour molecules (Burova et al., 2003; Tolstoguzov, 2003). The advantages of the new approach is clear; unlike the classical models, there is no need any longer to assume (i) specific binding sites for flavour molecules and water on macromolecular surfaces; (ii) stoichiometric binding reactions between flavour (f) and the binding site (s),  $f + s \rightarrow fs$ ; and (iii) equal binding constant for all the binding sites (Shimizu et al., 2017a; Shimizu et al., 2017b). Such unrealistic assumptions have led to many paradoxes and controversies in wide-ranging disciplines, including protein thermodynamics, colloid and surface science, and solution chemistry; the confusion could only be resolved by abandoning the unrealistic assumptions (Shimizu, 2004; Shimizu and Boon, 2004). As a further bonus, confusing ideas about “water structure” vanish from the approach, because the interactions are overwhelmingly direct: macromolecule to flavour molecule rather than macromolecule via some ill-defined chain of water structure to flavour molecule.

The new approach is based on the well-established fluctuation solution theory (FST) also called Kirkwood-Buff (KB) theory (after the early pioneers of this approach), though its application to biological macromolecules has been established only recently (Shimizu et al., 2017a; Shimizu, 2004; Shimizu, Stenner & Matubayasi, 2017b). With this method, flavour-macromolecule interactions can be quantified:

- (1) without any models and assumptions, and are derived directly from the first principles of molecular thermodynamics;
- (2) truly as non-specific, dynamic and long-ranged interactions through the use of molecular distribution functions;
- (3) via a remarkably simple methodology for going from experimental data to theoretical analysis.

Thus, replacing and updating the theoretical foundation has led to the following realizations and new possibilities that were beyond the reach of the classical binding models (Shimizu et al., 2017a):

- Prevention of misidentifying the true molecular mechanism, such as misinterpreting flavour-macromolecule repulsion as the increase of macromolecular hydration.
- Possibility to relate, collate and compare the data obtained from different experimental techniques in a unified rigorous theory.
- Quantifying non-specific interactions even from historic data, by considering binding models merely as fitting equations from which we can extract what is really going on.
- Comparing, via the same type of molecular-based theory, flavour-macromolecule interaction with other additives such as co-solutes, denaturants, stabilizers etc. which in turn provide interactions that modulate gelation and denaturation. Similarly, the same approach is used to describe the solubilisation of hydrophobic molecules (such as flavour) into aqueous solution.

In the following, we shall demonstrate how the new approach drastically simplifies the analysis of experimental data, and brings in long-awaited clarity in the interpretation. For brevity, we focus exclusively on the realistic case when the flavour and biological macromolecules are both dilute. For a generalization beyond this limit, which is straightforward, please refer to our recent review on this topic (Shimizu et al., 2017a).

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## Quantifying Non-specific Interactions via the Kirkwood-Buff Integral

Like any other interactions in the solution phase, flavour-macromolecule and flavour-water interactions can be defined most generally and rigorously (but also in a way that fits with our intuitions) in terms of the radial distribution function (RDF) which is a descriptor of how many molecules of type  $i$  are at any given distance from molecules of type  $j$ . The stronger the affinity between  $i$  and  $j$  the higher RDF becomes overall, whereas the more excluded  $j$  from  $i$  the lower RDF becomes overall. In the former scenario, the integrated RDF (called the KB integral, see Fig. 1) is less negative or even positive, whereas in the latter it is negative (Abbott et al., 2017; Shimizu et al., 2017b). These integrals are shown as  $G_{ij}$ . So, in the case of Protein interactions with Flavour molecules, this would be  $G_{PF}$  or  $G_{FP}$ , since the ordering is not important. Our previous tutorial review on solubilization by hydrotropes (Abbott et al., 2017) features a large set of free interactive “apps” to assist an intuitive understanding of KBIs and RDFs.

The negative contribution to KBIs, which even a strong positive contribution from the affinity in the solvation shall cannot overcome, comes from the excluded volume effect, i.e., the inability of two molecules to overlap (Fig. 1). Although excluded volume can be described in terms of abstract numbers such as entropy, its interpretation in terms of the RDF is more direct and insightful. In the cases where there is especially strong affinity between  $i$  and  $j$  sufficient to overcome the excluded volume effect, KBI becomes large and positive. KBI, on the contrary, is large and negative for molecules that are mutually incompatible (Abbott et al., 2017; Shimizu et al., 2017b).

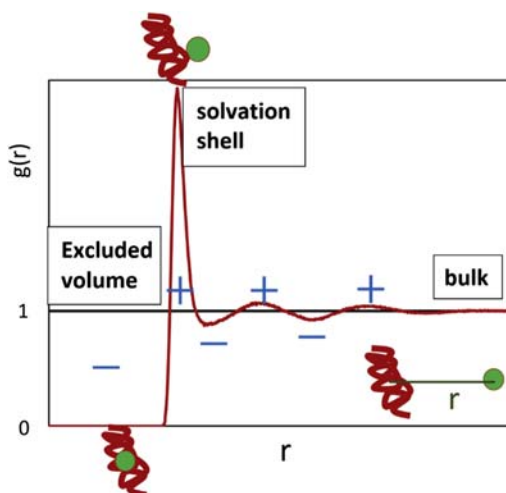
## Analysing Fresh Experimental Data

What follows is necessarily somewhat theoretical. However, for those who simply want access to the results, we have provided an “app”, <https://www.stevenabbott.co.uk/practical-solubility/kb-flavours.php>, that carries out all the analyses, showing the input data and the calculated values. The user can choose from a number of published datasets or load their own data once they have placed it into the simple format required by the app.

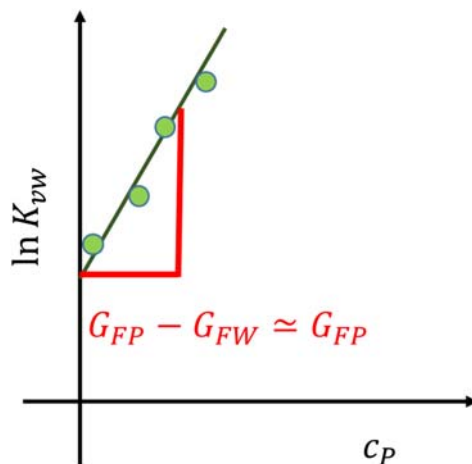
## Headspace Analysis

Let us consider an aqueous solution as a system consisting of water (W), protein (P) and flavour (F) molecules. Flavour molecules are volatile, and their solubility in water is relatively low. The question is how the vapour-water partition coefficient of the flavour molecule  $K_{vw} = c_F/c_F^{vap}$  ( $c_F$ : molar concentration of flavour molecule in the aqueous phase;  $c_F^{vap}$ : in the vapour phase) is affected by the presence of protein molecules. This question can be answered using the competition between the flavour-water and flavour-protein KBIs as (Shimizu, 2004; Shimizu and Matubayasi, 2014; Shimizu et al., 2017b)

$$\left( \frac{\partial \ln K_{vw}}{\partial c_P} \right)_{T,P,c_F \rightarrow 0; c_P \rightarrow 0} = G_{FP} - G_{FW} \quad (1)$$



**Figure 1** The physical meaning of the macromolecular-flavour Kirkwood-Buff integral (KBI), defined as  $G_{PF} = \int dr 4\pi r^2 [g_{PF}(r) - 1]$ , where the radial distribution function (RDF),  $g_{PF}(r)$  (red), depends on macromolecule-flavour distance  $r$ , and tends to 1 at large separation. The regions with  $g_{PF}(r) < 1$  signifies the less-than-bulk distribution of the flavour, which contributes negatively to the KBI, whereas the regions with  $g_{PF}(r) > 1$  signifies the more-than-bulk distribution of the flavour, which contributes positively to the KBI. The large negative contribution to KBI from small  $r$  is due to the excluded volume (i.e., the macromolecule and flavour molecules cannot overlap), if there is repulsion between the flavour and the macromolecule. See our previous tutorial review (Abbott et al., 2017) as well as the accompanying app <https://www.stevenabbott.co.uk/practical-solubility/rdf.php> for the interactive and intuitive understanding of the concept.



**Figure 2** A schematic explanation of the strategy to calculate the macromolecule-flavour affinity, characterised in terms of the KBI  $G_{FP}$ , from experimental data. The gradient of  $\ln K_{vw} = \ln (c_F/c_F^{vap})$  (vapour-solution partitioning determined from headspace measurements) against the macromolecular concentration  $c_P$  yields the KBIs. See main text for further explanations. An interactive web-based app (Shimizu et al., 2017a) for data analysis is available at <http://www.stevenabbott.co.uk/practical-solubility/kb-flavours.php>.

Eq. (1) immediately leads to the following set of procedures to determine the KBI difference,  $G_{FW} - G_{FP}$ :

- (i) Change and record protein concentration  $c_P$ ;
- (ii) At each protein concentration, measure
  - (a) the vapour phase flavour concentration  $c_F^{vap}$  from the headspace vapour measurements;
  - (b) the solution phase flavour concentration  $c_F$ ;
  - (c)  $K_{vw} = c_F/c_F^{vap}$  from (a) and (b)
- (iii) The slope (Fig. 2) yields  $G_{FW} - G_{FP}$ .

$G_{FW}$  and  $G_{FP}$  can both be determined independently. This requires an additional piece of information, i.e., the partial molar volume of the flavour molecule,  $V_F$ , from which  $G_{FW}$  can be determined directly using

$$V_F = -G_{FW} + RT \kappa_T \approx -G_{FW} \quad (2)$$

where the term containing  $\kappa_T$ , isothermal compressibility, makes a negligibly small contribution (Shimizu, 2004; Shimizu and Matubayasi, 2014; Shimizu et al., 2017b).  $V_F$  can be determined, in principle, from density measurements of the aqueous flavour solutions (Chalikian, 2003). However, this approach may not be suitable in practice because most flavour molecules have low aqueous solubility. Hence the reliable alternative is to estimate  $V_F$  using empirical formulae based on group additivity (Lepori and Gianni, 2000), which is quite good enough for the purpose.

## The Use of a Semi-permeable Membrane to Quantify Protein-Flavour Interactions

Now we consider protein molecules in dilution inside the semi-permeable membrane (Shimizu et al., 2017a; Timasheff, 2002b). Outside the semi-permeable membrane, we inject the flavour molecule, which can go through the semi-permeable membrane, but protein molecules stay inside the membrane. If we could measure the concentration of the species  $F$  both in the bulk phase ( $c_F$ ) and inside the semi-permeable membrane ("vicinity" concentration,  $c_F^{vic}$ ) at the protein concentration  $c_P$ , we could obtain the KBI directly from the following definition (Shimizu et al., 2017a):

$$G_{PF} = \frac{1}{c_F} \frac{c_F^{vic} - c_F}{c_P} \quad (3)$$

However, it is impossible in practice to determine  $c_F$  and  $c_F^{vic}$  directly from experiments. Instead, what is measurable experimentally is the increment of (or decrease in) flavour molecule concentration that accompanies the introduction of protein molecules inside the membrane (Timasheff, 2002a). Such an approach follows the well-established protocol for determining ligand binding on proteins using a semi-permeable membrane (Wyman and Gill, 1990). Such a measurement leads to the amount of flavour molecules bound to a mole of protein

$$\Gamma = \left( \frac{\partial m_F}{\partial m_P} \right)_{T,P,\mu_F} \quad (4)$$

where  $m_i$  is the molality of the species  $i$ . Molecular thermodynamics shows that this quantity can be expressed in terms of KBI as

$$\Gamma \simeq c_F (G_{PF} - G_{PW}) \quad (5)$$

under the approximation that flavour molecules are much smaller in general than proteins (Shimizu et al., 2017a).

To evaluate  $G_{PF}$ , we need to obtain  $G_{PW}$  via the partial molar volume of the protein in pure water,  $V_P$ , via  $G_{PW} = -V_P$ .  $V_P$  can either be measured experimentally using density meter or estimated from the molecular weight (Chalikian, 2003; Chalikian and Filfil, 2003).

## Recycling Old Literature Data

KBIs can be obtained directly from old papers that report the flavour-macromolecule binding constant  $K$  and the number of binding flavour sites  $n$ . This is despite the unrealistic assumptions that classical binding models employ (Shimizu et al., 2013; Shimizu et al., 2017b), yet it can serve as an excellent model for the fitting of experimental data (Arora and Damodaran, 2010; Damodaran and Kinsella, 1981a, 1981b; Guichard and Langourieux, 2000; Muresan et al., 2001; O'Neill, 1996; O'Neill and Kinsella, 1987b).

Old literature focuses predominantly on the amount  $\Gamma$  of flavour molecules bound to a mole of protein (Wyman and Gill, 1990), assuming  $n$  flavour-binding sites on the surface of a protein, each of which binds the flavour molecule with the binding constant  $K_b$ , as

$$\Gamma = \frac{nK_b c_F}{1 + K_b c_F} \quad (6)$$

Because it is often impossible to re-analyze historical data, we propose a practical way out to obtain KBIs from them (Shimizu et al., 2017a). This can be achieved by using the binding model (Eq. 6) as a fitting equation for  $\Gamma$ . From Eqs. (5) and (6), we obtain

$$G_{PF} - G_{PW} = \frac{nK_b}{1 + K_b c_F} \quad (7)$$

To evaluate  $G_{PF}$ ,  $G_{PW}$  can be obtained from the experimental or estimated partial molar volume of the protein in pure water,  $V_P$  (Chalikian and Filfil, 2003), using  $G_{PW} = -V_P$ , as before.

## Consequence of Flavour Binding on Macromolecular Stability

The presence of flavour, even in dilution, strongly affects protein denaturation temperature and sol  $\rightarrow$  gel transition temperature. The addition of flavour molecules commonly increases the transition temperatures drastically, which means there is a marked difference in flavour-macromolecule affinity between native ( $n$ ) and denatured ( $d$ ) states, or gel ( $g$ ) and sol ( $s$ ) states. Leaving the in-depth treatment to our recent review (Shimizu et al., 2017b), here we summarise the results. The change of free energy  $\Delta\mu_P^*$  that accompanies gel melting,  $g \rightarrow s$ , can be linked to the KBI difference,  $\Delta G_{PF} = G_{PF}^{(s)} - G_{PF}^{(g)}$  and  $\Delta G_{PW} = G_{PW}^{(s)} - G_{PW}^{(g)}$ , as

$$\frac{1}{RT} \left( \frac{\partial \Delta\mu_P^*}{\partial c_F} \right)_{T, P, c_P \rightarrow 0; c_F \rightarrow 0} = \Delta G_{PW} - \Delta G_{PF} \quad (8)$$

and the free energy change (the left hand side) can be obtained directly from the gel melting temperature ( $T_{g \rightarrow s}$ ) and  $\Delta H_{g \rightarrow s}$  the enthalpy change accompanying gel melting, as

$$\frac{\Delta H_{g \rightarrow s}}{RT^2} \frac{\delta T_{g \rightarrow s}}{\delta c_F} = \Delta G_{PW} - \Delta G_{PF} \quad (9)$$

The same approach is applicable also to protein denaturation,  $n \rightarrow d$ .

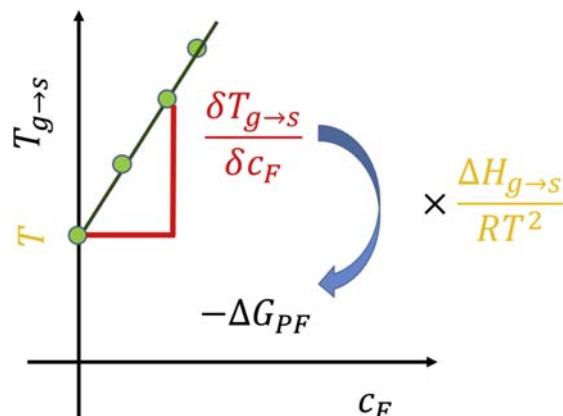
For practical purposes, the flavour concentration dependence of the melting temperature is sufficient for the determination of KBIs, when combined with the melting enthalpy in water (Burova et al., 2003; Tolstoguzov, 2003). In addition, compared to  $\Delta G_{PF}$ ,  $\Delta G_{PW}$  has been shown to be negligible, due to the usual smallness of the volume changes that accompany denaturation or gelation (Shimizu and Boon, 2004; Shimizu et al., 2017b). Eq. (9) can therefore be simplified further as

$$\frac{\Delta H_{g \rightarrow s}}{RT^2} \frac{\delta T_{g \rightarrow s}}{\delta c_F} \simeq -\Delta G_{PF} \quad (10)$$

This means that  $\Delta G_{PF}$  is determinable directly from the plot in Fig. 3.

## Concluding Remarks

That flavour molecules interact with food macromolecules in a non-specific manner has long been known. However, the lack of an appropriate theoretical language for non-specific interactions has forced the non-specific interaction to be modelled stoichiometrically, and hence unrealistically. This has made the experimental analysis of flavour-macromolecule interactions more complicated than it should ever be.



**Figure 3** A schematic explanation of the strategy to calculate the change of macromolecule-flavour affinity, characterised in terms of the KBI difference  $\Delta G_{PF}$ , from experimental data. The gradient of the gel-melting temperature ( $T_{g \rightarrow s}$ , or similarly the denaturation temperature) against the flavour concentration  $c_F$  yields the KBIs. See main text for further explanations. An interactive web-based app (Shimizu et al., 2017a) for data analysis is available at <http://www.stevenabbott.co.uk/practical-solubility/kb-flavours.php>.

Based on the principles of molecular thermodynamics (Shimizu, 2004; Shimizu et al., 2017b), non-specific interactions can now be captured and quantified directly from experiments without unrealistic assumptions. The analysis has been simplified drastically by making the stoichiometry assumption redundant. The collective and statistical nature of flavour-macromolecule affinity can now be captured in terms of the KB integrals. Such experimentally-determined KBIs enable us not only to quantify the interactions between different species but also their relative contributions to macromolecular solvation and transitions. Such a clarification has led to significant insights, such as the abandonment of the popular “water structure” hypothesis (Abbott et al., 2017; Shimizu et al., 2017b).

To make this short article as accessible as possible, we have focused exclusively on the simplest and most common case, i.e., the flavour and biological macromolecules are both dilute. However, systems beyond the dilute limit can be analysed equally straightforwardly through the general theory presented in our recent reviews (Abbott et al., 2017; Shimizu et al., 2017a; Shimizu et al., 2017b).

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## Relevant Websites

- <https://www.stevenabbott.co.uk/practical-solubility/kb-flavours.php> (Interactive web-based apps for food-flavour interactions).
- <https://www.stevenabbott.co.uk/practical-solubility/kb.php> (Interactive introduction to the fluctuation solution theory).
- <https://www.stevenabbott.co.uk/practical-solubility/the-book.php> (An interactive introduction to solvation and solubility).

# Flavor Enhancement Induced by Taste–Odor Interactions

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## Glossary

**Flavor** is a complex sensory impression which describes the overall perception generated after local processing and central integration (in the brain) of the signals resulting from the individual and combined effects of aromatics, taste sensations, trigeminal nerve stimulation and chemical feeling factors evoked by a substance in the oral cavity, including aroma/odor (detected by the olfactory epithelium in the nose), taste (detected by taste buds on the tongue), pain/irritation/hotness/temperature (detected by the trigeminal sensors in the mouth and nose), and mouthfeel/texture (detected by the force sensors in the mouth).

**Taste** is a sensory modality involving the oral perception of food-derived chemicals/substances, and describes the sensation produced when a substance on contact with the mouth and throat and reacts chemically with the taste receptor cells located on taste buds in the oral cavity, especially on the tongue. Several taste qualities have been defined or proposed including sweet, sour, salty, bitter, umami, astringent, pungent and kokumi, with the first five being more recognized. Umami and kokumi are Japanese words and represent ‘delicious taste’ and ‘rich taste’, respectively. Both can enhance the sensation of other tastes/flavors in different ways and to different extents. Umami is the sensation perceived upon the consumption of a food containing glutamate and ribonucleotides like inosinate and guanylate, whereas, Kokumi is a complicated sensation and describes continuity, mouthfulness, thickness, taste-enhancing and persistent/long-lasting characteristics associated with substances like calcium, protamine, glutathione and L-histidine.

**Smell or odor** is detected by the olfactory epithelium of the nose. The sense of smell gives rise to perception of odor via the mediator, the olfactory nerve. An odor/scent, pleasant or unpleasant, is caused by one or more volatilized molecules (chemical stimuli) at a low concentration and perceived by the sense of olfaction. The term “aroma” is often used to describe a pleasant odor, and sometimes the terms “odor” and “aroma” are used to represent the orthonasal olfactory perception and the retronasal olfactory perception, respectively. Like the sense of taste, the sense of smell is also a chemical sense although smell can work at a remarkably large distance as compared to taste. The smell process involves the travel of vaporized odor molecules in the air to reach the nostrils, dissolution of these molecules in the mucus, detection by olfactory receptor neurons in the olfactory epithelium underneath the mucus, transmission of the obtained sensory signal(s) to the olfactory bulbs located at the back of the nose (where the sensory receptors as part of the brain would send the signals to the brain centers).

## Introduction

Food sensory attributes such as smell, taste, colour and texture play vital roles in food preference, eating habits and life enjoyment. Among the sensory attributes, the flavor of food is the key determinant of food quality and acceptance. Flavor is an integrated sensation of food reflecting chemical and trigeminal nerve stimulation evoked by taste and odor substances. The perception of flavor is driven largely by smell and taste, with some contributions from trigeminal and tactile sensations, and visual and auditory cues. Compared to any other of senses such as taste and touch, the sense of smell is a lot more sensitive (up to 10,000 times more sensitive) and the recognition of smell is far quicker (almost immediate).

Food is a complex system consisting of nutrients and flavor compounds in a great variety (such as proteins, fats, sugars, non-volatile acids, volatile acids and carbonyl compounds). The volatile flavor compounds typically include aldehydes, ketones, alcohols, acids, esters, furans, pyrazines, amides and sulfur compounds. After all these substances are blended and processed into a certain format of food, a rich and characteristic flavor would be produced and perceived by humans.

## Contributions Made by Peptides Towards Taste or Flavor

Peptides are commonly found, as intermediates of protein synthesis and decomposition, in various foods such as meats, vegetables, pickled foods, dairy products and other types of food. Peptides are important nutrients and health-promoting bioactive substances that are mostly digestible and absorbable. They play important roles in food flavor and texture/mouth-feel through imparting their own sensory and other physico-chemical characteristics, and enhancing, and/or masking the sensory properties of food.

Umami peptides can be directly isolated from or obtained after hydrolysis of gluten (Schlichtherle-Cerny and Amadò, 2002), fish (Maehashi et al., 1999), ham (Aristoy and Toldrá, 1995; Sentandreu et al., 2003) and soy sauce (Lioe et al., 2006, 2007). Aspartame (Magnuson et al., 2007) and alitame (Kim and Shin, 2001) have already been developed as food sweeteners. The perception of the flavor of taste peptides is comprehensive. Peptides may exhibit complex taste profiles, and can make direct and indirect contributions



to the basic taste qualities of foods including sourness, sweetness, umami, bitterness, and saltiness. It was found that Arg-Pro, Gly-Arg-Pro and Arg-Pro-Gly were perceived with strong bitterness (Otagiri et al., 1985). Dipeptides (Gly-Asp, Ala-Glu and Glu-Leu) were reported to exhibit sour and umami tastes simultaneously (Roudot-Algaron et al., 1994). Furthermore, the eleven-amino acid peptide (Ala-Pro-Pro-Pro-Pro-Ala-Glu-Val-His-Glu-Val) was found to have an acid inhibitory function (Okumura et al., 2004).

### Interactions Between Taste Peptides and Other Tastants Co-existing in Foods

Taste peptides may exhibit a synergistic effect with other co-existing flavor substances (such as sodium glutamate, sodium inosinate, disodium guanylate, sodium succinate, sodium acetate and acidulant), or significantly enhance or alter their original flavor characteristics. Accordingly, the special flavor characteristics of taste peptides can be utilized not only for improving food palatability, but also as a safe substitute for high sodium condiments.

It is worth noting that a concrete taste substance may not only impart a certain taste or several tastes, but also influence the perception of other substances in a different taste type. Taste–taste interactions have been explored widely in sensory research, molecular biology investigations on taste receptors, and human psychophysical studies. Previous research showed that bitterness could be masked by umami substances (Noguchi et al., 1975). Taking umami taste as an example, intracellular  $\text{Ca}^{2+}$ -flux signal induced by salicin could be significantly suppressed by five umami soybean peptides (Glu-Asp, Glu-Glu, Glu-Ser, Asp-Glu-Ser and Glu-Gly-Ser) in hTAS2R16-expressing cells via bitter taste receptor(s) in a time-dependent manner, and in a noncompetitive mode (Kim et al., 2015). Park et al. (2002) reported that 0.3% NaCl could enhance the umami and sweetness of synthesized oligopeptides with the amino acid sequence originated from a Vietnamese fish sauce. It was also reported that sucrose and monosodium glutamate had positive effects on the umami taste of the octapeptide subfraction extracted and isolated from puffer fish muscle (Zhang et al., 2012).

Tastants such as sugar, salt or monosodium glutamate in small amounts can enhance the overall flavor of foods. Moreover, these compounds can interact with the food aroma compounds (Friel et al., 2000). The intensity of perceived apple and lemon flavors was found higher in the presence of a sour taste (Cayeux et al., 2003). The congruency between olfactory and gustatory stimuli seems to be an important criterion for taste-referred aroma enhancement in a food product. Taste-induced enhancement of aroma significantly depends on the consonance of aroma and taste stimuli e.g. the enhancing effect detected during the interaction of fruitiness and sweetness or sourness (Frank and Byram, 1988).

### Odor-Induced Flavor Enhancement

The aromas of food products are derived from a variety of volatile aroma substances, most of which have no or little nutritional value and possess poor thermostability. Food aroma and taste play important roles in appetite control: aroma and taste alone or in combination can influence appetite sensation and food intake. Aromas also indirectly improve the digestion and absorption of nutrients. The perception of aroma is more sensitive and complex than taste perception. Aroma perception is highly correlated with the molecular structure of aroma and takes effect even at a very low concentration (i.e. 1–1000 ppm). The threshold values and concentrations of aroma substances are important parameters that determine the ultimate effect of aroma.

Certain tasteless substances (especially volatiles) could also influence taste perception. Aroma is well-known for its considerable contribution to the perception of taste even in trace amount. The “odor-induced changes in taste perception” phenomenon have been examined in quite a few studies. However, minimal efforts have been made in examining odor-induced taste peptide enhancement.

Specific odor-induced influences take place in the perception of salt taste. Sodium salt is a commonly used condiment, which is indispensable for the human body. However, excessive consumption of sodium salt may lead to a number of diseases such as hypertension, cardiovascular disease, kidney disease and other serious diseases. High intake of sodium salt would affect the disease resistance of human cells and aggravate the burden of kidneys. It is of high importance to explore solutions to reduce the amount of sodium salt in foods without compromising food taste. Utilizing the interactions between odor and taste substances represents a feasible approach for reducing the intake of sodium salt. The enhancement of saltiness is related to the consistency of odor and the concentration of salt in aqueous solutions or solid foods. Research showed that the intensity of perceived salty taste was significantly increased by the sardine aroma when the salt concentration was at a low- or medium-level, but no significant change in salty taste was found at high salt concentrations (Nasri et al., 2011). Similarly, specified odors may induce enhancement of saltiness in low salt solutions, suggesting an approach through which the amount of sodium chloride in food can be reduced without losing any intensity of salty taste (Lawrence et al., 2009). Nasri et al. (2013) found that the effect of odor-taste interactions on potassium chloride was stronger than on sodium chloride. Accordingly, odor-sour-salty ternary systems may allow a desirable flavor improvement based on the same mechanism as that for odor-induced taste enhancement in real food systems. In solid cheese models, comté cheese and sardine odors were found to play a significant role in the enhancement of saltiness of either flavored or unflavored cheese (Lawrence et al., 2011). Syarifuddin et al. (2016) also indicated that odors could compensate for taste insufficiency in low-salt, low-sugar and low-oil cheese model through cross-modal interactions. Emorine et al. (2015) reported that the effect of ham odor-induced saltiness enhancement was independent of salt and aroma diffusion. It is known that the ham with an uneven salt distribution was saltier ( $P < 0.01$ ) than that with homogeneous salt distribution, but the unevenly distributed ham odor

could affect neither the perception of taste nor the perception of odor. Djordjevic et al. (2004) confirmed that soy sauce odor-induced saltiness enhancement and strawberry odor-induced sweetness enhancement were centrally mediated and also resulted from taste-smell interactions.

The well-selected odor, even at a subthreshold level, can markedly enhance the perception of taste. The effect of sweet-congruent odor on sweetness perception was detected by Labbe and Morgenegg (2007) using a continuous flow system with a constant sucrose concentration but different odor concentrations. The authors indicated that at a supra-threshold level, ethyl butyrate and maltol would have the highest and lowest sweetening properties, respectively. At a subthreshold level, ethyl butyrate could significantly enhance the sweetness of the sucrose solution, whilst maltol did not, probably due to the different odorant-tastant associations and respective sensitivity. The concentration of ethyl butyrate is not in direct proportion to the enhancement of sweetness at a subthreshold level, but an opposite trend was detected at a supra-threshold level. The odor-induced sweetness enhancement is correlated with the concentration of a sweet substance. Symoneaux et al. (2015) confirmed that enhancement of cider's sweetness would occur when the sugar content was in the range of 35–40 g L<sup>-1</sup>.

Caporale et al. (2004) found that the presence of cut grass odor was beneficial for inducing a higher intensity of bitterness and longer duration in virgin olive oil even when diverse stimuli were upheld. McCabe and Rolls (2007) observed that a higher intensity of pleasant flavor was achieved if vegetable aroma was combined with sodium glutamate. Moreover, integration of vegetable aroma and sodium glutamate was found to facilitate a greater stimulation of the medial orbitofrontal cortex and pregenual cingulate cortex than the sum of respective stimulation provided by vegetable aroma or sodium glutamate, as revealed by functional magnetic resonance imaging. Niimi et al. (2014) found that at the low- and middle-aroma concentrations, cheese flavor was increased with the concentration of monosodium glutamate, but decreased in the system with aroma at a high concentration. Cheese aroma could significantly enhance the perception of umami and bitterness, but had no effect on the intensity of sweetness, saltiness or sourness. Therefore, the perceptible interaction between taste and aroma of cheese was influenced by the type of taste and the concentration of aroma. The same type of odor-induced flavor enhancement may lead to totally different results in varied food systems. In a cocoa beverage, cocoa and vanilla odors would induce enhancement on bitterness and sweetness, respectively. But surprisingly, vanilla odor did not enhance sweetness, on the contrary, enhanced the bitterness of caffeinated milk (Labbe et al., 2006).

Odor-induced changes in taste perception was mediated centrally (Caporale et al., 2004), and correlated with chemical reactions and physical interactions between taste and odor components as well as their subsequent interactions with taste receptors (Sun-Waterhouse and Wadhwa, 2013). Based on the obtained psychophysical and neuroanatomical results, the cerebral cortex activity of the coordinated odor-taste combination was extremely higher than that of the incongruent integration, involving insula, frontal operculum, anterior cingulate cortex, and orbitofrontal cortex (Seo et al., 2013). Flavor perception results from the central integration, which depends on the previous experience with congruent taste-odor integration. Dynamic stability of taste attractors in gustatory cortical was regulated by feedback signals from orbitofrontal cortex to gustatory cortical, resulting in an enhancement or inhibition of taste perception caused by certain aroma (Shimemura et al., 2016). Another reason for such odor-induced taste enhancement could be the cognitive link between the taste and aroma features. Aromas may possess certain taste attributes. For example, vanilla odor tends to be associated with sweet perception, whilst sardines odor is likely related to salty perception (Lim et al., 2014). Thus, the congruency of taste and odor in taste-odor interaction is especially important (Small and Prescott, 2005). Lawrence et al (2011) reported that the carrot odor, which was not related to saltiness, did not enhance saltiness (Lawrence et al., 2011). When studies are carried out on such odor-induced taste enhancement, one should pay attention to a “dumping” effect (the taste rating is extended in the absence of related attributes e.g. the perceived olfactory enhancement on the taste scale was evaluated with no olfactory intensity scale available) (Lim et al., 2014). In these cases, reaction deviation should be considered and included.

## Conclusion

Taste, odor or aroma are commonly used terms in describing food sensory attributes. The relationships between these attributes, and between them and other sensory attributes, are rather complicated, which represents an ongoing and challenging research topic. This article highlights the importance of understanding the interactions among various taste and odor substances in improving both the sensory attributes and the healthiness of human diet and food, as well as the feasibility to utilize the approaches based on odor-induced taste enhancement for reducing the amounts of undesired or even harmful food additives in the final consumer foods.

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# Encyclopedia of Food Chemistry: Protein–Phenol Interactions

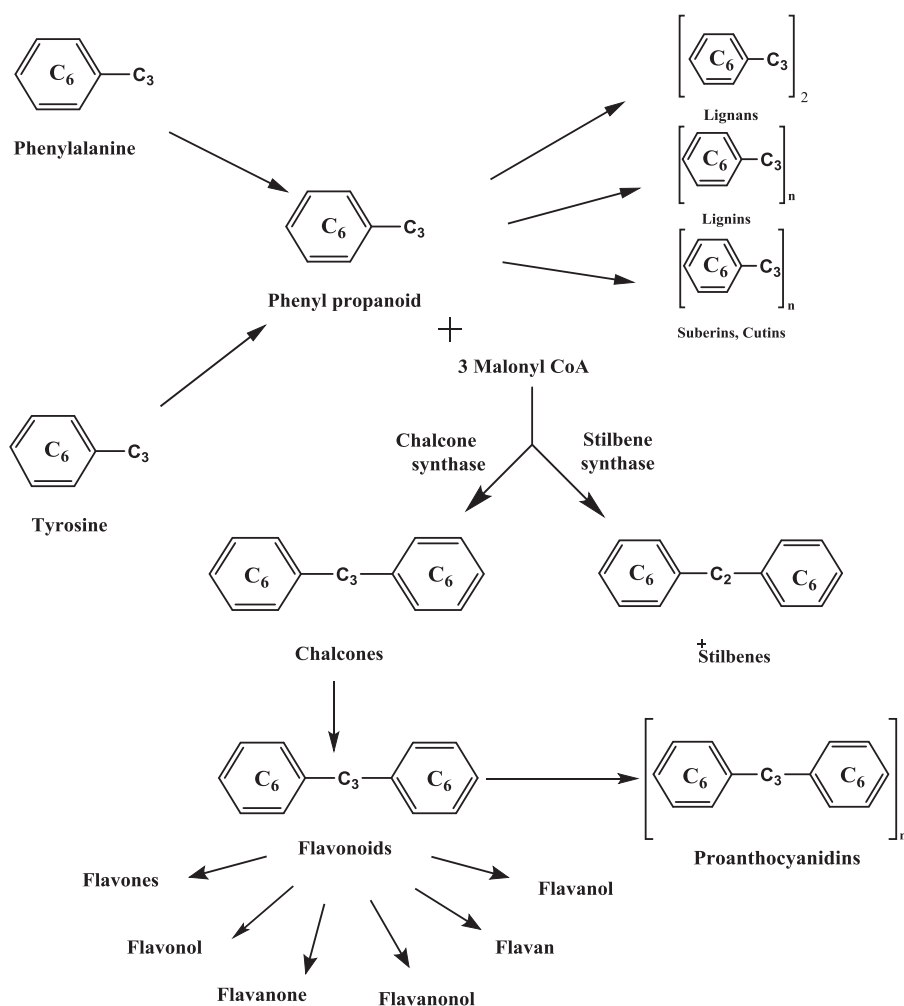
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## Introduction

Proteins in foods can form complexes with other food components, including polyphenols, which lead to vital changes in their structural, functional and nutritional properties (Shahidi, 2000; Ozdal et al., 2013; Bordenave et al., 2014; Shahidi and Ambigaipalan, 2015). Understanding of these changes as a result of interactions with phenolic compounds is essential in light of their scientific, industrial, and economic significance.

Phenolic compounds are plant secondary metabolites and are derived from phenylalanine and, to a lesser extent, tyrosine (Fig. 1) (Shahidi, 2000). Their chemical structures have an aromatic ring attached to one or more hydroxyl groups, including their functional derivatives (Shahidi and Ho, 2005). Due to the indigestion of plant foods these phenolic compounds can be found in animal tissues and non-plant materials (Shahidi, 2000). Plant phenolics may act as natural pesticides, providing protection against ultraviolet rays and also involved in pigmentation in plant foods, among others (Shahidi and Nacz, 2003; Martinez-Gonzalez et al., 2017). Generally, phenolic compounds can be classified into simple phenols, hydroxybenzoic acid and hydroxycinnamic acid derivatives, flavonoids, stilbenes, lignans and hydrolyzable as well as condensed tannins, the latter also known as proanthocyanidins (Nacz and Shahidi, 2006; Shahidi and Ambigaipalan, 2015; Martinez-Gonzalez et al., 2017). Proanthocyanidins exist as oligomers and polymers of flavonoids like flavan-3-ols whereas hydrolysable tannins are often found as glycosylated gallic acid (Ferreira and Li, 2000; Shahidi, and Ambigaipalan, 2015). More than 8000



**Figure 1** Phenolics compounds originating from phenylalanine and tyrosine. Adapted from Shahidi and Nacz (2003).

phenolic compounds have been identified in plant-based materials such as fruits, vegetables, cereal grains, legumes and oilseeds, among others (Ozdal et al., 2013). The simple phenols mainly include monophenols such as *p*-cresol, thymol and orcinol mainly isolated from fruits (Naczki and Shahidi, 2006; Ozdal et al., 2013). Diphenols such as hydroquinones are considered as the most widespread simple phenols (Shahidi and Ho, 2005). In addition, polyphenols present in foods may further be categorized according to their carbon atoms in conjunction with the basic phenolic skeleton (Ozdal et al., 2013). Hydroxybenzoic acids possessing the general structure C6–C1 (such as gallic, vanillic, syringic, and ellagic acids) and hydroxycinnamic acids which have C6–C3 (*p*-coumaric, caffeic, ferulic, sinapic, and chlorogenic acids) are the two main classes of phenolic acids, the latter being more common in food products compared to the former (Shahidi and Ho, 2005; Shahidi and Ambigaipalan, 2015).

The other prominent subclass of polyphenols is the flavonoids and they are ubiquitously found in plants. They have the general formula of C6–C3–C6 with flavonoid skeleton which consists of two benzene rings linked by a 3 carbon heterocyclic ring (Shahidi and Naczki, 2005; Martinez-Gonzalez et al., 2017). Flavonoids are further divided into subgroups such as flavones, flavanones, flavonols, flavanonols, isoflavones, flavanols (catechins), and anthocyanidins according to the substitution of the heterocyclic ring (Strat et al., 2016). Flavanols, also known as flavan-3-ols, are hydroxylated at C3 in the heterocyclic ring and often exist in the monomeric form or as oligomers/polymers (Strat et al., 2016). The compounds (+)-catechin (+C), (–)-catechin (–C), (–)-epicatechin (EC) are the major dietary flavanol monomers (Jakobek, 2015). Flavonols are different from flavones by possessing a hydroxyl group in the 3-position and can be regarded as 3-deoxyflavonol (Shahidi and Ambigaipalan, 2015). Flavonols are referred to as 3-hydroxyflavonones or dihydroflavonols and differ from flavonones by having a hydroxyl group in the 3-position (Shahidi and Naczki, 2003).

Phenolic compounds containing two benzene rings separated by an ethane bridge are called stilbenes and found abundantly in higher plants (Shahidi and Ho, 2005). Lignans are dimers of phenylpropanoid (C6–C3) units linked by the central carbons of their side chains and four major groups of linear lignans are formed according to their oxygenation such as lignans (butane derivatives), lignolides (butanolide derivatives), monoepoxylignans (tetrahydrofuran derivatives) and bipheoxylignans (Shahidi and Naczki, 2003). Coumarins are another group of polyphenols derived from lactones of *cis*-*O*-hydroxycinnamic and can be found in the free form or as glycosides, especially in the plant kingdom (Shahidi and Naczki, 2003). In general, most of the dietary polyphenols have been subjected to numerous *in vivo* and *in vitro* studies to demonstrate their interactions with macro- and micronutrients (Bordenave et al., 2014). All these interactions are influenced by various parameters, including temperature, pH, ionic strength, type and nature of the protein and phenolic compounds that are involved in the formation of bonding or complexes (Ozdal et al., 2013). The mechanism and type of these interactions also play a vital role as they influence the functional properties of both the protein and the phenolic compounds (Jakobek, 2015). Upon oxidation, phenolic compounds gain strong affinity towards macronutrients. Various studies have investigated the bioaccessibility and bioavailability of dietary phenolic compounds (Jakobek, 2015). From a nutritional point of view, bioavailability of phenolic compounds is the most important parameter in the evaluation of their physiological effects. Therefore, it is imperative to consider the nature and the significance of these interactions and their subsequent effects on food systems as a whole.

### Formation of Protein–Phenolic Interaction

Basically, four types of interactions are reported for phenolics and proteins and these are hydrogen bonding, ionic (electrostatic) interaction, covalent bonding and hydrophobic interaction (Bordenave et al., 2014; Ozdal et al., 2013; García-Estévez et al., 2017; Fernandes et al., 2017). Among them, hydrophobic and hydrogen bondings account for the main driving force for all these interactions (Jia et al., 2016). According to Shahidi and Naczki (2003), phenolics form complexes at both low and high concentrations of proteins due to the formation of hydrophobic interactions. This is further explained by hydrogen bonding via hydroxyl groups in the phenolic compounds and the carbonyl groups of the peptide bonds of proteins (Loomis and Battaile, 1966).

Interactions between protein and phenolic compounds can be found in different occasions such as phenolic compounds attached to the storage and physiologically active proteins present in plants, interactions of plant phenolic compounds with proteins present in food matrices, during digestion with enzymes in the gastrointestinal tract, after the digestion during absorption they may interact with plasma proteins and finally there may be a possibility to interact with the target tissues or organs in the human body.

Formation of these interactions can be divided into three stages. At initial stage after the addition of polyphenol, several polyphenol molecules may bind with the proteins; then higher amount of polyphenol can induce the formation of polyphenol-coated dimers, thus causing precipitation and finally with increasing the number of molecules larger complexes may be formed (Tang et al., 2017). These aggregates can either be soluble or insoluble (Bandyopadhyay et al., 2012). The interaction of the –OH groups of polyphenols with the protein chain or hydrophobic stacking of the aromatic groups of the protein and polyphenols lead to the formation of aggregates.

Hydrophobic interactions can occur between two aromatic rings where one could be hydrophobic amino acid (Le Bourvellec and Renard, 2012). This is also known as stacking interaction. Protein concentrations have a direct influence on this hydrophobic interaction as hydrophobic monolayer is formed at low protein concentrations while at high concentrations more hydrophobic surface layer is formed due to the complexation of polyphenols and proteins. This process also includes the cross-linking of protein molecules. Aggregation and precipitation occur as a consequence of all these steps. Ionic bonding or electrostatic forces



between the hydroxyl group of the phenol compounds and amino groups of protein residues takes place in such interactions (Martinez-Gonzalez et al., 2017).

Furthermore, Kroll et al. (2003) proposed five potential types of interaction between phenolics and proteins, namely hydrogen bonding,  $\pi$ -bonding, as well as hydrophobic, ionic, and covalent linkages. Compared to covalent linkages, non-covalent linkages are weaker since they do not involve sharing of electron pairs (Nagy et al., 2012; Jakobek, 2015). Most of these non-covalent interactions are reversible and are assumed to be stabilized by hydrogen bonding (Yuksel et al., 2010; Jia et al., 2016). In contrast, covalent bonds form irreversible interactions.

Reversible complexation takes place in a two-stage process, in the first stage the polyphenol and the co-substrate, are in equilibrium with soluble complexes via various non-covalent forces (Luck et al., 1994). In a second stage these soluble complexes may aggregate and precipitate from the solution upon which the position of this equilibrium changes. The precipitated complexes may then redissolve and this whole process is reversible under favorable conditions (such as absence of oxygen, metal ions, acid or base). The maximum protein–polyphenol interaction occurs at the isoelectric point of the protein (Trigueros et al., 2014) or optimum pH for the complex formation which is described as the pH at which maximum precipitation occurs (Naczek et al., 1996).

Different phenolic compounds can form complex with active groups of protein. In particular, natural phenolic compounds are prone to interact with active groups in protein molecules. This may affect biological, nutritional and functional properties of proteins including changes in surface hydrophobicity, solubility, gelling, emulsification, and antioxidant properties of the protein molecule (Ozdal et al., 2013; Bordenave et al., 2014; Jakobek, 2015; Shahidi and Ambigaipalan, 2015; Jia et al., 2016; Martinez-Gonzalez et al., 2017; Czubinski, and Dwiecki, 2017). As proteins are affected by their interactions with phenolic compounds, the biological activity and functionality of phenolics are also affected by their interactions with proteins.

### Protein Interactions With Phenolic Acids

Phenolic acids as low molecular weight plant phenols and are considered as important native food components with a wide range of structures and functions. Phenolic acids generally have one or more aromatic rings bearing one or more hydroxyl groups and in particular, presence of aromatic ring with hydroxyl group and carboxylic acids in phenolics increases their affinity to conjugate with major food components (Alu'datt et al., 2016). Unique array of phenolic acids which include derivatives of hydroxybenzoic and hydroxycinnamic acids are found to form complexes with proteins (Shahidi and Zhong, 2010; Shahidi and Chandrasekara, 2013). The interaction of phenolic acids with proteins occurs mainly by hydrogen bonds involving the hydroxyl groups of polyphenols and polar groups of proteins, as well as hydrophobic contacts between the planar ring surfaces of phenolic compounds and the rings of proline residues (Ferrer-Gallego et al., 2017). Moreover, the process of protein hydrolysis may weaken the protein–phenolic complexes by reducing the interactions between these compounds and by increasing the availability of the nucleophilic sites of peptides and of phenolic compounds (Guimarães Drummond e Silva et al., 2017) in millets (Chandrasekara and Shahidi, 2011), coffee beans (Narita and Inouye, 2011), flaxseed (Guimarães Drummond e Silva et al., 2017), salivary proteins (Ferrer-Gallego et al., 2017) and myofibrillar protein (Cao and Xiong, 2015).

### Protein Interactions With Flavanoids

Flavonoid–protein interactions are widely investigated with polyphenols such as flavan-3-ols, flavonols and proanthocyanidins (Xiao, and Kai, 2012; de Freitas and Mateus, 2012; Bordenave et al., 2014; Watrelot et al., 2016; Chaudhury et al., 2017). The formation of flavonoid–protein complexes is usually promoted by the food matrix (Czubinski, and Dwiecki, 2017). Protein–flavonoid complexation is mainly due to non-covalent interactions between the compounds of interest. These non-covalent interactions between flavonoids and proteins can further be divided into chemically specific or non-specific (Bordenave et al., 2014). Most of the specific bindings are between flavonoids and globular tertiary structure of protein such as hemoglobin or milk immunoglobulins (Xi, and Guo, 2007; Xiao et al., 2011).

Flavonols and flavones also have the potential to form specific non-covalent interactions with proteins. Hydrogen bonds between the hydroxyl groups of the polyphenol ligands and the catalytic residues of the binding site can directly be linked to the inhibitory activity of the flavonoids over human digestive enzymes (Lo Piparo et al., 2008). Flavonoids interact with dietary proteins and digestive enzymes irreversibly in the gut and are assumed to be transported in vivo bound to plasma proteins (Brunet et al., 2002). Non-specific, non-covalent interactions of flavonoids and proteins have often been detected in salivary proline-rich proteins (PRP), BSA, ovalbumin and casein (Bordenave et al., 2014). Moreover, higher affinity of PRPs is expected for PRP compared to BSA and other globular proteins as PRP possess a randomly coiled structure and globular proteins lack proline which is considered to be an important feature for polyphenol binding (de Freitas and Mateus, 2012).

Complexation of flavonoids with salivary PRPs is also reported to result in astringency. For example, complexation of oligomeric flavonoids, such as proanthocyanidins, and salivary PRP create aggregates that precipitate (Bordenave et al., 2014). Compared to oligomeric flavonoids, interaction between monomeric flavonoids and salivary protein form complexes that do not precipitate (likely soluble complexes) but result in astringency perception (de Freitas and Mateus, 2001). London interactions between the non-polar polarizable aromatic rings of flavonoids and non-polar polarizable parts of protein side chains operate in both of these scenarios either forming soluble or insoluble complexes (Bordenave et al., 2014).



## Protein Interactions With Stilbenes

Stilbenes, such as resveratrol (3,5,4-trihydroxystilbene), interact with different types of proteins and this complexation influences the binding sites, structure and the conformational changes of proteins. The protein-phenol complexation includes hydrophobic and amphiphilic ligands and may lead to structural transition of proteins. Particularly, structure-affinity relationships and the physicochemical properties-affinity relationships of phenol–protein interactions were found to be important in both food and pharmaceuticals (Bordenave et al., 2014).

## Protein Interactions With Lignins

Lignins are complex substances with aromatic rings, formed via polymerization of a mixture of the three monolignols *p*-coumaryl, sinapyl and coniferyl alcohols (Shahidi and Naczek, 2003). The phenolic group in the lignin is considered as an excellent hydrogen donor. It can interact with functional groups of proteins including carboxyl, hydroxyl, and amino groups to form covalent and hydrogen bonds (Pradyawong et al., 2017). In other words, they can interact with the side-chain of amino acids via quinone intermediates (Cong et al., 2013). Plant cell wall-localized proteins complex with the wall structural components, mainly lignins (Cassab, 1998). Particularly, amino acids with nucleophilic side chains can covalently attach to lignin (Cong et al., 2013). Moreover, it has been suggested that lignin interacts with protein to form a protein-lignin complex or network by hydrophobic interactions, and ionic and hydrogen bonds (Pradyawong et al., 2017). Lignins enhance the tensile strength, thermal stability, and Young's modulus by cross-linking with proteins (Doherty et al., 2011; Salas et al., 2012; Xiao et al., 2013). Understanding the nature of lignin–protein interactions is of fundamental importance as it plays a critical role in lignin-based value-added products such as bio-based adhesives.

## Protein Interactions With Coumarins

Coumarins are lactones of *cis*-*O*-hydroxycinnamic acid that exist in the free form or as glycosides (Shahidi and Naczek, 2003). Compared to other phenolic compounds, coumarins and their interaction with other macromolecules including proteins were studied mainly under anti-diabetic candidates (Ali et al., 2016), antibiotic agents (Gormley et al., 1996; Marcu et al., 2000) and other health beneficial components. Hydrophobic binding sites of these proteins were found to determine the capacity of binding aromatic and heterocyclic compounds to them and strong bindings of hydroxycoumarin derivatives that improved their therapeutic activities.

## Protein Interactions With Tannins

The defining characteristic of tannins is their ability to precipitate protein molecules (Hagerman et al., 1998). The interaction between proteins and tannins influences the appearance and taste of foods and beverages (Shahidi and Naczek, 2003; Ozdal et al., 2013), the function of ecological (Hagerman and Butler, 1991) and agricultural (Kumar and Singh, 1984) systems. Tannins interact with proteins mainly through hydrogen bond formation between the phenolic donor and the peptide acceptor (Hagerman et al., 1998; Nie et al., 2017). The nature of tannin/protein interactions is either covalent or non-covalent, based on whether the molecules were irreversibly bound to each other or not. In addition, as a consequence of the type of bonding, complexes formed can either be soluble or insoluble (Soares et al., 2012). There is an optimum tannin/protein ratio for which maximum precipitation occurs. It has been observed that the quantity of insoluble precipitates is lower for both higher and lower tannin/protein ratios. Besides, as protein structure plays an essential role in complex formation (Hagerman et al., 1998), binding sites of the proteins are important. For example, if protein has a fixed number of binding sites and tannin has more than one binding site, even though there is excess tannins, the final result is dictated by the number of binding sites in the protein. All the sites in proteins would be occupied by tannins and due to that tannin could not find available binding site for another protein when it is attached at one end. This results in smaller particles and less precipitation (less haze).

Proline-rich proteins (PRP) form strong hydrogen bonds with tannins (Hagerman et al., 1998; Soares et al., 2012). Conformation of these interactions of PRP are flexible and increase the accessibility of hydrogen bonding sites (Hagerman et al., 1998; Watrelot et al., 2017). The association between tannin and PRP is suggested to have face-to-face stacking of aromatic groups onto proline residues while interaction with globular proteins may involve only surface exposure of aromatic residues (de Freitas and Mateus, 2001). Complexation of tannins and salivary PRPs is due to their involvement in inducing the astringency in food products including wine, teas and some dairy products (Scollary et al., 2012; Thongkaew et al., 2014). These processes of creating complexation between polyphenol and protein is commonly utilized in food processing industries such as wine making which improves the haze stability and taste (Sarni-Manchado, and Cheynier, 2002; Nguela et al., 2016). Interactions of tannins with peptides and proteins also possess biological importance as they influence the absorption, metabolism and bioactivities of polyphenols in the human body including inactivation of digestive enzymes. Moreover, they may be responsible for plant defence mechanisms against predators (Sarni-Manchado and Cheynier, 2002).

**Table 1** Potential effects of phenol–protein interactions on nutritional and health related properties of foods

Property of the food	Source of food protein	Phenolic compound	Changes in functionality or modification	References
Allergic potential	peanut proteins	dietary polyphenols	direct inhibition of mast cell mediator release suppression of T cell proliferation, inhibition of cytokine secretion	Nowak-Węgrzyn and Sampson (2011) and Srivastava et al. (2002)
	milk and peanut proteins	green tea catechins	Increase the resistance of allergenic proteins towards the enzymatic hydrolysis	Astwood et al. (1996); Maleki et al. (2000)
	Egg white protein	green tea polyphenol	polyphenol decrease the allergenicity of egg white protein and modulate immunoreactivity	Foegeding et al. (2017)
Anti-inflammatory properties	milk protein	green tea polyphenol	accelerate protein gelation rate and produced firm $\beta$ -lactoglobulin gels improve the antiproliferative bioactivity of the polyphenols	Schneider et al. (2016); von Staszewski et al. (2011)
Emulsifying properties	zein protein	tannic acid	stabilize emulsions by a pickering emulsion gel mechanism	Zou et al. (2015)
	$\beta$ -lactoglobulin	green tea polyphenols	improve the stability of emulsion by producing surface active particles	von Staszewski et al. (2011)
Foam formation	$\beta$ -lactoglobulin whey protein isolate	catechin phenolic compounds from cranberry, blackcurrant and muscadine grape juices	improve foam formation and stability enhance foam formation and stability compared to whey protein isolate alone	Sarker et al. (1995) Schneider et al. (2016)

### Potential Effects of Protein–Phenol Interaction on Nutrition and Health

Protein–phenol interactions influence the sensory properties and as well as nutritional composition of the foods at the molecular level (Foegeding et al., 2017). These associations are likely to impact the functional properties of foods as they enhance the protection against chronic and degenerative diseases (Bordenave et al., 2014). Growing consumer interest and demand for foods rich in biologically-active, health-protective properties have encouraged the food application of protein-polyphenol complexation. Use of proteins as encapsulating agent for polyphenols (Foegeding et al., 2017), for stabilizing protein foam structures (Prigent et al., 2009), use of protein–polyphenol interactions to stabilize, deliver and improve biological activity of phenolic compounds (Bordenave et al., 2014) are some of the efforts made to functionalize food with protein-phenol combination and taking advantage of their association. However, according to nutritional view point, nutritional quality and bioavailability of phenolic compounds as well as proteins may be significantly effected by the protein-phenol complexation (Table 1) and thus rational design of food products would ensure efficient utilization of phenol–protein interactions.

### Methods Used for Analysis of Phenolic–Protein Interactions and Their Limitations

Analysis of the interactions between proteins and phenolic compounds is important as it has direct influence on nutritional and functional properties of food products. Most of the frequently used analytical methods to investigate protein–phenol interactions is based on spectroscopic methods. UV–Vis spectroscopy is an ideal technique to investigate molecular nature of the interactions of phenols and proteins. Generally, spectrophotometric methods are considered as common, easy, practical, and qualitative methods to observe conformational changes in proteins and can also be used for selective determination of phenolic compounds in the presence of other substances (Avan et al., 2016; Zhang et al., 2017). In addition, microscopic, thermodynamic, electrophoretic, chromatographic, and bioinformatics analyses are other analytical methods reported for this purpose (Ozdal et al., 2013; Rohn, 2014; Jakobek, 2015; Czubinski and Dwiecki, 2017). Fluorescence spectroscopy (FLU) and calorimetric studies (isothermal titration calorimetry and differential scanning calorimetry) are considered as ideal methods for investigating the interaction between polyphenols and digestive enzymes. However, main challenges are still associated with the development of effective methods that could be used for both quantitative and qualitative determination of protein–phenol interactions.

### Conclusion

Protein–phenol interactions can lead to both favourable and adverse effects on structure, functional and nutritional properties of proteins as well as phenolic compounds. The consequences of these interactions play an important role in influencing the functional properties and sensory attributes of food components. The protein-phenol complexation may be reversible or irreversible. Protein-phenol associations may be via covalent bonding or non-covalent interactions. Contradictory results have been reported for bioavailability of the phenolic compounds, factors affecting the degree of the interactions and determination

methodologies for protein-phenol complexation. Further studies are needed to provide a deeper understanding and also to explore this complex phenomenon in order to clarify the controversial observations already reported in the literature.

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# Analysis of Flavonoid-Protein Interactions by Advanced Techniques

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## Introduction

Flavonoids possessing three ring chemical structure (C6—C3—C6) are ubiquitous in plants such as cereals, legumes, vegetables, and fruits as their secondary metabolites. In plants, flavonoids perform pivotal roles such as controlling auxin transport, defending against external predators, and acting as UV filters (Kitamura, 2006). Recently, there has been an increasing interest in flavonoids by both researchers and consumers due to their remarkable bioactivities such as anticancer, anti-inflammation, and anti-virus as well as reducing cardiovascular disease and type-2-diabete (Lin et al., 2000; Panat et al., 2016; Watanabe et al., 2007; Kang et al., 2013; Chtourou et al., 2016).

Proteins, which are assemblies of 20 different kinds of amino acids, are essential macromolecules for the life. Proteins are involved in a variety of functions in plant metabolism such as the regulatory function which decides the influx of compounds to the inside of cell or organelle at the surface of membrane or vice versa and play the structural role in which proteins help make the rigid formation of cellulose fiber at the cell wall. In addition, proteins serve as the main nutritional storage sources that are used for the development and growth of seeds. Proteins act similar roles in biological systems; that is, they act as a building material for tissues (collagen), render catalytic activity (enzymes), or are involved in the mechanisms for muscle movement (myosin and actin) (Foegeding, 2015).

In foods, a variety of macronutrients and micronutrients are present together, which causes numerous chemical interactions during storage and food processing. Among them, flavonoid-protein interaction is a common chemical reaction, leading to major changes in physico-chemical characteristics of proteins. For example, the interaction of flavonoid and protein affects solubility, thermal stability, and digestibility of proteins (Labuckas et al., 2008; Rawel et al., 2001). In addition, the structural changes of essential amino acids by the reaction with flavonoids influence to the nutritional quality of proteins (Kroll et al., 2003). The flavonoid-protein interaction also affects the antioxidant potential and the bioactivity of flavonoids mostly in a negative way due to the loss of hydroxyl groups upon the formation of covalent or non-covalent interaction between flavonoids and proteins (Duarte and Farah, 2011; Burg-Koorevaar et al., 2011; Serafini et al., 2009; Blauz et al., 2008). For instance, the interaction of milk proteins with flavonoids in coffee/tea decreases the antioxidant capacities of flavonoids in ABTS radical cation scavenging capacity and ferric reducing antioxidant power (FRAP) assays (Sanchez-Gonzalez et al., 2005; Ziyatdinova et al., 2010). Furthermore, the complexation of walnut proteins, mostly glutelin and globulin, with walnut flavonoids reduced the DPPH radical scavenging ability of flavonoids. The binding of milk proteins with flavonoids in cocoa also suppressed the antioxidant potential as revealed in ABTS radical cation scavenging capacity, ferric reducing antioxidant power (FRAP) assays, and DPPH radical scavenging ability (Belščak et al., 2009; Labuckas et al., 2008).

The interaction between flavonoids and proteins has been well investigated using several advanced techniques such as infrared (IR) spectroscopy, fluorescence spectroscopy, electronic absorption spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy, which exploits the property of elementary particles such as electron, nucleus and photon based on the quantum mechanics. For example, the interaction of  $\alpha$ - and  $\beta$ -caseins with tea flavonoids such as (+)-catechin, (–)-epicatechin, (–)-epigallocatechin and (–)-epigallocatechin gallate observed by using Fourier-transform infrared spectroscopy (FTIR), fluorescence spectroscopy, and electronic absorption spectroscopy (Hasni et al., 2011). Therefore, such advanced techniques are successfully employed for understanding the interaction of flavonoids and proteins.

Electron paramagnetic resonance (EPR) spectroscopy is also a useful means for the determination of chemical reaction between molecules. However, the limitation of this technique is related to the fact that it is only applicable to the radical or resonance possessing molecules, thus EPR spectroscopy could only be used for monitoring the reaction of flavonoids with free radicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH). Hence, this allows one to evaluate electron donating potential of flavonoids. Thus, EPR spectroscopy has successfully been used to determine the antioxidant potential of plant-based foods such as lentils, millets, and fruit seeds (Yeo and Shahidi, 2017; Alshikh et al., 2015; Chandrasekara and Shahidi, 2010; Ayoub et al., 2016) and could potentially be used to determine changes in their reactivity upon complexation with proteins.

## Chemistry of Flavonoid-Protein Interaction

Flavonoids and proteins can chemically interact during food processing and storage by forming reversible and irreversible interactions. The reversible interaction of flavonoid-protein is formed via non-covalent bonds such as hydrogen bond, hydrophobic interaction and van der Waals forces (Charlton et al., 2002a,b; Jobstl et al., 2004; Poncet-Legrand et al., 2006; Prigent et al., 2003; Richard et al., 2005, 2006; Siebert, 2006). This weak interaction can be readily broken by a relatively low level of energy. Conversely, the irreversible interaction, which is covalently bound, is not dissociated even under alkali or acidic hydrolysis. The formation of irreversible covalent bond accompanies a complex reaction mechanism, which is initiated by the oxidation of flavonoids. The oxidation of flavonoids, especially two hydroxyl groups of the B-ring in flavonoids, leads to the formation of o-quinone (O'Connell and Fox (1999)). The o-quinone can then react with thiol and amino groups of proteins through the nucleophilic 1, 4-Michael



**Table 1** Analysis of flavonoid-protein interaction using advanced techniques

Analytical technique	Flavonoid	Protein	References
Infrared (IR)	rutin	gelatin	Yan et al. (2011)
	(+)-catechin, (–)-epicatechin	$\alpha$ -, $\beta$ -casein	Hasni et al. (2011)
Fluorescence	(–)-epigallocatechin	$\beta$ -lactoglobulin	Wu et al. (2011)
	quercetin	whey protein	Mehanna et al. (2014)
	apigenine, kaempferol, quercetin and myricetin	soyprotein (soy glycinin)	Rawel et al. (2002)
	(+)-catechin, (–)-epicatechin	$\alpha$ - and $\beta$ -caseins	Hasni et al. (2011)
Electronic absorption	quercetin	human serum albumin	Zsila et al. (2003)
	(–)-epigallocatechin	$\beta$ -lactoglobulin	Kanakis et al. (2011)
	(+)-catechin, (–)-epicatechin	$\alpha$ - and $\beta$ -caseins	Hasni et al. (2011)
Nuclear magnetic resonance (NMR)	5,7-dihydroxy 4'-methoxy isoflavone,	Bovine serum albumin, soy glycinin, and $\beta$ -lactoglobulin	Ali (2002)
	(–)-epicatechin	salivary proline-rich proteins	Baxter et al. (1997)
	(+)-catechin, (–)-epicatechin	salivary proline-rich proteins	Delius et al. (2017)

addition which subsequently leads to the formation of irreversible covalent bond between flavonoids and the proteins (Le Bourvellec and Renard, 2012). The irreversible interaction between flavonoids and proteins has been reported in the literature. For instance, Rawel et al. (2002) demonstrated the irreversible interaction of soyprotein with apigenine, kaempferol, quercetin and myricetin. In addition, O'Connell and Fox (1999) and Tantoush et al. (2011) explored the irreversible covalent bond between  $\beta$ -lactoglobulin and sour cherry flavonoids. However, most of the research work on the determination of covalent bond between flavonoids and proteins have been carried out in an indirect way such as protein precipitation using turbidimetry and nephelometry, thus this chapter concentrates more on non-covalent interactions that are usually determined by advanced techniques such as IR, fluorescence and electronic absorption spectroscopy, as well as NMR (Table 1).

## Analysis of Flavonoid-Protein Interaction by Advanced Techniques

### Infrared (IR) Spectroscopy

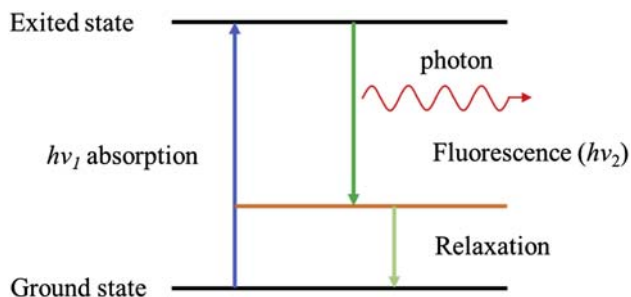
Infrared spectroscopy is a simple and feasible technique widely used in diverse areas including food science. This advanced technique exploits the fact that the specific movement of molecules such as rotation and vibration absorbs in the infrared wavelength range through the resonance. Infrared is defined as electromagnetic radiation having wavelengths in the range of 0.8–1000  $\mu\text{m}$  ( $14000\text{--}10\text{ cm}^{-1}$ ), which is relatively longer than visible, ultraviolet, and X-ray. The energy of infrared wavelength can be absorbed by the molecular movements such as rotation and vibration as mentioned earlier. For instance, the oscillation (stretching frequency) at the hydroxyl group of a molecule absorbs wavelength at  $3610\text{--}3670\text{ cm}^{-1}$ . Thus, IR spectroscopy allows one to identify functional groups and their changes during chemical reaction. The interaction of flavonoid-protein leads to the alteration of functional groups such as hydroxyl and amide due to the covalent or non-covalent interaction between them. This can be detected using IR spectroscopy.

Yan et al. (2011) found the complexation of gelatin with rutin using Fourier-transform infrared spectroscopy FTIR. Hasni et al. (2011) demonstrated the complexation of  $\alpha$ - and  $\beta$ -caseins with tea flavonoids using FTIR, in which they reported two casein amid bands, namely band I at  $1656\text{--}1652\text{ cm}^{-1}$  for C=O stretching and band II at  $1544\text{--}1500\text{ cm}^{-1}$  for C–N stretching, then found the shift of these bands after the complexation of flavonoid with casein. Wu et al. (2011) studied the complexation of (–)-epigallocatechin (EGC) and  $\beta$ -lactoglobulin using FTIR; they also observed the shift of amide I (mainly C=O stretch) and II band (C–N stretch coupled with N–H bending mode), i.e. amide I band moved from  $1639.37$  to  $1641.30\text{ cm}^{-1}$  and the amide II band moved from  $1541.01$  to  $1542.93\text{ cm}^{-1}$ . They concluded that the band shift may be due to the alteration in secondary structure of  $\beta$ -lactoglobulin upon complexation with (–)-epigallocatechin (EGC). In another study, the complexation of whey protein with quercetin was also measured by FTIR; the electrostatic interactions such as hydrogen bond between whey proteins and quercetin contributed to the stabilization of ketone groups and peptide bonds and these were reflected in the changes of FTIR spectrum. That is, band I at  $1648\text{--}1649\text{ cm}^{-1}$  for the C=O stretching vibrations and band II at  $(1536\text{--}1547)\text{ cm}^{-1}$  for the N–H bending vibrations were shifted upon complexation of whey protein with quercetin (Mehanna et al., 2014).

### Fluorescence Spectroscopy

Fluorescence is defined as the emission of wavelength by a molecule after excitation by light and electromagnetic radiation. Individual molecules possess their own intrinsic energy level (ground state) and they are excited by absorbing specific strength





**Figure 1** The excitation of a molecule by absorbing wavelength and yielding fluorescence.

of energy which can elevate to the upper energy level. The excited unstable molecules tend to be stabilized by releasing energy via rotation, vibration, and translational motion. Furthermore, some molecules release energy in a wavelength (normally lower energy level than the excitation wavelength), and the released wavelength is via fluorescence (Fig. 1). Fluorescence spectroscopy observes the changes of excitation wavelength of target fluorescence compounds, which is induced by the alteration of electron density of the molecules, hence it has been widely used for the investigation of chemical interaction between molecules including flavonoid-protein complexation.

The binding of flavonoid-protein has been successfully explored by using fluorescence spectroscopy. The alteration of the quantum yield of fluorescence provides useful information about the chemical interaction between molecules (Tayeh et al., 2009). For example, the intrinsic tryptophan fluorescence such as Trp-37, Trp-66 in  $\alpha_{s1}$ -casein and Trp-109 in  $\alpha_{s2}$ -casein is used for the detection of chemical interaction of casein protein with flavonoids (Kumosinski et al., 1993). Rawel et al. (2002) reported the interaction of soyprotein (Soy glycinin) with apigenine, kaempferol, quercetin and myricetin using fluorescence spectroscopy in which the intensity of the emission wavelength of soyprotein at 338 nm was decreased after interaction. Hasni et al. (2011) studied the interaction of  $\alpha$ - and  $\beta$ -caseins in milk with tea flavonoids such as (+)-catechin, (–)-epicatechin, (–)-epigallocatechin and (–)-epigallocatechin gallate by using intrinsic fluorescence of tryptophan in casein; they determined the binding constant between flavonoids and caseins, and the results showed a higher constant value in (–)-epigallocatechin and (–)-epigallocatechin gallate than (+)-catechin and (–)-epicatechin due to the presence of more hydroxyl groups. Moreover, the shifted emission band observed after complexation also allowed more detailed explanation of the interaction. The downward shift was found in catechin- $\alpha$ -casein and epicatechin- $\alpha$ -casein complex (from 350 nm to 334 and 343 nm) due to the tightening of protein structure by hydrogen bonding. Fluorescence spectroscopy was also employed to determine the binding of quercetin, rutin, and isoquercetin with proteins such as human serum albumin, bovine serum albumin, and soy glycinin through the alteration of excitation and emission energy levels (Rawel et al., 2005).

### Electronic Absorption Spectroscopy

Electronic absorption spectroscopy, referred to as UV/Visible spectroscopy, is routinely used in analytical chemistry. The principle of electronic absorption spectroscopy relates to the absorption of UV/visible wavelength by the chromophore of molecules; that is, an electron in the chromophore such as  $\pi$ -bond absorbs the photon having UV/visible wavelength through resonance and this leads to the excitation of the electron to the higher energy level. Especially, the electron in the outer shell of molecules is responsible for the absorption of UV/visible wavelength, which is longer and low energy wavelength than X- and  $\gamma$ -ray. Thus, electronic absorption spectroscopy is a useful means to detect the changes of electronic state of molecules resulted from the interaction with other molecules such as flavonoid-protein interaction.

Until now, the electronic absorption spectroscopy has been successfully adopted in understanding the interaction of flavonoid and protein. Zsila et al. (2003) found a strong bathochromic shift of approximately 30 nm upon the interaction of quercetin to the human serum albumin at 374 nm. Besides, the band broadening at that wavelength was also observed upon complexation, which occurs by the interactions between molecules such as dipole-dipole, H-bond, and ionic interaction, hence it is explained as the non-covalent interaction of quercetin to the amino acid residue of human serum albumin. Kanakis et al. (2011) studied the interaction of  $\beta$ -lactoglobulin with (–)-epigallocatechin (EGC); they found the changes of spectrum upon complexation of  $\beta$ -lactoglobulin with (–)-epigallocatechin (EGC). Hasni et al. (2011) found the interaction of milk  $\alpha$ - and  $\beta$ -caseins with tea flavonoids using UV-visible spectroscopic method by yielding the binding constant from the reciprocal plot and the result showed that the stability of casein-catechin and casein-epicatechin was similar due to the same number of hydroxyl groups, however the complexation of casein with (–)-epigallocatechin (EGC) and (–)-epigallocatechin gallate (EGCG), possessing more hydroxyl groups, revealed a better stability than the catechin- and epicatechin-casein complexation.

### Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy that exploits the magnetic characteristic of certain atomic nuclei is a representative means to identify the chemical structure of molecules. The nuclear magnetic resonance provides key information about the structure, dynamics, reaction

state, and chemical environment of molecules; thus, the nuclei spin of atom in molecule is influenced by neighboring atom(s) and the surrounding environment, leading to scrutinizing the chemical structure of target molecules (Le Bourvellec and Renard, 2012). The chemical shift, which is the resonant frequency of a nucleus compared to the standard compound in a magnetic field, is varied depending on the electron shielding from an atom or functional groups in molecule. Thus, the chemical interaction of flavonoid with protein, which accompanies the alteration of electron shielding, can be explored using NMR spectroscopy by monitoring the alteration of chemical shift. The NMR spectroscopy allows accurate determination of interaction sites, mechanism of interaction, and even the dissociation constants upon the interaction of flavonoids and proteins (Murray et al., 1994; Charlton et al., 2002a,b; Baxter et al., 1997; Wroblewski et al., 2001). The interaction of flavonoids and protein have been widely investigated by using NMR. For example, Delius et al. (2017) studied the interaction between salivary proline-rich proteins and flavonoids by  $^1\text{H}$ -NMR spectroscopy, in addition Baxter et al. (1997) investigated the interactions of flavonoids and a salivary proline-rich protein using NMR in which they found that flavonoids with more hydroxyl groups interact with the salivary proline-rich protein in a multidentate fashion as well as self-polymerization. On the other hand, beyond the advantages of NMR spectroscopy such as giving precise binding sites, the number of bonding sites, and the interaction of molecules with environments (i.e., complexation of flavonoid-protein), it has a serious drawback in the application of NMR spectroscopy. That is, the sample for the application to the NMR should be prepared in a high concentration to gain good resolution of peaks for the correct interpretation. However, many flavonoids and proteins have a low solubility at high concentrations, especially in aqueous medium, which makes it difficult to apply to the NMR system.

## Conclusion

The interaction between flavonoids and proteins is responsible for the formation of both reversible and irreversible interactions. The reversible interaction of flavonoid-protein is produced through non-covalent bonds such as hydrogen bond, hydrophobic interaction and van der Waals forces, whereas irreversible interactions occur by covalent bonding that is not dissociated by even alkali or acid hydrolysis process. The structural changes upon interaction of flavonoids with proteins have been successfully investigated using advanced techniques such as infrared (IR) spectroscopy, fluorescence spectroscopy, electronic absorption spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy. Each technique has its own advantages and disadvantages in the application for the analysis of flavonoid-protein interactions, thus it is imperative to consider the condition of samples prepared and research environment to obtain the required results.

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# Covalent Interactions Between Proteins and Phenolic Compounds

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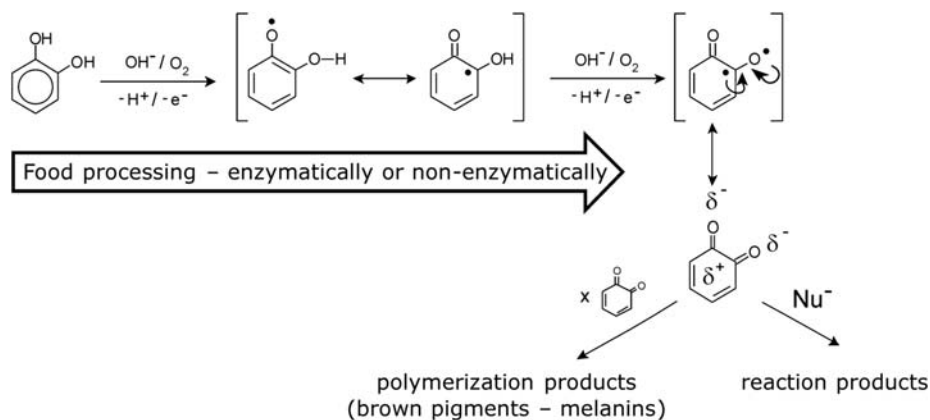
## Introduction

Phenolic compounds are secondary plant metabolites that plants use for coloring, UV shielding, as phytoalexins, or repellents (Friend, 1979; Dixon and Paiva, 1995). As part of the human diet, they are hypothesized to affect human health either in a positive way or in some cases lead to adverse implications (Scalbert et al., 2005; Crozier et al., 2009). However, in most studies chemical transformations of phenolic compounds during plant food preparation have not been considered carefully, and compounds are often regarded as pure isolates, even inert. In realistic scenarios and because of high reactivity of phenolic compounds, their interaction with other compounds present cannot be excluded. Often such interactions already take place in the plant, as endogenous mechanism for activating or inhibiting a certain function (e.g., glycosylation for enhancing solubility and transport). However, due to the high number of other compounds present, a whole bunch of nontargeted reactions should be considered as well. With regard to food processing - conditions of higher temperature, or certain treatments affecting the plant cell structure (e.g., peeling, cutting) - even more reaction products can be expected, as compounds "meet" each other that have been not related in the untreated plant before. In the further course, after consumption of a plant-based food, interactions and reactions in the human body are possible, as well.

Among the primary compound classes, proteins seem to be the most interesting ones, as they can interact with phenolic compounds on different occasions. From a chemical point of view, protein-phenol interactions can occur in different ways. Similar to the typical protein-protein interactions, hydrogen and ionic bonding, hydrophobic and aromatic interactions are key mechanisms. Moreover, covalent bonds are of special interest, as such bonds are mainly irreversible and influence the (chemical) properties of both reaction partners enduringly.

## The Covalent Reaction of Phenolic Compounds With Proteins

A key step of the reaction of phenolic compounds with proteins is the capability of the phenolic compounds to be easily oxidized enzymatically or nonenzymatically (Cilliers and Singleton, 1991; Friedman, 1996) and being therefore highly reactive (Fig. 1). Primarily, the resulting ortho- or para-quinoid structures are reacting with other quinones to produce dark melanin pigments (Nicolas et al., 1994; Friedman, 1997; Robards et al., 1999). This is valid for most of the phenolic compounds. In case of mono-hydroxyl phenolic compounds such as ferulic or coumaric acid, the reaction proceeds via the formation of semiquinones. The free radicals can then participate in nonenzymatic reactions, including polymerization, hydration, and disproportionation (Thurston, 1994; Selinheimo et al., 2007). But also during thermal treatments (i.e., during food processing), the formation of quinones is a key element and will lead to (smaller) degradation products that may also serve as substrates for follow-up reactions such as polymerization (browning) (Buchner et al., 2006). All the previously mentioned reactions are prerequisites for generating electrophilic species capable of underlying a nucleophilic (Michael-type) addition (Fig. 1). In case of the proteins, they provide nucleophilic side chains as preferred reaction partners. Especially  $\epsilon$ -amino groups of lysine residues, thiol groups, or the indole group of tryptophan are the most reactive ones. In one of the earliest investigations, Mason and Peterson (1955) reported that the reaction of the quinone proceeds rapidly at a neutral pH, as demonstrated for proline and nucleoprotamine. Besides, imidazole and disulfide groups have



**Figure 1** Oxidation of a catechol moiety as a prerequisite for the formation of reaction products. Reprinted from Food Research International, vol. 65, Part A, Rohn, S., November 2014. Possibilities and Limitations in the Analysis of Covalent Interactions between Phenolic Compounds and Proteins, pp. 13–19, with permission from Elsevier. Permission was requested and publisher (Elsevier) agreed (License Number 4207161081661).

also been reported to be reaction partners (Vithayathil and Murthy, 1972; Loomis, 1974; Matheis and Whitaker, 1984a,b; Kroll et al., 2003).

### Where Does This Happen?

A review of the literature reveals the following locations or categories for the interactions of phenolic compounds: (1) interactions in the plant with storage and physiological active proteins; (2) interactions of plant phenolic compounds with food proteins during food processing, resulting in changes of food texture; (3) interactions may further occur with food proteins or enzymes in the course of digestion in the gastrointestinal tract; (4) during digestion, proteins/enzymes secreted in the gastrointestinal tract may serve as the interaction partners, as well; (5) after absorption, plasma proteins can be the target; and finally (6) interactions possibly occur with proteins in target tissues/organs in the human body.

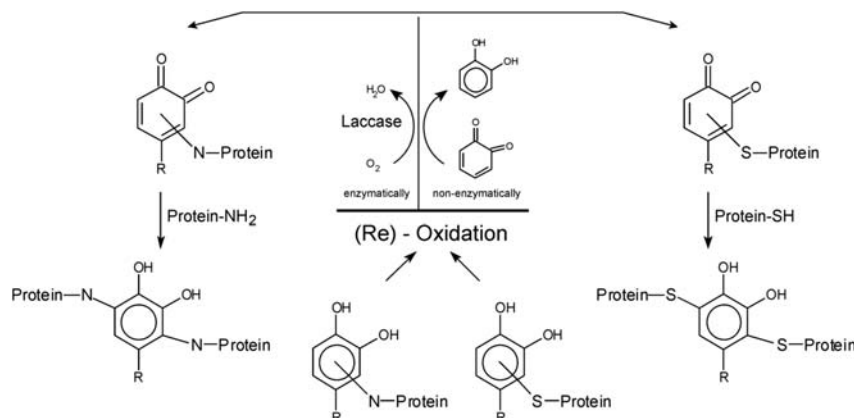
### Physicochemical Consequences

From the point of view of the chemical structure, the conformation of a protein is influenced by the posttranslational modifications. As described above, such reactions almost occur nontargetedly, which means that depending on the initial protein structure/conformation, the most accessible protein side chains react primarily, before the protein refolds and uncovers to further generate possible reaction sites. This further means that every protein might be affected specifically depending on the conditions (phenolic substrates, pH value, temperature, amino acid sequence, isoelectric point). A prediction of the degree of derivatization seems to be impossible, which is a severe aspect when one tries to characterize/analyze such protein derivatives. A change in the protein structure leads to a corresponding change in the hydrophobic–hydrophilic properties of the protein derivatives, which is accompanied by a change in the solubility and a shift of the isoelectric point. These changes in turn may influence certain technofunctional properties (e.g., emulsion-, foaming properties) (Prigent et al., 2003; Gonzalez-Perez et al., 2002; Gonzalez-Perez and Vereijken, 2007).

With regard to color, it has been shown with smaller reaction products that the implementation of the aromatic rings leads to a color shift. Under conditions of autoxidation of caffeic acid esters and in presence of an amino compound the formation of benzacridine derivatives was observed (Namiki et al., 2001). The reduced form of the derivative was found to be yellow and further with characteristic products of green and blue color (Namiki et al., 2001; Yabuta et al., 2001). Several green trihydroxy benzacridine derivatives were identified during the alkaline sunflower meal extraction. There, the presence of chlorogenic acid in the meal leads to the formation of *o*-quinones (Bongartz et al., 2016). Larger reaction products turn brown as observed during alkaline sunflower protein processing (Sabir et al., 1974).

### Protein Cross-linking

When taking a look at the reaction mechanism, it is obvious that the phenolic compounds remain reactive after the addition to a protein side chain. This means that they are able to be oxidized again, and depending on the structural/conformational characteristics of the protein, a second binding of a further protein molecule can occur (Fig. 2). This protein cross-linking leads to dark-colored protein complexes of high molecular weight (Sabir et al., 1974).



**Figure 2** Protein cross-linking as a result of the reaction of oxidized phenolic compounds with protein side chains, followed by a second enzymatic or nonenzymatic reoxidation of the phenolic compounds. Reprinted from Food Research International, vol. 65, Part A, Rohn, S., November 2014. Possibilities and Limitations in the Analysis of Covalent Interactions between Phenolic Compounds and Proteins, pp. 13–19, with permission from Elsevier. Permission was requested and publisher (Elsevier) agreed (License Number 4207161081661).

## Nutritional Consequences

### Phenolic Compounds

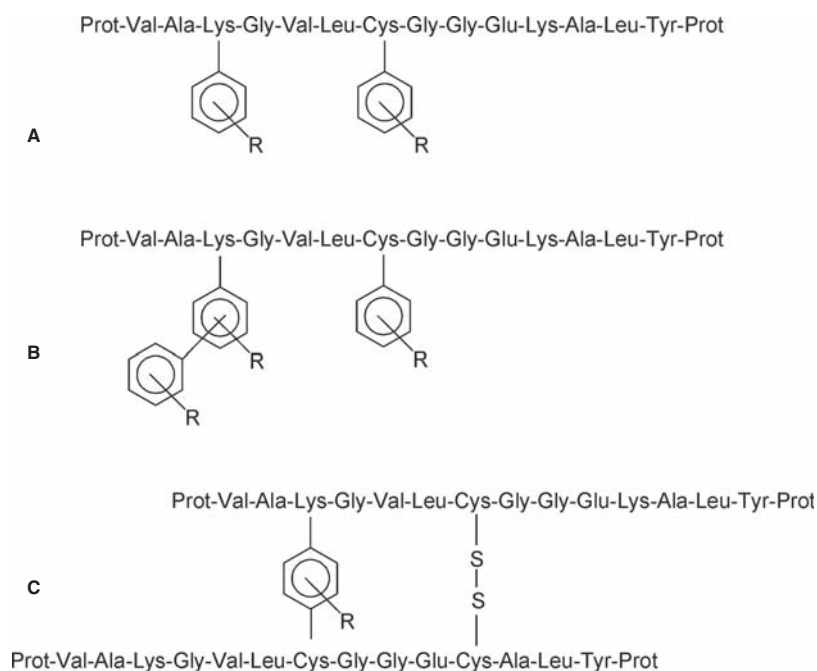
Resulting from their reaction with proteins, properties of the phenolic compounds might be affected as well, e.g., the antioxidant activity of the phenolic compounds declined as a result of the covalent attachment to the protein (Rohn et al., 2004, 2005).

### Proteins

The derivatization of proteins (with phenolic compounds) influences the biological activity of proteins/enzymes (Alberghina, 1964; Rohn et al., 2001, 2002, 2004, 2005). Indispensable amino acids such as lysine and tryptophane are preferred reaction partners; thus a loss of nutritional quality of the proteins can be observed (Davies et al., 1978; Petzke et al., 2005; Rohn et al., 2006). For example, a rat growth and nitrogen balance study showed that a protein derivatization with chlorogenic acid affected the nutritional quality of beta-lactoglobulin (Petzke et al., 2005). Proteolytic digestion of such protein derivatives showed that at a lower degree of derivatization, the tryptic degradation was adversely affected, whereas the peptic digestion declined with increasing modification. In vitro experiments illustrated that the digestion of derivatized lysozyme with enzymes of the gastrointestinal tract was adversely affected (Rawel et al., 2001a).

### Phenolic Polymers or Phenol-Protein Reaction Products?

As planned by nature, the phenolic compounds preferably react with themselves prior to the reaction with the amino group. Usually this leads to dark-colored melanins observed during the enzymatic browning of plant tissues. In the presence of nucleophilic amino groups and taking the mechanism proposed by Namiki and coworkers into account, a dimerization of phenolic compounds prior to their interaction with proteins during heating and at alkaline pH values seems to be a precedent reaction (Namiki et al., 2001; Yabuta et al., 2001). This was also already shown for chlorogenic acid in simple model mixtures with selected amino acids (Prigent et al., 2003). With regard to proteins and protein cross-linking, complexity can be even higher as more or less oligomerized phenolic compounds might serve as substrates for the reaction with the proteins. Consequently, three types of reaction products have to be considered (Fig. 3A–C): In the first case, there are simple reaction products, where single phenolic compounds are bound to single specific amino acid side chains (Fig. 3A). Secondly, the phenolic compounds form oligomers of varying molecular weight before being bound to the amino acid residues (Fig. 3B). Finally, there is protein cross-linking that can occur via monomeric or even



**Figure 3** (A–C) Different reaction product profiles. Simple reaction products, where single phenolic compounds are bound to single specific amino acid side chains (A); mixed reaction products consist of bound oligomers besides monomers (B); complex cross-linked proteins and variations thereof (C). Reprinted from Food Research International, vol. 65, Part A, Rohn, S., November 2014. Possibilities and Limitations in the Analysis of Covalent Interactions between Phenolic Compounds and Proteins, pp. 13–19, with permission from Elsevier. Permission was requested and publisher (Elsevier) agreed (License Number 4207161081661).



oligomeric phenolic structures. Further, the occurrence of neoformed disulfide bridges is also possible due to the various redox reactions of the phenolic compounds and the transformation of the protein structure/conformation (Fig. 3C).

Recently, two types of covalent protein–phenol reaction products have been described in honey. With the use of size-exclusion chromatography it was possible to reveal that oligomerization of phenolic monomers seems to occur prior to protein binding (Brudzynski et al., 2013). As obvious, a mix of all kinds of reactions might be possible under realistic conditions of food processing, which makes the analysis of phenol–protein reaction products a huge challenge.

### Analysis of the Phenol–Protein Reaction Products

Analysis of phenol–protein adducts is quite challenging, as the phenolic compounds remain reactive after the addition to the protein. Understanding the conditions required for the formation of covalent bonds or the biological consequences of the covalent modification is hindered by the lack of suitable analytical methods for evaluating the reaction products and the paucity of methods to purify the products for a complete characterization (Hagerman, 2012). Strategies and methodologies are not yet satisfactory.

Similar to the analysis of posttranslational modifications determined in biochemistry and physiology (e.g., glycosylations, phosphorylations), the analysis of nontargeted reaction products of phenolic compounds and proteins is challenging. It could be rated as an even greater challenge, because most of the biochemical posttranslational modifications are catalyzed by specific enzymes that can be taken into account for analyzing the reaction products. In case of phenol–protein reactions occurring during food processing, consumption, and digestion with multiple influencing factors described earlier (oxidation conditions, chemical structure of the substrates, pH value, temperature, protein sequence and structure, etc.), the obtained products are of a far higher variety.

From simple adducts to more complex intermediates up to cross-linking products, an analytical strategy has to be developed, which fits the specific goals of analysis. When investigating protein–phenol adducts, there are several possibilities. From all the aspects mentioned so far, it seems to be of further interest in knowing to which extent a protein is modified.

Indirect methodologies focus on the estimation of modified amino acids, analyzing the extent of phenolic compound bound, or evaluation of the resulting change in the properties of both compounds. For example, there are numerous (coloring) reagents to determine free amino groups in a protein. One of the approaches appropriate for phenol–protein adducts is the derivatization with trinitrobenzenesulfonic acid in a sodium dodecylsulfate solution of the samples (Adler-Nissen, 1972). For modified whey proteins, it has been shown that the amount of free amino groups declines resulting from the modification with different plant phenolic compounds (Rawel, Kroll and Hohl, 2001b; Rohn et al., 2001). From such data it is possible to give a rough estimation on the degree of modification. With regard to nutritional value, it is possible to determine the amount of (bio)available lysine (James and Ryley, 1986). For the analysis of modified thiol groups there are also several methods available, e.g., the total thiol groups can be determined by a fluorescence method, using monobromobimane as a derivatization reagent (O’Keefe, 1994). Similar to the analysis of free amino groups, a reaction of phenolic compounds does also lead to a decline in the amount of free thiol groups. Application of denaturing and reducing reagents such as sodium dodecylsulfate and dithiothreitol leads to full unfolding of the modified protein and allows evaluating if disulfide bridges have been involved in the reaction. Denaturation and fluorescence measurement can also be used for the estimation of a tryptophan modification (Jackman and Yada, 1989). A possible reaction of oxidized phenolic compounds with the heterocyclic N-atom of tryptophan has also been discussed previously (Hurrell and Finot, 1984).

The consequences of covalent reactions between phenolic compounds and proteins can be evaluated at hand of a series of changes in selected protein properties (Kroll et al., 2003). Chromatographic and electrophoretic behaviors (e.g., changes of the isoelectric point) are affected. The hydrophobic–hydrophilic properties and solubility are also modified, and these in turn may influence certain functional properties (e.g., emulsion-, foaming properties) of the protein derivatives (Prigent et al., 2003; Gonzalez-Perez et al., 2002; Gonzalez-Perez and Vereijken, 2007).

Direct analysis is used to identify the specific binding site in a protein structure. Information about specific binding sites in the protein sequence to predict, correlate, or even model resulting effects on the protein conformation with the corresponding change of its properties would be very valuable. However, there are certain limitations. As already described, main reasons are the complexity of reaction products formed and the remaining reactivity of both reaction partners: proteins and phenolic compounds. Several analytical techniques, such as capillary electrophoresis, electrospray mass spectrometry, high-performance affinity chromatography, NMR spectroscopy, fluorescence quenching, and multispectroscopic methodologies, have been developed to characterize protein–phenol interactions (Le Bourvellec and Renard, 2012; Xiao and Kai, 2012). However, identification of specific binding sites of small molecules within a protein sequence and the corresponding conformational position is still challenging. Innovative approaches are necessary to combine the abovementioned techniques. This has to be done in parallel by using more than one methodology to evaluate/correlate the data sets. A further option is the direct coupling of two techniques (“hyphenation”). A very good example for the latter approach and a promising alternative tool for the analysis of phenol–protein interactions is high-performance thin-layer chromatography (HPTLC). A few years ago, coupling of TLC and MALDI-TOF-MS was already successfully applied to analyze (unmodified) peptides (Gusev et al., 1995a,b). One-dimensional (1D) and two-dimensional (2D) HPTLC were already successfully applied to mapping of peptides (Pasilis et al., 2008a,b). Intact proteins and protein derivatives can be separated to a certain extent, as well (Biller et al., 2015).

Due to the possibility of developing HPTLC plates in two dimensions using two different solvent systems, 2D HPTLC enables protein or peptide separation with high resolution. In addition to the first dimension, which already can give good separation, a development in the second dimension can be added. Proteins/peptides, which are similar in polarity and molecular weight, may be detected as one band in 1D HPTLC. During 2D HPTLC such a band of several analytes can be separated, and each peptide can be detected as a single spot, which is similar to 2D gel electrophoresis but with far higher separation possibilities. The influence of the reaction between selected phenolic compounds on the proteins or the peptide profile in terms of quantity, polarity, UV activity, and antioxidant activity prior to or after proteolytic digestion can be studied with HPTLC. When modified proteins or peptides are separated, there are different possibilities for their detection on the plate. Usually, various staining reagents can be applied to reveal specific properties of the peptides. To observe changes in color and UV activity, detection under visible and UV wavelengths is performed. Staining with fluorescamine and ninhydrin is particularly used for peptide detection. Antioxidant activity of peptides or even modified peptides can be measured with solutions of stable radicals (Tschersch et al., 2013).

## Conclusions and Outlook

Reactions between phenolic compounds and proteins might severely influence the properties of both compound classes. Although covalent modifications occur only to a small extent, specific protein functions in plants, food, or the human body might be significantly affected. The consequences such as toxicological risks, when such protein derivatives are consumed or when such protein derivatives are formed in the human organisms, are not clearly understood and evaluated, yet. When comparing with reactions such as the Maillard reaction, which is also a very complex cascade of reactions, there may be health risks as well as advantages.

Analysis of the reaction products is challenging, as the reaction product mixture is quite complex depending on simple or complex modifications with protein cross-linking, where it is not sure, yet, how the cross-linking develops (e.g., in phenolic compound containing melanoidins). With regard to the kind of phenolic compound (small phenolic acids, flavonoids, or complex procyanidins or tannins), the more complex the phenolic compound, the more complex the reaction products will be. However, the oxidation of a catecholic moiety has to be the primary step. The phenolic compounds remain reactive during the course of reaction, and oligomers or degradation products of the phenolic compounds can be substrates for follow-up reactions. Analytical techniques have been highly improved in the last decade but are still inefficient to identify the binding sites at hand of strategies of traditional protein chemistry. Proteomics (*bottom-up* or *top-down* approaches) is also not fully applicable. Innovative strategies have to be developed, which not only focus on the protein but also taking into account the properties of the phenolic reaction partner. This does not necessarily mean that more modern devices have to be developed. Instead, the combination of traditional and hyphenated techniques, such as the coupling of HPTLC with mass spectrometry, might initiate a *neoproteomics* approach, directly targeting the phenol–protein reaction products.

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# Interactions Between Proteins and Polyphenols in Beer

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## Glossary

Haze in beer is essentially the variably-sized suspended particles that scatter light. Substances such as proteins, polyphenols (tannins), polysaccharides, yeast cells, bacteria, and foreign material can all contribute to the haze in beer. Two forms of postpackaging haze are often observed: chill haze (which results from precipitated proteins in a cooled beer) and permanent haze (which is visible in beer at room temperature). Hop haze is a permanent haze as a result of aggressive dry-hopping. Haze in beer may indicate bacterial infection (which is often accompanied by off-flavors).

## Introduction

Beer is one of the most widely consumed beverages worldwide because of its unique sensory attributes (including clear frothy appearance and fresh taste) as well as high nutritional value. With a few notable exceptions, consumers generally expect packaged beer to be clear and bright, thus beers should remain clear until they are purchased and consumed. However, as beer is inherently unstable, haze may develop inevitably during a long period of storage, and its formation is a quality indicator of physical instability. Despite the importance of both biological and flavour stability, the shelf life of beer is determined mainly by its physical stability (Steiner et al., 2010). The formation of haze or turbidity in clear and bright beer is regarded as not only a serious quality defect but also a potential hazard, thereby would decrease the likelihood of repeat purchasing of the product (Robinson et al., 2004). Nowadays, the brewers aim to delay the onset of haze so that any haze produced is imperceptible until after beer's intended shelf life.

## Haze Formation in Beer

Beer is a complex mixture of more than 450 constituents including proteins, carbohydrates, polyphenols, fatty acids, nucleic acids, amino acids, etc (Steiner et al., 2011). Some of these ingredients in bright beer tend to aggregate and form haze with different particle sizes and appearances. Glenister (1975) classified the haze particles in beer as native particles, process particles, and foreign particles according to the source of haze, whereas, Bamforth (2011) divided haze into precipitates, bits, haze, and invisible haze in the light of the size of haze. Among the contributors to haze formation, protein–polyphenol interaction is believed to be the most frequent cause (Bamforth, 1999; Siebert, 2006). There are two forms of postpackaging haze in beer, “chill haze” and “permanent haze” (Nadzeyka and Altenhofen, 1979). Chill haze develops when beer is chilled to 0 °C, which involves a reversible non-covalent association of small-polymerized polyphenols with proteins. When the beer is warmed to 20 °C, this haze can be redissolved into beer and the beer becomes clear and bright again. Permanent haze is present in beer at all practical temperatures, which initially results from non-covalent bonding between polypeptides and polyphenols, and soon after, covalent bonds are formed to yield non-reversible insoluble complexes (Siebert et al., 1996a). It is worth noting that both types of protein–polyphenol haze would normally develop and eventually exist in beer even when it is properly stabilized.

## Proteins Responsible for the Haze Formation in Beer

Beer contains two types of proteins: those must be retained (because of their role in foaming), and those should be eliminated or minimized (otherwise causing haze). Approximately 500 mg/L of proteinaceous substances occur in the form of polypeptides within the 5–100 kD size range, and are the products of barley proteins that have been modified chemically and enzymatically during brewing (Steiner et al., 2010). There is often far more proteins in beer than what is needed to form a haze (as low as 2 mg/L for haze formation) (Bamforth, 1999; Chapon, 1994; Steiner et al., 2010). The proteins in beers are considered as the main contributors to haze formation, and protein composition and the extent of protein modification (e.g. glycation, coagulation and aggregation) throughout malting and brewing are of particular importance. Indeed, the haze isolated from beers consists of about 40%–75% of proteins, polyphenols (in combination with proteins) and 2%–15% of carbohydrates (Steiner et al., 2010). Generally, the haze active (HA) proteins found in beer are derived from barley hordein, and their contributions to haze formation depend greatly on their proline content. The proline residues are involved in the sites where the polyphenols are attached to the protein. HA proteins also contain considerable glutamine residues, and adjacent proline and glutamine tend to enable unusually strong binding to polyphenols (Siebert, 2009). Asano et al. (1982) found that proteinaceous fractions responsible for chill haze in beer were derived mainly from malt hordein, and had molecular weights of 1–40 kDa and appreciable amounts of proline. Loisa et al. (1971) suggested that the acidic proteins originated from the albumins and globulins of barley were responsible for chill haze formation. Nadzeyka and Altenhofen (1979) proved that proline and glutamic acid-rich hordeins with the molecular weight within 10–30 kDa were the main initiators of haze development. Outtrup (1989) emphasized the importance of hydrophobic amino acid

residues in proteins that contributed to the formation of haze, and thought that the polypeptides in beer mostly related to physical instability were those derived from the hordein of barley and rich in proline and glutamine. Some researches proposed that polypeptides with an isoelectric point between 3 and 5 and relatively high molecular weights are particularly haze-active (Matsuzawa and Nagashima, 1990; Mussche, 1990). Moreover, 3%–7% of beer glycoproteins have been found to be involved in haze formation, with the most important glycoproteins in the size of 16.5 kD and 30.7 kD, and with a characteristic compositions of proline, glutamic acid, and hexose (Leiper and Miedl, 2009).

### Polyphenols Responsible for the Haze Formation in Beer

Phenolic compounds represent a group of chemical substances containing at least one phenol unit, which can be divided into monophenolic compounds (including phenolic acids or their decarboxylation products), non-flavonoid polyphenols (including stilbenes, chalcones, lignans and (hydrolyzable) tannins), and flavonoids and condensed tannins (such as flavones, flavonols, flavanols, flavanones, flavan-3-ols and condensed flavan-3-ols (proanthocyanidins)) (Aron and Shellhammer, 2010; Callemien and Collin, 2010; de Freitas and Mateus, 2012). Phenolic components in beer originate from malt and hop. Although hop is normally added much less (100 times lesser) than malt, hop contributes about 30% of total beer polyphenols and is the exceptional source of catechins and proanthocyanidins. It is worthy to note that not all the beer polyphenols are involved in physical instability and participate in haze formation, and oxidized flavonoids can have a strong influence in this aspect. Also, the structures of phenolic compounds could evolve during beer production and storage. HA-polyphenols should be able to effectively crosslink HA-proline-rich proteins to form a stable network causing precipitation (Siebert, 2006). It has been shown that the binding of phenol to protein depends to a great extent on the number and location of the hydroxyl groups on an aromatic ring (McManus et al., 1985). Polyphenols with a higher degree of polymerization are found to be more haze-active than those with a lower molecular weight, with one aromatic ring bearing two hydroxyl groups being sufficient for their interactions with proteins (Gramshaw, 1967; Siebert and Lynn, 1998). Polymerization degree appears to be a more determinant factor than the number of hydroxyl groups (Mulkey and Jerumanis, 1983). Accordingly, monophenols are hardly attached to proteins, and metadiaphenols have weak affinities for proteins, whereas, *ortho*-diphenols and vicinal triphenols exhibit moderate and relatively strong binding affinities, respectively (Siebert, 2006). Indeed, Mulkey and Jerumanis (1983) found that the haze-forming activity of polyphenols increased with molecular complexity (trimers > dimers > monomers). Moreover, Asano et al. (1984) reported that the flavan-3-ol monomers, (–)-epicatechin and (+)-catechin and (+)-gallocatechin, can bind to but do not crosslink with HA proteins, and phenolic acids had no haze-forming capacity. Interestingly, oxidized flavanols can initiate chill haze and, once condensed or polymerized into proanthocyanidins, they participate in the formation of permanent haze. In fact, flavanoids in beer are known constituents of permanent haze, and proanthocyanidin oligomers possess two or more binding sites within the same molecule, allowing their crosslinking with HA proteins. Trimer, tetramer and higher proanthocyanidin oligomers are difficult to survive over the course of the brewing process, whilst the proanthocyanidin dimers such as procyanidin B3 and prodelfinidin B3 have been shown as the main haze-active polyphenols in beer (Siebert and Lynn, 1998).

### Interactions Between Proteins and Polyphenols in Beer

Beer contains smaller amounts of haze-active polyphenols than haze-active proteins, and haze more likely occurs near pH 4.0 than at pH 3.0 or above pH 4.2. According to Siebert (2009), the basic mechanism underlying the interaction between proteins and polyphenols is that a polyphenol molecule with at least two binding sites attaches to two proteins and bridges are then formed between the polyphenol and two proteins to yield a 3-membered structure. Additional polyphenol molecules are able to form an attachment with this 3-membered structure, followed by further binding to additional protein molecules. This construction process would continue until the complex eventually grows so large that it is no longer soluble in beer. Some authors claim that the protein–polyphenol interaction is reversible at least initially (rather than covalent bonding) because heating can often disperse such initial protein–polyphenol hazes. Moreover, both hydrogen bond acceptors and non-polar solvents (but not salt) are found to interfere with haze formation in beer (Asano et al., 1982; Siebert et al., 1996b). As a result, hydrogen bonding and hydrophobic effect (but not ionic bonding) play an important role in the protein–polyphenol interaction (Siebert and Lynn, 2008). Protein–polyphenol interaction in a model or real beer system is mostly influenced by the concentrations of proteins, polyphenols, polysaccharides, alcohol content, oxygen, pH, temperature, ionic strength and the presence of metal ions (Siebert et al., 1996a,b). Siebert et al. (1996a,b) have conducted a detailed study on protein–polyphenol interaction in model system, in which the amount of haze was found to increase up to a peak and then decline when protein (polyphenol) content remained constant and polyphenol (protein) concentration was increased. An increase of pH would also cause a similar change in the amount of haze formed (which would increase firstly, and then decline), and the pH at which a maximum haze occur is much lower than the isoelectric point of gliadin. Meanwhile, Siebert et al. (1996a,b) noticed that alcohol had little effect on haze formation at the normal pH of beer, and the intermediate protein/polyphenol ratios resulted in the largest particles. In addition to such influencing factors investigated by Siebert and Lynn (2001), parameters such as oxygen, shaking, temperature, light, polysaccharides and (heavy)



metals will significantly contribute to colloidal instability caused by interactions between proteins and polyphenols (Callemien and Collin, 2010; Leemans et al., 2003; Collin et al., 2013). Temperature has a significant influence on haze formation in beer as an increase in temperature raises the rate of the protein–polyphenol reactions. Thus, pasteurization or storage at a higher temperature would accelerate haze formation (Stewart, 2004). Oxidation or the presence of oxygen has a great effect on beer haze formation, oxidation probably promotes the polymerization of polyphenols that cross-link with proteins to form haze (Bamforth, 1999). Indeed, monomeric polyphenols, such as catechin, would exhibit a higher potential to initiate haze when they are polymerized through oxidation (O'Rourke et al., 1998). Metal ions, particularly ferrous ion, can promote the formation of colloidal haze because the free radicals generated by Fenton and Haber-Weiss reactions in beer would attack proanthocyanidins to produce oxidation products with higher affinities for proteins (Kaneda et al., 1990). In addition, movement of beer accelerates haze formation because of rapid interaction of colloids, and light encourages oxidation and consequently causes haze formation (Callemien and Collin, 2010; Stewart, 2004). A number of polysaccharides have been associated with beer hazes or flocs, which include starch, mannan and beta-glucans (Siebert, 2009).

## Conclusion

In this review, we provide an overview of the current status of research on the interaction between proteins and polyphenols in beer and associated mechanisms. So far, the physical instability of beer is under control in both research and industrial settings. However, much research effort is still needed to maximize the nutritional profile of beer, especially nutrients like proteins and the health-promoting polyphenols, whilst minimizing haze formation.

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# Interactions Between Milk Proteins and Polyphenols in Model Systems or Complex Dairy Matrices

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## Overview

Polyphenols are secondary metabolites of plants and consist of one or more aromatic rings with one or more hydroxyl substitutions. They include different sub-classes such as flavanones, flavones, flavan-3-ols, flavon-3-ols, isoflavones, and anthocyanidins according to the differences in oxidation levels and substitutions on the heterocyclic pyran ring (C-ring) (Huvaere and Skibsted, 2015). Besides the main differences in carbon skeleton, modifications on primary and secondary structural elements (e.g. glycosylation, acylation, methylation etc.) causes great differences in structure and biological functions leading to complexity in polyphenol studies. Among polyphenols, flavonoids and phenolic acids have important places in human diet and are most abundantly found in fruits, vegetables, cocoa, coffee, tea, wine and beer. The intense research focus on polyphenols over the last decades is mainly due to their possible health promoting effects. The health benefits of polyphenols have been reported to result from the antioxidative and radical scavenging properties of these compounds which eventually prevent the formation of free radicals and resultant oxidation and damage that are associated with neuro-degenerative and cardiovascular diseases and common chronic diseases including cancer (Wang et al., 1996). On the other hand, bioavailability of phenolic compounds and their metabolites is affected by several factors such as food processing conditions and interactions with other molecules (salivary proteins, digestion enzymes etc.) (Cheynier, 2005). Bioavailability of polyphenols were reported to be higher when they are consumed with food rather than in form of supplements (Williamson, 2017). Therefore, their interactions with macromolecules in food are critical for nutrition studies.

The interactions of polyphenols with proteins have been extensively studied. Milk proteins are classified into two main groups namely caseins and whey proteins. Caseins are characterized with high proline content and amorphous structure. Main casein fractions found in milk are  $\alpha_{s1}$ -casein,  $\alpha_{s2}$ -casein,  $\beta$ -casein and  $\kappa$ -casein. These fractions in milk are held together by hydrophobic interactions, H-bonds and calcium phosphate bridges to form casein micelles.  $\beta$ -Lactoglobulin,  $\alpha$ -Lactalbumin, bovine serum albumin (BSA), and immunoglobulins are major whey proteins in milk and contain varying number of disulfide bonds which contribute to a compact spherical shape. Although the amount of whey proteins in milk is much lower than that of caseins, the nutritional and functional properties make whey proteins valuable components of milk. Milk protein-polyphenol studies initially gained attention due to some dietary habits such as consumption of tea or coffee with milk. Moreover, there is a growing interest in natural bioactive delivery systems in pharmacological studies. Special structural/functional characteristics and nutritional importance of milk proteins make them appropriate candidates as delivery tools for polyphenols. From the food science and technology point of view, the nature and consequences of milk protein-polyphenol interactions are critical for functional dairy product formulations. Utilization of polyphenols for improving techno-functional properties of dairy products has been under consideration in the last 20 years. Although the interactions of polyphenols with milk proteins in model systems and various dairy matrices have been recently reviewed and discussed in detail (Yildirim-Elikoglu and Erdem, 2017), the competitive binding of polyphenols to other macromolecules in complex dairy product matrices needs to be investigated. In addition, the conflicting findings have been revealed from a number of studies due to a wide range of structural differences and methods used to evaluate the binding. Development of standard methods for the determination of interactions and consequences of binding reactions seems essential for future studies.

## Mechanism of Interactions in Model Systems

Polyphenols can react with proteins both reversibly and irreversibly. Irreversible interactions involve covalent bond formation upon enzymatic or nonenzymatic oxidation of phenolic compounds to *o*-quinones which may lead to protein cross-linking. Contrarily, weak interactions such as hydrophobic, H-bonding and van der Waals attractions are responsible for reversible protein-polyphenol reactions. Over the years, a number of studies on milk protein-polyphenol interactions have been reported in model systems and the mechanisms behind these interactions were investigated. The majority of the studies pointed out the non-covalent interactions between milk proteins and polyphenolic compounds (Wu et al., 2011; Yuksel et al., 2010; Prigent et al., 2009; Ojha et al., 2012; Kanakis et al., 2011), while covalent interactions were less frequently reported (Gallo et al., 2013; Rawel et al., 2001).

Whey proteins in milk are of special interest especially as delivery tools for bioactive compounds due to their nutritious value, low cost and availability. A wide variety of phenolic compounds including phenolic acids, flavonoids, phytoalexins and extracts of polyphenol-rich foodstuffs have been studied extensively for molecular interactions with  $\beta$ -Lactoglobulin. The predominating forces between  $\beta$ -Lactoglobulin and polyphenols were reported to be H-bonding, hydrophobic interactions and van der Waals attractions (Wu et al., 2011; Ojha et al., 2012; Jauregi et al., 2016). On the other hand, irreversible covalent bond formation between  $\beta$ -Lactoglobulin and cocoa polyphenols was also reported (Gallo et al., 2013).  $\alpha$ -Lactalbumin, the second pre-dominant whey protein in milk, has been studied to a lesser extent. Noncovalent interactions were reported between complexes formed between

$\alpha$ -Lactalbumin and kaempferol, genistein, resveratrol, curcumin or green tea polyphenols (Mohammadi and Moeeni, 2015a, 2015b; Al-Hanish et al., 2016). BSA is a large globular protein and reported to bind various small compounds including fatty acids, flavor compounds and some surfactants. This protein is widely preferred for bioactive compound binding studies due to the homology between human serum albumin (HSA) and BSA. Binding of tea polyphenols, including oligomeric tannins and procyanidins, to BSA have been studied extensively (Lei et al., 2017; Watrelot et al., 2015; Karonen et al., 2015). Similar to other whey proteins, BSA and polyphenol interactions were reported to be dominated by hydrophobic interactions and electrostatic attractions which might be stabilized by H-bond formation (Bourassa et al., 2010; Fu et al., 2016; Papadopoulou et al., 2005; Kitson, 2004).

The interactions between casein micelles and polyphenols are of special interest for the evaluation on consumption behaviors of polyphenol-rich products along with milk. Besides nutritional studies, casein micelles and individual casein fractions have also been studied for the evaluation of polyphenol binding as an alternative approach for bioactive compound/drug delivery systems. Caseins found in milk are characterized with a high proline content and flexible structures which make them amenable for polyphenol binding. Various polyphenolic substances were reported to bind to caseins predominantly via hydrophobic effects and H-bridges (Moeiniafshari et al., 2015; Bohin et al., 2012; Mehranfar et al., 2013). Although the binding affinity and characteristics have been reported to depend on several factors, weak attractions dominate the interactions between polyphenols and milk proteins, whereas irreversible interactions are rarely identified under specific conditions.

### Effect of Structural Features and Environmental Conditions on Binding

The nature and strength of polyphenol binding to proteins have been reported to be affected by a number of factors among which the chemical structures of proteins and polyphenols are of great importance.

The conformation and structure of proteins greatly affect the binding properties of polyphenols. In general, proteins with a regular secondary structure show lower affinity to polyphenols compared to randomly structured proteins. Besides, amino acid composition of protein molecule is known to affect its interactions with polyphenols. Proteins with more prolyl residues and proline repeats possess strong affinity to polyphenolic substances due to the attraction forces between proline and the hydroxyl group of polyphenols (Murray et al., 1994). Therefore, caseins in milk have a great potential as delivery tools for polyphenols. Especially,  $\beta$ -casein could bind to several phenolic compounds with high affinity because of the high number of proline residues and hydrophobic nature of  $\beta$ -casein (Hasni et al., 2011; Bourassa et al., 2013). However, the amino acid composition and prolyl residues might not be the critical factors in some cases where other factors play more significant roles.  $\beta$ -Lactoglobulin has three possible binding sites for small ligands and these ports were reported to bind various polyphenols strongly (Riihimäki et al., 2008; Hemar et al., 2011; Gholami and Bordbar, 2014). Free cysteine (Cys) residue in  $\beta$ -Lactoglobulin was also able to react with polyphenols and alkylation of Cys residue may hinder binding (Gallo et al., 2013).  $\alpha$ -Lactalbumin is a metalloprotein which is known for its ability to bind several metal cations. The binding of several polyphenols (such as curcumin, resveratrol, genistein and kaempferol) to  $\alpha$ -Lactalbumin was reported in literature. The hydrophobic cleft of BSA showed high affinity to some polyphenols. Additionally, the neighborhood of Tyr-134 residue on the surface was reported to be a possible binding site for polyphenols. Although sophisticated molecular methods have revealed specific sites on milk proteins with higher affinity, nonspecific protein-polyphenol complexation also occurs especially for caseins lacking a defined globular tertiary structure.

The type and chemical structure of polyphenols also significantly affect the characteristics of polyphenol-protein interactions. A number of studies revealed that the affinity of polyphenols for protein binding is enhanced with increasing molecular weight and bulkiness of the polyphenol (Soares et al., 2007). Besides molecular weight, differences in hydroxylation, methylation and glycosylation may also change the binding affinity of polyphenols. Methylation of the polyphenols may cause a slight inhibitory effect on their reactions with milk proteins (Xiao et al., 2011). Similarly, glycosylation was reported to affect these interactions in a negative way which might be due to steric hindrance and hydrophilic character of the attached carbohydrate group. However, a number of studies showed that an increase in the number of hydroxyl groups on polyphenol structure (ring A or ring C) significantly promotes the binding reactions (Kanakakis et al., 2011; Hasni et al., 2011; Xiao et al., 2010). The small changes in molecular structure may have an important consequence in binding characteristics and biological functions of polyphenols.

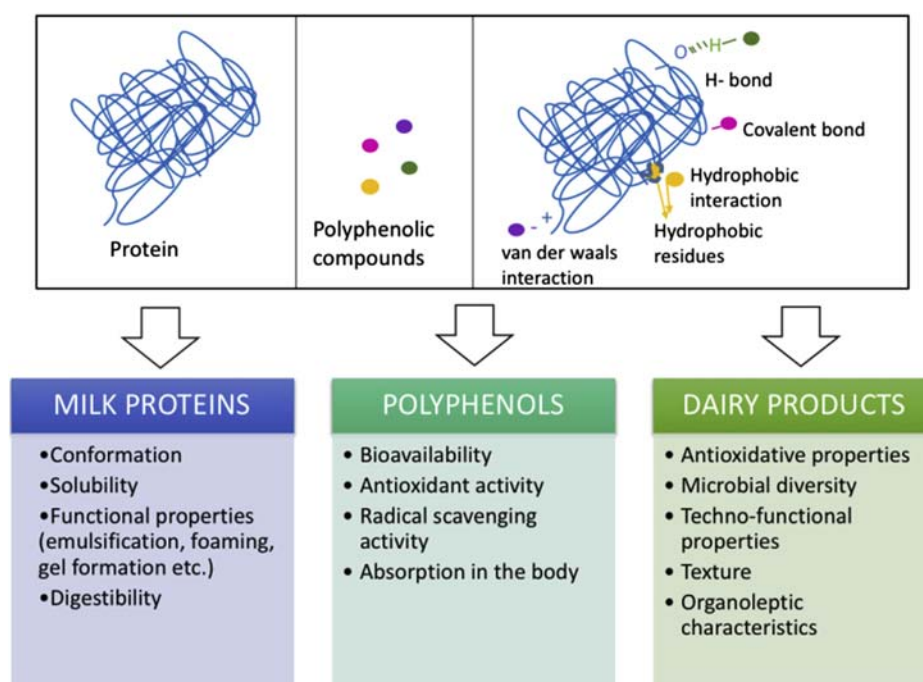
Although the structural differences of reacting molecules play a key role in protein-polyphenol reactions, environmental factors such as temperature, pH and ionic strength may also affect these interactions. The pH of the reaction environment becomes critical especially when the electrostatic interactions are pre-dominating. Polyphenols were reported to bind stronger to proteins when the pH closes to the isoelectric point of the protein molecule (Wang et al., 2007). High temperature is generally considered to improve binding affinity of polyphenols unless the temperature is near or above the denaturation point of proteins. In case of thermal denaturation, conflicting results were obtained for milk proteins. A group of researchers claimed that exposure of hydrophobic ports due to protein denaturation leads to stronger interactions between epigallocatechin gallate (EGCG) and BSA (Wang et al., 2007), whereas, other studies revealed an impaired binding to quercetin or chlorogenic acid after heat denaturation of BSA (Rawel et al., 2005). In addition, denaturation of  $\beta$ -Lactoglobulin was reported to promote the binding of polyphenols (Liang and Subirade, 2012). As seen from these contradictory results, the effect of thermal denaturation could be varying with the type of polyphenols and/or dominating interactions. Therefore, making a general estimation on temperature-dependent protein-polyphenol interactions does not seem possible.

## Consequences of Binding

Binding reactions of milk proteins and polyphenols can lead to significant alterations in the properties of proteins and polyphenols or polyphenol-enriched dairy product characteristics, as illustrated in Fig. 1. Irreversible interactions between proteins and polyphenols have been reported to cause more pronounced changes though irreversible interactions are less common compared to weak interactions. One of the most frequently reported effects of polyphenol binding on proteins is the change in the secondary or tertiary structure of the protein molecule. Changes in structural features of milk proteins may affect further binding reactions of proteins. The alterations in protein structure caused by polyphenol binding seem to be affected by factors such as structure, concentration and binding affinity of polyphenols, pH of the reaction environment, and the state of the protein molecule (native or denatured), which leads to conflicting results (Stojadinovic et al., 2013; Zorilla et al., 2011).

Some researchers could not find any differences in the conformations of BSA,  $\beta$ -Lactoglobulin and  $\beta$ -casein upon binding to several flavonoids (Papadopoulou et al., 2005; Bohin et al., 2012; Gholami and Bordbar, 2014). On the other hand, partial stabilization (Wu et al., 2011; Kanakis et al., 2011; Skrt et al., 2012) and destabilization (Kanakis et al., 2013) of milk proteins resulted from changes in their secondary or tertiary structures when interacted with polyphenols have been recently reported. Besides structural changes, milk protein-polyphenol interactions may also lead to alterations in functional properties of proteins, some of which are essential for the production of dairy products. Formation of protein aggregates upon interactions with polyphenols, due to the occurrence of crosslinking bridges mainly via H-bonds, have been revealed by several studies. Therefore, the solubility of proteins is considered to be reduced by their interactions with polyphenols. On the other hand, a study using a model system to evaluate the solubility of BSA and  $\alpha$ -Lactalbumin asserts that the solubility of proteins remained unchanged upon binding to phenolic acids (Prigent et al., 2003). Enhanced foaming ability and foam stability of whey proteins such as  $\beta$ -Lactoglobulin,  $\alpha$ -Lactalbumin and BSA were also discussed upon their binding to polyphenols (Prigent et al., 2009; Sarker et al., 1995). From the nutritional point of view, digestibility of milk proteins when consumed/interacted with polyphenols is of significant importance. However, the effect of polyphenol binding on digestion of milk proteins has been evaluated to a lesser extent. The susceptibility of  $\beta$ -Lactoglobulin to *in vitro* gastric and pancreatic digestion was reported to be altered upon its interactions with polyphenols.

As perceived responsibility for the main health benefits, antioxidative properties of polyphenols have been one of the most important aspects for protein-polyphenol studies. *In vitro* and *in vivo* antioxidant assays can be used for evaluation of any changes induced by protein-polyphenol interactions. Recent studies have revealed that the observed effects of polyphenols were lower than expected due to their low absorption and transformation to the metabolites that do not act as powerful antioxidants. Therefore, most of the biological functions of polyphenols proven in *in vitro* studies, might not be observed *in vivo*. The majority of *in vitro* studies revealed the masking effect of noncovalent interactions between milk proteins and polyphenols on the antioxidant and radical scavenging activities (Ryan and Petit, 2010; Staszewski et al., 2011). *In vivo* approaches pointed out two opposite consequences of polyphenol binding for bioavailability and absorption of polyphenols: either a negligible effect or a diminishing effect (Serafini et al., 1996; Langley-Evans, 2000; Roura et al., 2007; Keogh et al., 2007). The conflicting findings might arise from



**Figure 1** Schematic representation of binding forces between milk proteins and polyphenols and possible related changes.

different methodologies used for the evaluation of biological activities of polyphenols. Although a lot of effort has been made in order to reveal the effects of milk protein-polyphenol interactions on the bioavailability of polyphenols, understanding these mechanisms at the molecular level with a description is still lacking.

## Interactions in Complex Dairy Matrices

The interactions between different polyphenols and individual milk proteins have been extensively studied in model systems. However, complex dairy matrices require more extensive studies, as other macromolecules could interfere with polyphenol-protein interactions. Moreover, the dynamic nature of dairy product matrices is a limiting factor for proper elucidation of these interactions leading to conflicting results. Use of polyphenols as fortification agents for a variety of dairy products has been proposed formerly (O'Connell and Fox, 2001). The studies were primarily based on the improvement of food structure, quality and novel functional properties of food formulations.

Research on the interactions between milk protein and polyphenols has been mainly conducted on the effects of the binding on biological activities of polyphenols and antioxidative properties of functional dairy products rather than the mechanistic understanding of binding in complex matrices. Improvements of radical scavenging activity and antioxidative properties of several dairy products such as yogurt and cheese were reported through fortification with polyphenols (Najgebauer-Lejko et al., 2011; Rashidinejad et al., 2013; Han et al., 2011; Trigueros et al., 2014). Polyphenol-protein interactions were found to improve the thermal stability of caseins probably via the interactions of *o*-quinons with amino acid residues to prevent, eventually, protein aggregation (O'Connell et al., 1998). However, yogurt samples upon polyphenol addition had poor gelation properties and increased syneresis which could mainly result from the alterations in the strength and nature of the bonds and subsequent formation of a three dimensional network. The formation of covalent bonding (mainly S-S bridging) during acidification was found to decrease when polyphenols were present in yogurt formulations (Vega and Grover, 2011). Interestingly, addition of polyphenols to drinking yogurt samples resulted in different behaviors for differently formulated yogurts, which reveals the importance of the interactions between molecules, other than milk proteins and polyphenols, in complex matrices (Sun-Waterhouse et al., 2013). The rennet-coagulation properties were also reported to be altered in the presence of polyphenols due to protein-polyphenol interactions (Haratifar and Corredig, 2014), although detailed information on the effects of these interactions on coagulation process and associated mechanisms is needed. The textural analysis of Cheddar cheese enriched with green tea extracts revealed a firmer structure, as compared to the control cheese, pointing out enhanced para-casein micelle interactions during coagulation of the enriched cheese. Furthermore, the primary proteolysis, resulting mainly from the residual chymosin and plasmin activities, seemed to be promoted as well (Giroux et al., 2013).

## Conclusions

The possible interactions between milk proteins and polyphenols may lead to a number of changes in several properties of the interacting molecules and the dairy products fortified with phenolics. Generally, weak noncovalent attractions dominate the interactions between polyphenols and milk proteins. The binding affinity and characteristics of such interactions are affected by a number of factors. Model system studies carried out with molecular methodologies revealed the mechanisms behind these reactions. However, due to the high structural diversity of polyphenols and proteins, conflicting findings have been obtained. Investigation and consequences of these reactions in dairy products would be even more complicated due to the presence of other possible interactions and the complex dynamic nature of these matrices. The widely discussed alterations caused by polyphenol-protein interactions include structural/functional changes in protein structure and antioxidative properties of polyphenols. However, these changes might depend on other environmental influences and structural parameters of dairy products as well. Despite the extensive studies on polyphenols and milk proteins, the structural mechanisms associated with the obtained consequences of these interactions are not clearly known yet. More research using different approaches would be essentially required in order to predict the potential of using polyphenols as ingredients for dairy products in technological and nutritional aspects.

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## Interactions of $\beta$ -Lactoglobulin With Small Molecules

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### Glossary

**$\beta$ -Lactoglobulin** One of the most abundant milk proteins that was proposed to be a transport protein for various small molecules.

**Calyx site** A large barrel-shaped hydrophobic core of  $\beta$ -lactoglobulin that was proposed to be the main binding site for hydrophobic molecules.

### Introduction

$\beta$ -Lactoglobulin is a globular protein that is present in the milk of many mammalian species including ruminants, such as cows and sheep, and some non-ruminants, such as pigs and horses (Kontopidis et al., 2004; Sawyer and Kontopidis, 2000).  $\beta$ -Lactoglobulin is the major whey protein in milk. For example, it accounts for about 10% of the total milk proteins in cow's milk. Surprisingly,  $\beta$ -lactoglobulin is not present in the milk of humans and other species including some rodents, rabbits, camels and llamas.

Although  $\beta$ -lactoglobulin was discovered and isolated more than 80 years ago, to date, no functions have been conclusively ascribed to the protein (Kontopidis et al., 2002; Pérez and Calvo, 1995).  $\beta$ -Lactoglobulin has been proposed to be a transport protein for various small molecules. These include, but are not limited to, fatty acids, polyphenols and retinols. The purpose of this review is to summarise recent studies on the binding between  $\beta$ -lactoglobulin and its proposed ligands. The review focuses on binding interactions with  $\beta$ -lactoglobulin in its native structure at physiologically relevant temperatures. Heat-induced aggregates or adducts between  $\beta$ -lactoglobulin and small molecules are out of scope of the review.

### Structure of $\beta$ -Lactoglobulin

Bovine  $\beta$ -lactoglobulin contains 162 amino acids with a molecular weight of 18.4 kDa (Brownlow et al., 1997; Kontopidis et al., 2004).  $\beta$ -Lactoglobulin exists as a dimer (Fig. 1) at pH 2.5 to 7.5 in the presence of salts (which represents almost all the conditions of food systems and manufacturing) (Mercadante et al., 2012). Genetic polymorphism of  $\beta$ -lactoglobulin has been reported (Threadgill and Womack, 1990). For example in cow's milk, the most common variants of bovine  $\beta$ -lactoglobulin are variants A and B. The differences between the two variants are the two mutations that occur at positions 64, in which an aspartic acid (variant A) is replaced by a glycine (variant B), and 118, in which a valine residue (variant A) is replaced by an alanine (variant B). Structurally, bovine  $\beta$ -lactoglobulin contains one  $\alpha$ -helix and nine antiparallel  $\beta$ -strands ( $\beta$ A– $\beta$ I). Eight of these strands ( $\beta$ A– $\beta$ H) form the cylindrical shape central calyx of the protein (Fig. 1). The structure of  $\beta$ -lactoglobulin is further stabilised by two disulphide bonds that occur between Cys66 and Cys160, and between Cys106 and Cys119.

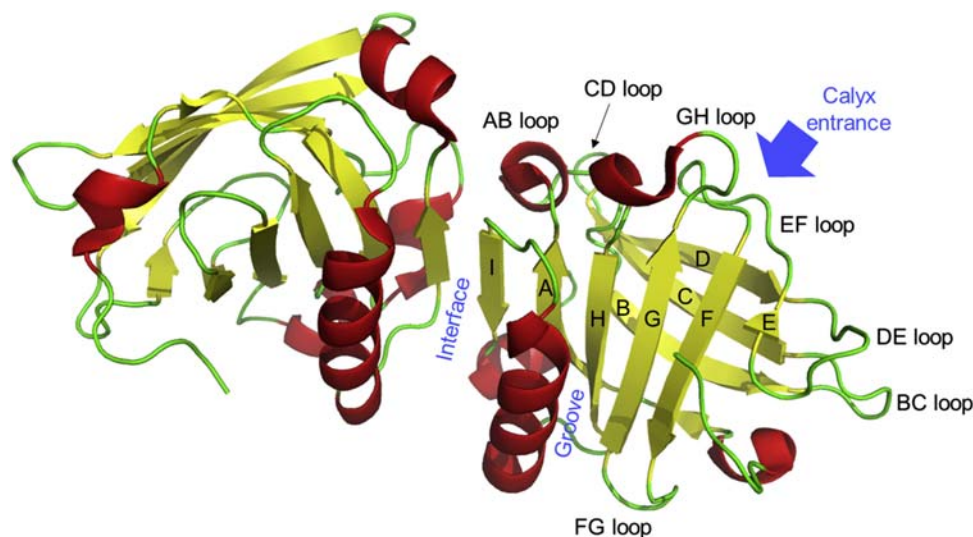
$\beta$ -Lactoglobulin does not have a compact hydrophobic core (Kontopidis et al., 2002, 2004). The two hydrophobic walls of the calyx cylinder are approximately 15 Å in length and have a volume of 315 Å<sup>3</sup>. This structure was proposed to be the main binding site for small compounds with high hydrophobicity (Fig. 1). A pH-triggered dynamic mechanism that involves the opening and closing of the loop on top of the calyx (between  $\beta$ E and  $\beta$ F) was proposed in order to control the binding and release of ligands. Apart from the internal core of the calyx, two other binding sites have also been proposed (Fig. 1). These two binding sites are located at the dimer interface and in the hydrophobic region between  $\alpha$ -helix and the  $\beta$ -barrel of the protein. These sites may be important for the binding of hydrophilic molecules, although the binding pockets remain undetermined.

### Binding Between $\beta$ -Lactoglobulin and Small Molecules With High Hydrophobicity

The binding interactions between hydrophobic ligands (such as fatty acids and fat soluble vitamins) and  $\beta$ -lactoglobulin are well characterised.

#### Fatty Acids

Oleic acid was the first ligand of  $\beta$ -lactoglobulin to be reported (Davis and Dubos, 1947). Since then, the binding of different fatty acids to  $\beta$ -lactoglobulin has been studied extensively. A high substrate promiscuity was observed. Recent studies have focused on the structural basis of fatty acid's binding to the protein.



**Figure 1** Dimeric structure of  $\beta$ -lactoglobulin. The three ligand binding sites are highlighted in blue. These include the calyx site, the dimer interface, and the hydrophobic groove between the  $\alpha$ -helix and the  $\beta$ -barrel. The EF loop was proposed to act as a pH-sensitive gate to the calyx site.

### Structural Basis for Fatty Acid Ligand Binding

Several studies have been conducted to understand the relationship between the structure of the fatty acid ligands and their binding potency to  $\beta$ -lactoglobulin. To date, over 50  $\beta$ -lactoglobulin structures were reported in the protein data bank (PDB), and many of these structures were co-crystallised with long chain fatty acids. These structures showed that the aliphatic chain of fatty acid ligands binds to the calyx core in a linear conformation, and the carboxylic group of the fatty acids interacts with the polar residues (i.e. Lys60, Glu62 and Lys69) of the protein (Loch et al., 2012, 2013a,b). However, this binding mode is not universal for all fatty acids. Structural and computational studies showed that, depending on the aliphatic chain length, the interaction(s) between the carboxyl group of the fatty acid ligands and the polar amino acids on the top of the calyx may not always survive (Qin et al., 1998; Evoli et al., 2014; Deepa et al., 2017). This is likely due to the competition between the polar interactions of fatty acid's carboxyl group and the protein, and the hydrophobic interactions of fatty acid's aliphatic chain and protein's calyx core (Loch et al., 2011). By using X-ray crystallography and taking caprylic and capric acids as examples, it was found that the shorter fatty acid caprylic acid binds, through its carboxylic group, to the upper part of the calyx near the polar residues, whilst the longer fatty acid capric acid is bound deeper in the calyx through hydrophobic interactions (but does not interact with the polar residues at the top of the calyx) (Loch et al., 2011). The importance of hydrophilic and hydrophobic interactions in the binding of fatty acid ligands with  $\beta$ -lactoglobulin was also confirmed through molecular dynamics studies (Bello et al., 2012). This competition between hydrophobic and polar interactions also appears to have an impact on the binding affinity of the fatty acids. Using electron paramagnetic resonance (EPR) spectroscopy and spin-labelled stearic acids, Guzzi et al. showed that binding affinity is correlated to the length and size of the fatty acid hydrocarbon chain. In particular, the highest affinity is observed when the fatty acid hydrocarbon chain fills fully the hydrophobic calyx cavity of  $\beta$ -lactoglobulin (Guzzi et al., 2012).

### Number of Binding Sites

The main binding site for fatty acid ligands in  $\beta$ -lactoglobulin is the calyx core, which was demonstrated by X-ray crystallography in 1999 (Wu et al., 1999). Molecular modelling studies also showed that the calyx is the main binding site for fatty acid ligands (Evoli et al., 2014). Several surface binding sites have also been proposed, although the number of such surface sites was not well defined. Molecular modelling studies showed that at least two binding sites are located on the surface of  $\beta$ -lactoglobulin, although these sites were predicted to bind fatty acid ligands with lower affinities than the calyx site (Evoli et al., 2014). Le Maux et al. reported that  $\beta$ -lactoglobulin may have more than 7 binding sites in its native form, and even more when it is in its covalent dimeric or oligomeric forms by using isothermal titration calorimetry (ITC) and intrinsic fluorescence (Le Maux et al., 2013). Incredibly, using ITC, Fang et al. showed that  $\beta$ -lactoglobulin may have 32 binding sites for fatty acid ligands (Fang et al., 2015). Overall, computation and experimental evidence suggests that these external sites may be involved in transient and relatively short-lived  $\beta$ -lactoglobulin-fatty acid interactions, which may be related to the functions of the protein as fatty acid transporter.

### Conformational Change Upon Fatty Acid Ligand Binding

No major global structural changes of  $\beta$ -lactoglobulin upon fatty acid binding were observed experimentally or computationally (Fang et al., 2015; Bello et al., 2012). However, local changes may occur upon fatty acid binding. Such changes are inferred from a recent study by Liu et al. (2017), in which  $\beta$ -lactoglobulin was able to interact with both fatty acids and curcumin at the same time. More importantly, the authors showed that the presence of fatty acids appeared to enhance the binding affinity of

curcumin, thus inferring localised conformation change. However, experiments also showed that the formation of curcumin-fatty acid- $\beta$ -lactoglobulin ternary complexes did not cause changes in the secondary structure of the protein. Further experiments are needed to understand fully this synergetic behaviour (Liu et al., 2017).

## Lipid-Soluble Vitamins

### Vitamin A<sub>1</sub> (Retinol)

One of the proposed biological roles of  $\beta$ -lactoglobulin was the transport of retinol. Retinol is a fat-soluble vitamin that participates in the regulation of important cellular functions including cell growth, proliferation and differentiation (Sawyer and Kontopidis, 2000). The binding of retinol to  $\beta$ -lactoglobulin was found to be pH dependent. At pH 7, at which  $\beta$ -lactoglobulin is a dimer, there are two apparent binding sites for retinol (which are the calyx and the surface sites), whilst at pH 2, there is only one set of binding sites at the surface of the protein for retinol (Ahmadi et al., 2015). Interestingly, the binding of retinoid was found to induce a change in the secondary structure of the protein, with a decrease in  $\beta$ -sheet and an increase in turn structure, which, as a result, destabilises the protein (Belatik et al., 2012). Further experiments are needed to understand the significance of such conformational, oligomeric and/or structural changes.

### Vitamin D

Vitamin D<sub>3</sub> is a fat-soluble vitamin found to bind to both the calyx and surface hydrophobic pocket of  $\beta$ -lactoglobulin. The binding of vitamin D<sub>3</sub> to  $\beta$ -lactoglobulin appeared to improve the stability of the vitamin when the vitamin is exposed to UV light (Diarrassouba et al., 2014). The resistance of  $\beta$ -lactoglobulin-vitamin D<sub>3</sub> complex to protease-catalysed denaturation was also increased. The authors also showed that the  $\beta$ -lactoglobulin-vitamin D<sub>3</sub> complex has led to improved bioavailability of vitamin D<sub>3</sub>, as the complex managed to cross cell membranes (Diarrassouba et al., 2014). These findings are useful in food applications, offering the possibility for using the  $\beta$ -lactoglobulin-vitamin D<sub>3</sub> complex to improve the intake of vitamin D<sub>3</sub> (Diarrassouba et al., 2014).

### Vitamin E

$\alpha$ -Tocopherol, or vitamin E, is a fat-soluble vitamin that was found to bind to both the surface and calyx sites of  $\beta$ -lactoglobulin at neutral pH. However, at acidic pHs,  $\alpha$ -tocopherol is released from the internal cavity but is still bound to the surface of  $\beta$ -lactoglobulin (Liang and Subirade, 2012). The interaction of  $\beta$ -lactoglobulin with lipophilic  $\alpha$ -tocopherol did not disrupt the secondary or tertiary structures of  $\beta$ -lactoglobulin.  $\beta$ -Lactoglobulin appeared to increase the solubility of  $\alpha$ -tocopherol by forming a  $\beta$ -lactoglobulin- $\alpha$ -tocopherol complex (Liang et al., 2011).  $\beta$ -Lactoglobulin was also found to protect  $\alpha$ -tocopherol against decomposition (Liang et al., 2011). Similar to vitamin D<sub>3</sub>, this study shows the potential for  $\beta$ -lactoglobulin to act as a carrier to improve the intake and bioavailability of the vitamin.

## Binding of $\beta$ -Lactoglobulin to Polyphenols

The binding of polyphenols to  $\beta$ -lactoglobulin has been intensively studied due to the antioxidant activity of the polyphenols against reactive oxygen species. The binding site for a polyphenol was proposed to be located in the hydrophobic calyx of  $\beta$ -lactoglobulin and on the surface of the  $\beta$ -lactoglobulin. Similar to the binding of fatty acid ligands, the hydrophobic interaction in the calyx is often considered specific and generally has high affinity, whilst the interaction at the surface is generally weaker and nonspecific.

### Structural Basis of Polyphenol Binding to $\beta$ -Lactoglobulin

The structural basis of the binding of polyphenol to  $\beta$ -lactoglobulin has been studied extensively both experimentally and computationally. Polyphenols are often found to bind inside or at the entrance of the calyx cavity of  $\beta$ -lactoglobulin through a combination of hydrophobic interactions, hydrogen bonding and  $\pi$ - $\pi$  stacking interactions (Cheng et al., 2017; He et al., 2016; Wu et al., 2013a,b). pH may influence where the polyphenolic ligand binds to  $\beta$ -lactoglobulin, the centre or surface of the calyx site. By using curcumin as a model system, Li and co-workers found that at pH 6.0 (Li et al., 2013), curcumin tended to bind on the external surface of the calyx, whilst at pH 7.0 it tended to bind inside the central cavity. This is apparently due to the dynamic opening and closing of the loop at the top of the calyx site. At the neutral pH, the loop between  $\beta$ E and  $\beta$ F is in the opening conformation. This enables hydrophobic polyphenols to access to the internal calyx cavity. At acidic pH, however, the Glu89 residue is protonated. This shifts the EF loop to the closed conformation. The closing of the gate stops the polyphenol molecule from entering the core structure, thus the molecule tends to bind on the external surface of the calyx at such pHs (Li et al., 2013).

Polyphenols were also found to bind to the surface cleft of  $\beta$ -lactoglobulin (Wu et al., 2013a,b). For example, resveratrol, a natural polyphenolic compound that is commonly found in red wine and grapes, was found to bind to the outer surface of  $\beta$ -lactoglobulin near Trp19 and Arg124 at both neutral and acidic pHs (Liang et al., 2008; Liang and Subirade, 2012).

Not surprisingly, the structure of the polyphenols may have an effect on their binding affinity to  $\beta$ -lactoglobulin. For example, the position of the hydroxyl groups was found to be important. Hydroxylation at the 3-position would increase the affinity of the phenolic acids whilst hydroxylation at the 2- or 4-positions may have a negative effect (Wu et al., 2017). One potential application

of  $\beta$ -lactoglobulin is to deliver bioactive polyphenolics. A thorough understanding of the structural–activity relationship at the calyx site of  $\beta$ -lactoglobulin would facilitate the development of such applications.

### Protein Dynamics Upon Polyphenol Binding

The binding of polyphenols to  $\beta$ -lactoglobulin appears to cause changes to the protein's structure. For example, the binding of cyanidin-3-O-glucoside, a polyphenol that binds the  $\beta$ -lactoglobulin calyx site, causes a decrease in  $\alpha$ -helix and an increase in  $\beta$ -sheet of the protein, as revealed by UV–vis spectroscopy, fluorescence spectroscopy and circular dichroism analyses (Cheng et al., 2017). In contrast, the binding of epicatechin to  $\beta$ -lactoglobulin appeared to cause little change in the protein's  $\alpha$ -helix content, and the  $\beta$ -sheets were altered in a manner that implies the transition from dimer to monomer. The authors argued that this finding reflects the binding of epicatechin to the dimerisation interface of the  $\beta$ -lactoglobulin dimer, although further experiments are needed to prove conclusively this hypothesis (Nucara et al., 2013). A thorough understanding of the structure of polyphenols, their binding sites on  $\beta$ -lactoglobulin, and whether the binding interaction causes a conformational change, is important, in order to use  $\beta$ -lactoglobulin as a carrier for these bioactives.

### Applications of Polyphenol- $\beta$ -Lactoglobulin Complexes

The ability for  $\beta$ -lactoglobulin to complex with polyphenols has led to several potential applications in the food industry. It was found that the polyphenol- $\beta$ -lactoglobulin complex is more stable to pepsin and pancreatic digestion in the gastrointestinal tract, thus enhancing the protein's stability to digestion. However, this strong complex can also mask the total antioxidant capacity of the polyphenol (Stojadinovic et al., 2013). A similar observation was made by Libardi et al. using vanillin as an antioxidant (Libardi et al., 2011). A balance between stability and antioxidant capacity is important to tune the application of the polyphenol- $\beta$ -lactoglobulin complex.

Interestingly, the  $\beta$ -lactoglobulin-catechin complex showed decreased allergenicity compared to  $\beta$ -lactoglobulin on its own, even though the overall structure of the protein remained unchanged (Wu et al., 2013a,b). Further research in the structure–activity relationship of  $\beta$ -lactoglobulin, in the absence and presence of polyphenol binding, may enable us to find out the cause of  $\beta$ -lactoglobulin-induced allergenicity.

### Binding of $\beta$ -Lactoglobulin to Water-Soluble Small Molecules

Apart from hydrophobic molecules and polyphenols, many studies have shown that  $\beta$ -lactoglobulin also binds and interacts with polar, or water-soluble, molecules. We summarise recent studies on water-soluble vitamins and lactose as below.

#### Water-Soluble Vitamins

Folic acid is a vitamin that is essential for a variety of physiological processes. However, folic acid is unstable and readily decomposes under UV irradiation. It was postulated that the binding of folic acid to  $\beta$ -lactoglobulin may enhance the stability of the vitamin. The binding of folic acid to  $\beta$ -lactoglobulin was therefore investigated by UV–visible light absorption and scattering, fluorescence and molecular docking techniques. Folic acid was found to bind to the hydrophobic surface groove between the  $\alpha$ -helix and the  $\beta$ -barrel of  $\beta$ -lactoglobulin. Fluorescence analysis showed that the  $\beta$ -lactoglobulin-bound folic acid has increased photostability and thermal stability (Liang and Subirade, 2010, 2012; Liang et al., 2013). This indicates the potential of  $\beta$ -lactoglobulin in carrying and delivering water-soluble vitamins.

#### Lactose

The binding of lactose, which is the most abundant sugar in milk, to  $\beta$ -lactoglobulin was studied by molecular docking (Domínguez-Ramírez et al., 2013). Lactose is known to modify covalently the lysine residues of  $\beta$ -lactoglobulin at higher temperatures, a process that is known as glycation or glycosylation. However, the interaction of lactose with  $\beta$ -lactoglobulin at room temperature in the protein's native state remains unclear. The molecular docking study showed that lactose did not bind to  $\beta$ -lactoglobulin in its monomeric form, however, binding interactions were possible with  $\beta$ -lactoglobulin in its dimer form. Lactose did not bind to the calyx core, a result that is not surprising due to the hydrophobic nature of the calyx core. Instead, it was predicted that the dimeric interface of  $\beta$ -lactoglobulin is the most likely binding site for lactose. The docking model suggests that lactose forms hydrogen bonds with residues from both monomers of  $\beta$ -lactoglobulin (K141 and H146 from one monomer, and D137 and R148 from the other monomer). Nevertheless, it is worth noting that the binding of lactose to  $\beta$ -lactoglobulin at room temperature still needs to be confirmed experimentally.

## Conclusions

$\beta$ -Lactoglobulin is the second most abundant protein in milk. However, its function remains poorly defined, and the mapping of its small molecule interactome is incomplete.  $\beta$ -Lactoglobulin appears to be promiscuous in its ligand selectivity, and is able to interact with a large number of hydrophobic and hydrophilic molecules. This review covered recent studies about the binding of fatty acids, fat-soluble vitamins, polyphenols, as well as some water-soluble bioactive molecules. The ability for  $\beta$ -lactoglobulin to interact and bind different molecules has enabled the development of different applications in the food industry. For example, recent studies showed that  $\beta$ -lactoglobulin has promising potential as a carrier for bioactives in food applications. We hope this review will stimulate interest in further reading and research into this area.

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# Polyphenol-Protein Interactions and Changes in Functional Properties and Digestibility

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## Introduction

In last decades, the global trend has been shifted from synthetic materials to natural sources (the so-called 'back to nature'). There is an increasing consumer demand for healthy diets and nutritious foods which leads to a huge commercial market for functional foods. Functional foods are foods that can provide health promoting benefits beyond their nutritional value. Food fortification is one of the major approaches for developing functional food products. This process increases the content of one or more nutrients and bioactive substances, whether or not they are normally present in the food, for the purpose of correcting and/or improving the nutritional quality of the food and provide desired biological benefit(s) with minimised risk to health (Allen et al., 2006).

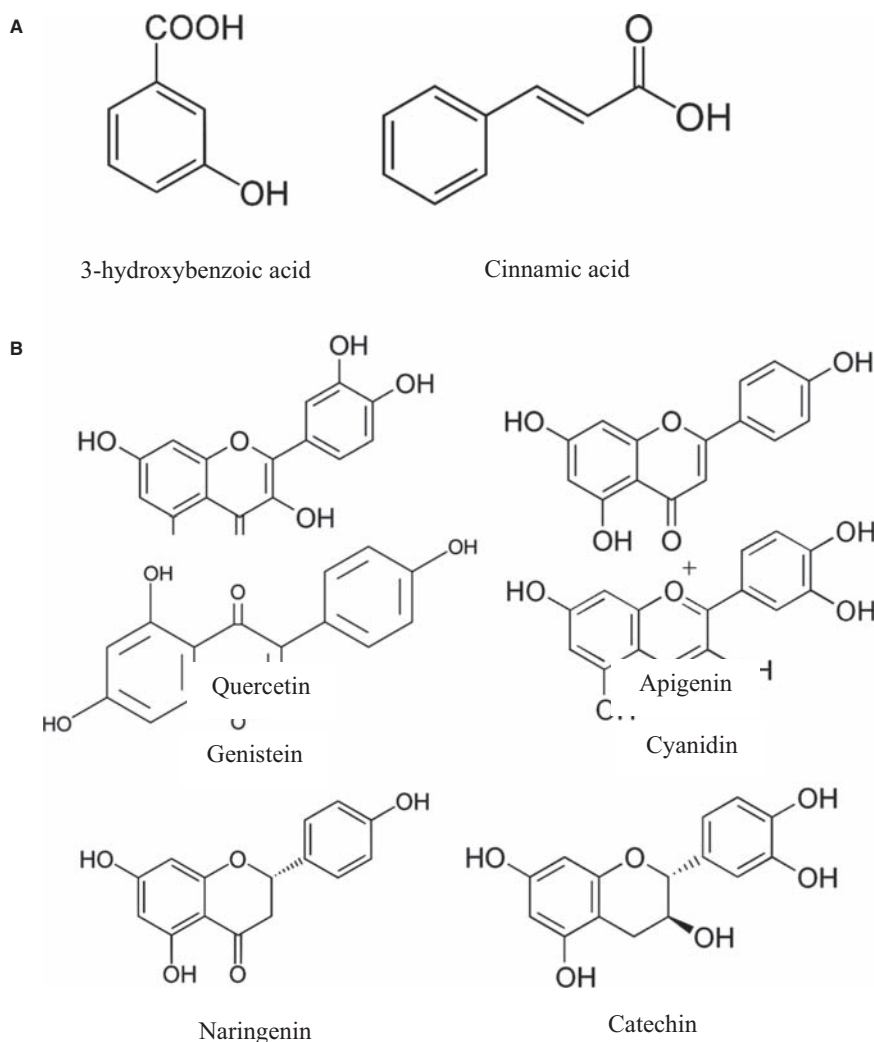
Phenolic compounds with more than 6000 identified substances represent the largest group of secondary metabolites in plant foods. Increasing interests in these compounds is largely related to their impacts on the organoleptic and nutritional qualities of fruits and vegetables, their roles in plant growth and metabolism and, more recently, their physiological activities in humans (Kroll et al., 2003). Functional foods with elevated levels of antioxidants including phenolic compounds are of high demand because of their health-promoting functions such as antioxidative, hepatoprotective, antibacterial, anti-inflammatory and anticancer activities to reduce the risk of chronic diseases (Siro et al., 2008; Yildirim-Elikoglu and Erdem, 2017). However, food fortified with phenolic compounds may lead to changes in the nutritional, chemical and rheological properties of the fortified food. Phenolics are able to interact with food matrix components which affect the biological impact of the fortified food (Umar Lule and Xia, 2005; Seczyk et al., 2015).

In this chapter, chemistry behind protein-phenolic interactions is summarized along with the factors affecting the interactions between proteins and phenolic compounds. Changes resulting from protein-phenolic interactions in proteins and phenolic compounds are also reviewed. Furthermore, processing effects on protein-phenolic interactions and characterization of the formed polyphenol-protein complex will also be described.

## Chemistry Behind Protein-Phenolic Interactions

Phenolic compounds, as the major secondary metabolites in plant foods, comprise more than 8000 identified substances (Guo et al., 2009). They have a common structural feature, an aromatic ring bonded to at least one hydroxyl group (Kroll et al., 2003), and are classified according to the number of carbon atoms in their structure. Flavonoids, phenolic acids, and less common stilbenes and lignans, are phenolic compounds occurring in plants (some common phenolic acids and flavonoids are shown in Fig. 1A and B) (Crozier et al., 2009; Czubinski and Dwiecki, 2017).

Phenolic compounds in food undergo chemical changes during food processing and can interact with protein macromolecules during food production and processing, and even after food consumption (i.e. during digestion in the gastrointestinal tract) (Buitimea-Cantúa et al., 2017; Le Bourvellec and Renard, 2012; Parada and Aguilera, 2007). The molecular weight and structural flexibility of a phenolic compound, as well as the number of hydroxyl groups and type of side chain have been identified as the key factors that affect phenolic-protein interactions (Buitimea-Cantúa et al., 2017; Czubinski and Dwiecki, 2017). Phenolic compounds with a higher molecular weight and a higher abundance of hydroxyl groups (providing more than one site for interaction) are thought to have a higher affinity for protein (Dubeau et al., 2010; Frazier et al., 2010; Jakobek, 2015; Xiao et al., 2011). Phenolic-protein interactions can be grouped into non-covalent interactions (reversible) and covalent interactions (mostly irreversible) (Prigent, 2005; Rawel and Rohn, 2010; Ozdal et al., 2013). Five types of non-covalent interactions were reported, including hydrogen bonds, electrostatic interactions, hydrophobic interactions, van der Waals interactions, and  $\pi$  bonds (Ali, 2013; Frazier et al., 2010; McRae and Kennedy, 2011; Poncet-Legrand et al., 2006; Prigent, 2005; Rawel and Rohn, 2010; Siebert, 2006). Among these, hydrophobic interactions and hydrogen bonds are the primary non-covalent forces that mediate phenolic-protein interactions. Amino acids, including alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, cysteine and glycine, may interact with phenolic compounds through hydrophobic interactions. Moreover, phenolic compounds are excellent hydrogen donors that form hydrogen bonds with the carboxyl group of proteins (Jongberg et al., 2015; Mulaudzi et al., 2012; Xiao et al., 2011). Consequently, hydrogen bonds may take place between the nitrogen or oxygen molecule of amino acids (e.g., lysine, arginine, histidine, asparagine, glutamine, serine, threonine, aspartic acid, glutamic acid, tyrosine, cysteine and tryptophan) and the hydroxyl groups of phenolics (Ali, 2013; Prigent, 2005; Rawel and Rohn, 2010). Interestingly, the ability of phenolic compound to produce a quinone radical mediates the formation of a covalent bond (Jongberg et al., 2015). Under alkaline conditions and in the presence of oxygen, the phenolic compounds are susceptible to enzymatic and non-enzymatic oxidation reactions causing the generation of highly reactive quinone radicals. As the second step of oxidation, the quinone undergoes condensation reactions (reacting with another quinone) to form a dimer, a high molecular weight brown colored pigment named as tannin, which can further react with amino acids in a polypeptide chain through covalent bonding



**Figure 1** Structures of the major phenolic acid (A), and flavonoid (B) compounds.

(Felton et al., 1989). As the third step, the dimers are re-oxidized and crosslinked to another polypeptide chain (Arts et al., 2001; Buchner et al., 2006). A schematic representation of the mechanism of phenolic-protein interactions is given in Fig. 2. Examples of non-covalent and covalent phenolic-protein interactions are provided in Table 1.

### Factors Affecting the Interactions Between Proteins and Phenolic Compounds

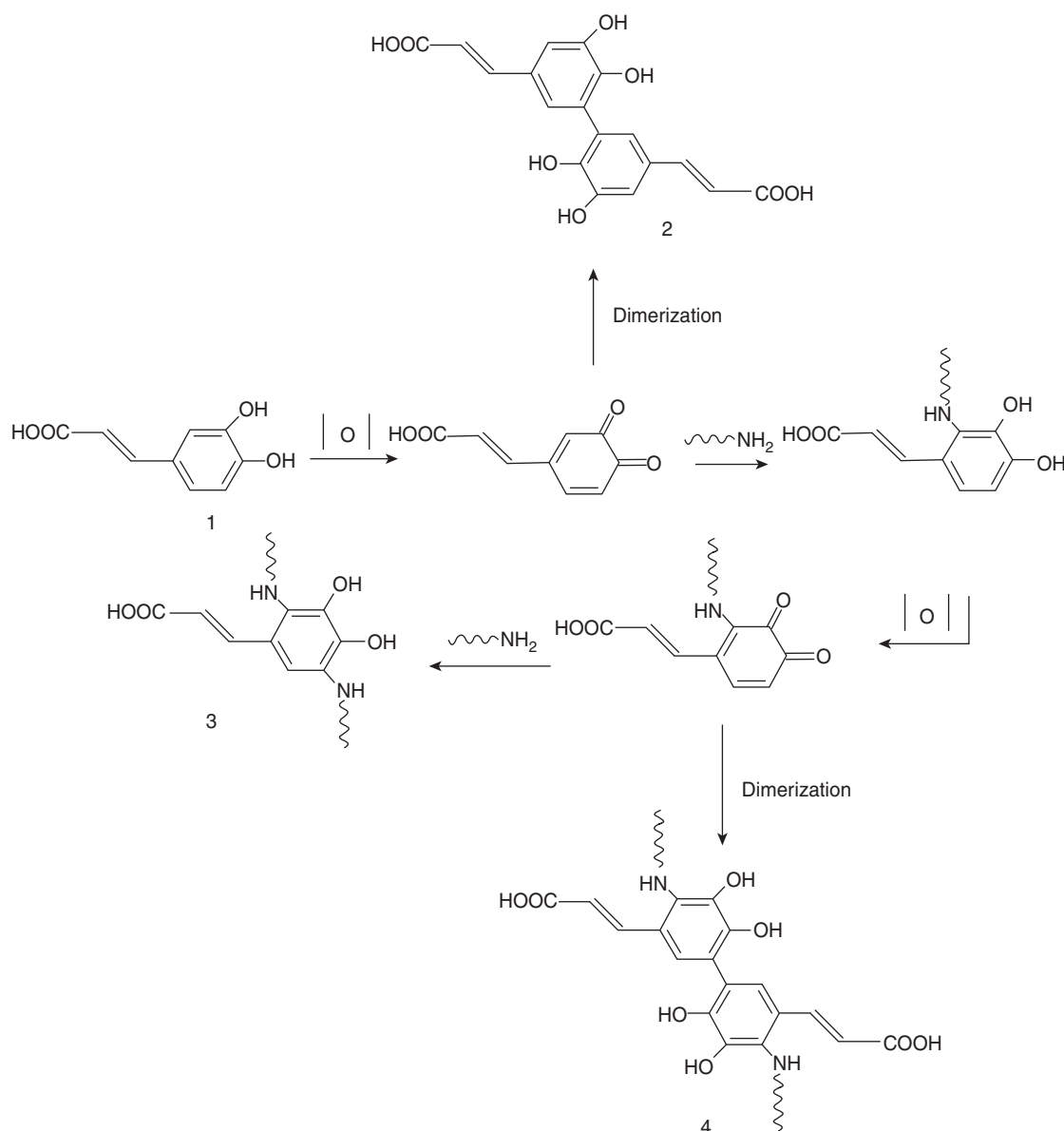
While there exist a variety of factors that influence protein-phenolic interactions including environmental factors such as temperature, pH, salt concentration and presence of certain reagents (Kroll et al., 2003; Ozdal et al., 2013), type of protein and structure of phenolic compound have been reported as the two main factors (Czubinski and Dwiecki, 2017)

#### Type of Protein

The hydrophobicity, isoelectric point and amino acid sequence of proteins influence strongly their binding affinity to phenolic compounds (Prigent et al., 2003). For instance, bovine serum albumin (BSA), which is more hydrophobic than  $\alpha$ -lactalbumin and lysozyme, exhibited higher binding affinity to chlorogenic acid (Naczek et al., 1996). Additionally, proteins that have relatively higher molecular weights may exhibit higher affinity for phenolics (Hagerman, 1989). The size of polypeptides and proteins also played a role in their affinities for polymeric proanthocyanidin (Siebert, 1999).

#### Structure of Phenolic Compounds

Phenolic compounds may differ in their molecular weight, degree of hydroxylation, methylation, glycosylation and hydrogenation, all of which play a role in the formation of protein-phenolic complexes. Polyphenols with higher molecular weights likely have



**Figure 2** Proposed reactions of a phenolic acid compound with amino side chains of polypeptides (Strauss and Gibson, 2004).

a higher binding affinity to proteins (Dubeau et al., 2010). Compared with some low molecular weight phenolics, protocatechuic acid and caffeic acid showed the strongest binding affinity for BSA whilst *p*-hydroxybenzoic acid had the lowest (Bartolome et al., 2000). The monoglycoside forms of flavonoids had stronger binding affinities for milk proteins compared to their polyglycoside forms. For quercetin and its glycosides, the aglycone form caused a stronger interaction with BSA (Martini et al., 2008). Methylation was reported to decrease the binding affinity of flavonoids for milk proteins, whereas, hydroxylation of the rings A and B in flavones and flavonols improved the binding affinity. Hydrogenation of flavonoids on their C2 = C3 double bonds led to reduced binding affinities for milk proteins (Xiao et al., 2011).

### Other Factors

Temperature is an important environmental factor that influences the binding affinities of phenolic compounds for proteins. The binding affinity of 5-O-caffeoylquinic acid for 11S protein of sunflower seed and BSA decreased with increased temperature (Prigent et al., 2003; Sastry and Rao, 1990). This was associated with the changes in hydrogen bonding between phenolic compounds and proteins (Sastry and Rao, 1990).

Another influencing environmental factor is pH. Increased precipitation of protein-polyphenol complexes was observed at pH values close to the isoelectric point of protein, due to a lower solubility of these complexes at this pH. Thus, the optimum pH for precipitation is close to protein isoelectric point, which varies among different proteins (Naczek et al., 1996, 2006). Higher binding

**Table 1** Non-covalent (reversible) and covalent (irreversible) interactions between phenolic compounds and proteins

<i>Non-covalent (reversible) interactions</i>					
<i>Interaction mechanism</i>	<i>Protein</i>	<i>Phenolic compound</i>	<i>Effects on protein and/or phenolic compound</i>	<i>Assessment method</i>	<i>References</i>
Hydrogen bonding	$\alpha$ -Lactalbumin Lysozyme Bovine serum albumin	Procyanidins of various degree of polymerization (DP)	Procyanidins having medium DP may play a role in; • Undesirable decrease in protein solubility • Improvement in foam stability	ITC	Prigent et al. (2009)
Hydrogen bonding	Bovine serum albumin	Ferulic acid	Increase in protein thermal stability	FD and CD spectroscopy, ITC	Ojha et al. (2012)
Van der Waals	Bovine $\beta$ -lactoglobulin	(–)-Epigallocatechin	Change of native conformation	FD, CD and FTIR spectroscopy	Wu et al. (2011)
Forces + Hydrogen bonding	$\beta$ -casein in milk	Green tea flavonoids (catechins)	Decrease in number of protein surface hydrophobic sites	Fluorometry analysis, ITC	Yuksel et al. (2010)
Hydrophobic binding	Walnut proteins	Walnut phenolics	Decrease in protein solubility of walnut flour obtained from whole kernels	SDS-PAGE	Labuckas et al. (2008)
Hydrophobic and hydrophilic interactions	Milk $\beta$ -lactoglobulin	Tea polyphenols	Increase in structural stabilization of protein	FD, CD and FTIR spectroscopy	Kanakis et al. (2011)
Hydrogen bonding and hydrophobic interactions	$\beta$ -lactoglobulin	Tea, coffee, and cocoa polyphenols	• Decrease in antioxidant activities of protein and polyphenols • Reduction in protein digestibility	FD and CD spectroscopy	Stojadinovic et al. (2013)
Hydrogen binding, hydrophobic and dipole–dipole type interactions	Bovine milk whey protein (i.e. $\alpha$ -Lactalbumin, Lactoglobulin and Lactoferrin)	Rosmarinic acid	• Decrease in antioxidant potential of rosmarinic acid • Increase in protein stability	Radical quenching assay (ABTS), optical density, liquid chromatography (HPLC and FPLC), DLS, and zeta-potential, FTIR, and DSC	Ferraro et al. (2015)
Hydrophobic interactions	Bovine $\alpha$ -lactalbumin (ALA)	Green tea epigallocatechin-3-gallate (EGCG)	• Increase in biological activity of EGCG	CD and FD spectroscopy, ITC	Al-Hanish et al. (2016)
Hydrophobic interactions	Egg white proteins; ovalbumin and lysozyme	Tea polyphenols	• Conformational and second structural change of proteins • Easier digestion of proteins at low pH; whereas reduced digestion of proteins at high pH	FD spectroscopy and FTIR	Shen et al. (2014)

<i>Covalent (irreversible) interactions</i>					
<i>Interaction mechanism</i>	<i>Protein</i>	<i>Phenolic compound</i>	<i>Effects on protein and/or phenolic compound</i>	<i>Assessment method</i>	<i>References</i>
Covalent bonds	Soy protein	Chlorogenic-, caffeic-, and gallic acid, flavones, apigenine, kaempferol, quercetin and myricetin	<ul style="list-style-type: none"> <li>• Reduction in lysine, cysteine and tryptophan residues in soy proteins,</li> <li>• The isoelectric points shifted to lower pH,</li> <li>• Increase in molecular weight,</li> <li>• More hydrophilic surface on soy protein,</li> <li>• Changes in the solubility of the protein derivatives</li> </ul>	CD spectroscopy, DSC	<a href="#">Rawel et al. (2002)</a>
Covalent bonds	Fish myofibrillar protein	Caffeic acid, catechin, ferulic acid and tannic acid	<ul style="list-style-type: none"> <li>• Enhanced mechanical properties of films from myofibrillar proteins</li> <li>• Different effects of phenolics on transparency and color of protein films</li> </ul>	Texture profile analysis, color measurement, light transmission, SDS-PAGE	<a href="#">Prodpran et al. (2012)</a>
Covalent bonds	Gelatin	Gallic acid and rutin	<ul style="list-style-type: none"> <li>• Increased gel strength</li> <li>• Increased thermal stability and decreased swelling (with rutin)</li> <li>• Modified physicochemical properties in gels treated with rutin</li> </ul>	Texture profile analysis, rheometry, DSC, swelling tests, scanning electron microscopy, X-ray diffraction, FTIR	<a href="#">Yan et al. (2011)</a>
Non-disulphide covalent linkages	$\beta$ -lactoglobulin	Sour cherry anthocyanins	<ul style="list-style-type: none"> <li>• Decreased protein allergenicity</li> <li>• Increased protein digestibility</li> </ul>	SDS-PAGE, isoelectrofocusing, immunoblotting, size-exclusion and reverse-phase chromatography, mass spectrometry, digestibility, antioxidant activity	<a href="#">Tantoush et al. (2011)</a>
Cross-linking	Gelatin (Type A)	Phenolic acid, quercetin, rutin	<ul style="list-style-type: none"> <li>• Enhanced mechanical strength, reduced swelling, and fewer free amino groups in gelatin gels cross-linked with phenolics</li> <li>• Denser polymeric networks</li> </ul>	Free amino groups analysis, gel rigidity, swelling, dynamic light scattering	<a href="#">Strauss and Gibson (2004)</a>
Cross-linking	Porcine plasma protein	Tannic acid, caffeic acid, ferulic acid	<ul style="list-style-type: none"> <li>• Increased tensile strength</li> <li>• Increased water vapor permeability</li> </ul>	Mechanical properties, water vapor permeability	<a href="#">Nuthong et al. (2009)</a>
Covalent interactions	$\beta$ -lactoglobulin	Coffee-specific phenolics	<ul style="list-style-type: none"> <li>• Enhanced antioxidative properties of proteins</li> <li>• Increased protein thermal stability</li> <li>• Higher protein stability against UV light, when emulsified with lutein esters</li> </ul>	MALDI-TOF-MS, TEAC, Far-UV and Near-UV, CD, DSC, SDS-PAGE	<a href="#">Ali et al. (2013)</a>

ITC: Isothermal Titration Calorimetry; FD: Fluorescence Dichroism; CD: Circular Dichroism; FPLC: Fast Protein Liquid Chromatography; FTIR: Fourier Transform Infrared; SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; DLS: Dynamic Light Scattering; DSC: Differential Scanning Calorimetry; HPLC: High Performance Liquid Chromatography; MALDI-TOF-MS: Matrix-Assisted Laser Desorption/Ionization—Time-of-Flight—Mass Spectrometry; TEAC: Trolox Equivalent Antioxidant Capacity Assay.



affinities of crude tannin canola extract for BSA, fetuin, gelatine and lysozyme, and also, ferulic acid and chlorogenic acid for BSA, were observed all at pHs near the isoelectric points of the proteins (Naczek et al., 1996; Rawel et al., 2005). However, binding of (–)-epicatechin to BSA was not affected by pH (Charlton et al., 2002; Frazier et al., 2006; Papadopoulou et al., 2005).

Protein-phenolic interactions may also be influenced by other environmental factors such as type and concentration of salt. High salt concentrations may lead to dissociation of oligomeric proteins, causing reduced amounts of binding points. Accordingly, increased salt concentration caused a decrease in binding strength of chlorogenic acid to 11S protein of sunflower seed (Sastry and Rao, 1990).

## Changes Resulting From Protein-Phenolic Compound Interactions

### Changes in Protein Properties

#### Structural Properties

The effects of protein-phenolic compound interactions on the structural properties of proteins can be studied using techniques such as differential scanning calorimetry (DSC), circular dichroism (CD), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Matrix-Assisted Laser Desorption/Ionization–Time-of-Flight–Mass Spectrometry (MALDI-TOF-MS), etc. Changes in the secondary and tertiary structures of protein may be reflected as percent changes in  $\alpha$ -helix,  $\beta$ -strand,  $\beta$ -turn and unordered structures (Ali, 2013). The conformation of caseins ( $\alpha$ - and  $\beta$ -caseins) was altered upon their interactions with tea polyphenols ((+)-catechin, (+)-epicatechin, (+)-epigallocatechin and (+)-epigallocatechin gallate (EGCG)), with large percent reductions in  $\alpha$ -helix and  $\beta$ -sheet structures and an increase in random coil structure (Hasni et al., 2011). In the presence of tea polyphenols,  $\beta$ -lactoglobulin conformation was changed to obtain greater structural stabilization with percent increases in  $\beta$ -sheet and  $\alpha$ -helix structures (Kanakis et al., 2011).

Protein-phenolic interactions could also cause the formation of high molecular weight fractions (Ali, 2013; Ozdal et al., 2013). Prigent et al. (2003) reported an increase in molecular weight upon the interaction of  $\alpha$ -lactalbumin or lysozyme with chlorogenic acid. Interactions between soy proteins and phenolic compounds like chlorogenic acid, caffeic acid or gallic acid also resulted in high molecular weight fractions (Rawel et al., 2002). Furthermore, the interaction with phenolic compounds could enhance thermal stability of proteins (Madhan et al., 2005; Tsai and She, 2006) and decrease surface hydrophobicity (Ali, 2013).

#### Effects on Functional Properties

The overall quality and sensory attributes of foods may be affected by functional properties of proteins (Ali, 2013) such as solubility, foaming ability, emulsification, antioxidative capacity, elasticity, color and flavor binding properties.

Protein solubility determines the functionality of proteins in food systems and is affected by phenolic compounds. Several studies reported a decreased solubility of proteins including lysozyme, walnut proteins and potato proteins as a result of their interactions with phenolic compounds (Prigent et al., 2003; Relkin and Shukat, 2012; Van Koningsveld et al., 2002), possibly due to the induced cross-linking of proteins, and altered net charges and hydrophilic/hydrophobic properties of protein molecules (Rawel et al., 2002). The covalent (irreversible) interactions of proteins with phenolic compounds were found to enhance the network formation in gelation gels with improved mechanical strength and higher thermal stability (Strauss and Gibson, 2004). Proteins that are modified with phenolic compounds can be applied as emulsifying agents in food systems, to decrease emulsion stability and increase size of oil droplets (Aewsiri et al., 2009).

#### Effects on Nutritional Properties and Digestibility

The nutritional value of food proteins depends on several factors including protein source, amino acid composition, ratio of essential amino acids, sensitivity to hydrolysis during digestion, processing conditions, etc (Ali, 2013). Several studies reported decreased nutritional value and digestibility of proteins after their interactions with phenolic compounds (Emmambux and Taylor, 2003; Kroll et al., 2003; O'Connell and Fox, 1999; Rawel et al., 2002; Rubanza et al., 2005). The interaction of milk proteins with caffeic acid during heating caused a decrease in the amount of available lysine and thiol groups (O'Connell and Fox, 1999). This has been linked to the occurrence of quinones resulting from caffeic acid oxidation and the subsequent formation of complexes with amino acids (Kroll et al., 2003). Reduced contents of lysine, cysteine and tryptophan were observed after the interactions of soy protein with several phenolic compounds such as chlorogenic acid, gallic acid, caffeic acid and quercetin (Rawel et al., 2002). The presence of condensed tannins in high-tannin sorghum would cause decreased sorghum protein digestibility (possibly due to the complexation of sorghum condensed tannins with the major sorghum protein, kafirin) (Emmambux and Taylor, 2003). Similarly, sea buckthorn procyanidins can cause the precipitation of proteins and inhibition of digestive enzymes, thereby changing the digestibility of proteins (Arimboor and Arumugan, 2011).

### Changes in Phenolic Compound Properties

#### Effects on Antioxidant Properties

The antioxidant activity of phenolic compounds may be affected by their interactions with proteins. A number of relevant studies have been published with most focusing on the interactions of phenolics from tea, coffee and cocoa with milk proteins (Ozdal et al., 2013). The interactions of tea phenolics including epigallocatechin gallate (EGCG) and gallic acid with  $\beta$ -casein significantly

reduced their antioxidant potential (Arts et al., 2002). Black tea with milk had a lower total polyphenol content than that without milk (Sharma et al., 2008). Addition of increasing amounts of milk to tea was negatively related to total antioxidant capacity (Ryan and Petit, 2010). The catechin-protein interactions could be strong causing reduced antioxidant capacity in milk chocolate, due to decreased recovery of catechin from the food matrix (Belscak et al., 2009). Masking effect of milk on antioxidant potential of different coffee brews was also reported (Niseteo et al., 2012; Sanchez-Gonzalez et al., 2005; Ziyatdinova et al., 2010).

### Effects on Bioavailability

Protein-phenolic interactions can affect the bioavailability of phenolic compounds. Consumption of coffee with milk resulted in a decreased amount of adsorbed chlorogenic acid into the bloodstream (Duarte and Farah, 2011). Interestingly, the bioavailability of cocoa phenolics was found unaffected by milk proteins under certain conditions (Roura et al., 2007). The plasma concentrations of cocoa polyphenols were not influenced by the presence of milk powder although the absorption of cocoa polyphenols was slightly (but not significantly) increased (Keogh et al., 2007). Burg-Koorevaar et al. (2011) also did not find a difference in plasma catechin concentrations upon consumption of tea with or without milk. The addition of bovine, soy or rice milk to tea samples increased the *in vitro* recovery of total tea catechins, whilst no significant difference was detected in *in vivo* (in humans) catechin bioavailability of tea samples with or without milk (Green et al., 2007). Similarly, no significant effects of milk proteins on *in vivo* bioavailability of tea and coffee phenolics were reported (Hollman et al., 2001; Leenen et al., 2000; Reddy et al., 2005; Renouf et al., 2010). In terms of blueberry polyphenols, their interactions with milk proteins led to a lower *in vivo* antioxidant potential and decreased caffeic acid absorption (Serafini et al., 2009). Contradictory results were obtained regarding the effects of milk proteins on the bioavailability of cocoa polyphenols e.g. decreased absorption of (–)-epicatechin into the bloodstream was attributed to its interaction with milk proteins (Serafini et al., 2003), whilst no effect of milk on flavanol absorption was reported (Schramm et al., 2003; Schroeter et al., 2003).

### Processing Effect on Protein-Phenolic Interactions

Besides the effect of food matrix, processing also initiates and changes the protein-phenolic interactions, and the stability and bioaccessibility of bioactive compounds. Such processing-induced effects are affected by food type, nature and location of phenolic compounds in food, and type, intensity and duration of processing (Chandrasekara et al., 2012; Rodriguez-Roque et al., 2015). Quite a lot of studies investigated protein-phenolic interactions and delivery of bioactive compounds in functional bread and dairy products, while some examined commercially available food products fortified with plant extracts. Swieca et al. (2013) enriched the bread with phenolics by partial replacement of flour with onion skin (OS) during dough preparation. The authors found that the phenolic contents in baked bread dough at 230 °C for 30 min, although increased with the level of onion skin supplement, were significantly lower than expected (the amount initially added) due to the masking effect of protein-phenolic interactions. The degree of this masking effect depended on the amount of added OS, and *in vitro* protein digestibility exhibited a reduction from 78.4% (for control breads) to 55% (for breads with a 4% OS supplement). Under the same processing conditions, Swieca et al. (2014) found that enrichment of bread with quinoa leaf (QL) phenolic-rich increased its total phenolic content and antioxidant activity with such increases all lower than expected, possibly due to the inhibition of reactive groups of phenolics by bread proteins via interactions. The authors also reported significant decreased protein digestibility caused by QL phenolics with the lowest occurring to bread fortified 3% QL phenolics.

Zhu et al. (2016) added black tea extracts (up to 175 mg gallic acid equivalent/gram wheat flour) to wheat flour to prepare Chinese Steam Bread (CSB). Black tea extract supplement significantly increased CSB phenolic content and antioxidant activities of in a tea extract concentration-dependent manner, although the increases in phenolics, flavonoids and antioxidant activities of CSB extracts were less than a half of the original black tea extracts. Thermal breakdown during steaming process, oxidation throughout the production, redox interactions and binding between tea polyphenols and gluten proteins all were possible contributors.

Sivam et al. (2011) enriched breads were produced by replacing the same amount of flour with apple, blackcurrant and kiwifruit phenolic extracts (APE, BPE and KPE, respectively) (at 3% w/w) in the presence or absence of apple pectin. The total extractable phenolic contents of fortified breads after baking were largely retained after bread making possibly due to the protection of the phenolic compounds against degradation by wheat proteins. The type of phenolics would affect their binding to wheat proteins resulting in different impacts on the final products. Sivam et al. (2012) further reported that addition of BPE and pectin caused altered conformations and extractability of wheat flour proteins in model bread systems (which simulated real finished breads): decreases in  $\beta$  turns and  $\alpha$ -helix structures but moderately increased intermolecular H bondings, and reduced amounts of unextractable HMW proteins.

Gazzola et al. (2012) studied the roles of proteins, polysaccharides, and phenolics in haze formation in white wine. Chitinases and thaumatin-like protein (TLP) proteins and total polyphenols derived from Chardonnay wine were purified and then added to the base wine. After a heating/cooling cycle (70 °C for 1 h and 25 °C for 15 h), there was only a limited effect of phenolics on Chitinases' aggregation behavior. The phenolics had varied effects on isoforms of TLP protein and the resulting aggregation behavior was likely affected by the types of proteins and molar ratio of phenolic/protein.

The effects of addition of pea protein isolate (PPI) and green tea extract (GTE) on rice-substituted fried noodle were investigated (Song and Yoo, 2017). Adding PPI caused decreased dough viscosity parameters of the substituted rice noodle, whilst addition of

PPI together with GTE recovered these values remarkably and had a positive effect on cooking loss of substituted noodles. The researchers concluded that the protein-phenolic interaction occurring to co-addition of GTE and PPI has a great potential for the fried rice-substituted noodle production in terms of higher antioxidant activity and desirable textural properties.

Fortification of milk products with polyphenols has been a desirable approach for bioactive compound delivery. Veena et al. (2015) reported that the addition of *Asparagus racemosus* (shatavari) extract led to a pH decrease thereby increasing the heat stability of enriched milk but decreasing rennet coagulation time.

Rodriguez-Roque et al. (2015) reported that the concentration and bioaccessibility of phenolic compounds in fruit juice-based beverages increased or did not change after high-intensity pulsed electric fields (HIPEF) or high-pressure processing (HPP) treatments, due to the interactions of phenolics with other food components released, breakdown of cell wall structure, or increased activities of enzymes (which would take part in phenol biosynthesis). In contrast, thermal treatment reduced the bioaccessibility of most phenolic compounds. Fortification the fruit juice-based beverages with bovine milk or soy milk might lead to a decrease in antioxidant activity of phenolics possibly owing to with their interactions with milk proteins.

The effects of adding blackcurrant extracts and pure cyaniding, before and after fermentation, on drinking yoghurt were studied by Sun-Waterhouse et al. (2013). A significantly increased total extractable phenolic content (up to 4 times higher) of fortified yoghurts obtained via the pre-fermentation approach, as compared to that via the post-fermentation approach, indicates enhanced extractability of polyphenols via fermentation. The properties of fortified drinking yoghurts including rheological behaviours such as elastic property ( $G'$ ) of yogurt samples were affected by the type of polyphenol added, type of pectin used in the yoghurt formulation, and method for polyphenol addition (pre- or post-fermentation), via specific intermolecular interactions.

Han et al. (2011) and Rashidinejad et al. (2015) investigated the effects of various natural extracts and their individual phenolic compounds on cheese making properties and final product characteristics. They found that the free radical scavenging activity of cheese samples fortified with phenolics was found higher than control cheeses, and phenolics changed the coagulation properties of milk proteins mainly due to the decrease in pH or milk protein-phenol interactions.

In summary, phenolic fortification may improve the health-promoting properties of food products, however, the interactions of phenolic compounds with food components especially proteins could influence antioxidant efficiency and protein digestibility.

## Characterization of Polyphenol-Protein Complex Formation

To understand the changes in bioavailability and digestibility caused by phenolic-protein interactions, it is important to characterize proteins and polyphenols in the same food system at molecular level. A range of techniques based on different mechanisms have been used for this purpose and compliment each other in terms of providing information related to the interaction between protein and phenolics.

### Fluorescence Quenching

Fluorescence quenching method helps to study non-covalent binding between proteins and phenolics based on the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule. Fluorescence quenching can be dynamic (owing to collisional encounters between the fluorophore and quencher), or static (owing to the formation of a ground-state complex between the fluorophore and quencher) (Lakowicz, 1999; van de Weert and Stella, 2011). Stern-Volmer equation is a tool to determine quenching constants to measure the binding affinity between the phenolics and proteins. Fluorescence quenching method is widely used, however, this technique does not provide any direct information about the molecular changes of proteins nor polyphenols (Bandyopadhyay et al., 2012). Zhang et al. (2014) used this technique to determine the binding constants, binding sites and types of binding force between milk whey protein and some phenolic acids, and found that the mechanism was of static quenching by complex formation.

### Circular Dichroism Spectroscopy

The three dimensional structure of proteins is changed upon binding to polyphenols, and CD spectroscopy gives information about the change in secondary structure in the far UV region (190–250 nm) and the tertiary structure in the near UV region (250–390 nm). He et al. (2016) reported that via binding to malvidin-3-O-glucoside (MG), the secondary structure of  $\beta$ -lactoglobulin was changed: a decrease in  $\alpha$ -helix, turn and random coil while an increase in  $\beta$ -sheet.

### Fourier Transform Infra-Red Spectroscopy

FT-IR spectroscopy measures effectively and efficiently the wavelength and intensity of the absorption of IR radiation by a sample (e.g. a very small amount of proteins), thus, is a reliable tool for examining protein chain conformation and hydrophobic contact in the polyphenol-protein complex. Sivam et al. (2012) aimed to develop fruit polyphenol-enriched breads and detected, using FT-IR, the changes in bread gluten conformations related to Amide I and II bands caused by blackcurrant polyphenol fortification.

### Mass Spectrometry

Mass spectrometry can identify the complexes formed between phenolics and proteins, via separating the charged particles according to their mass (more exactly of the mass/charge ratio, noted  $m/z$ ) (Chen and Hagerman, 2004). This technique requires a very small amount of sample, and can give information about the mechanism and stoichiometry of the adduct(s) formed although it is worth noting the possible difference between the adducts detected in a gas phase by MS and that specifically in solutions (Sarni-Manchedo and Cheynier, 2002). Using ESI-MS, Dias et al. (2016) identified the sequence of peptide fractions of wheat gliadin and demonstrated the binding between a food tannin (procyanidin B3) and wheat-derived peptide fractions.

### Dynamic Light Scattering

Contrary to other protein precipitation methods, DLS is a technique operating at a nano scale to provide information about size and shape (sphere or rod) of a sample (including the aggregates resulted from interactions), based on the relaxation rate of particles that scatter light in solution. The limitation of this technique is closely associated with the degree of turbidity of a sample (Le Bourvellec and Renard, 2012). Using DLS, Karefyllakis et al. (2017) reported a neutral pH caused the formation of sunflower protein isolate-chlorogenic acid (SFPI-CGA) adducts via hydrogen bonding-facilitated self gathering.

### Nuclear Magnetic Resonance

NMR spectroscopy determines the chemical structure of molecules by measuring the resonance of protons under a magnetic field and can give precise information about the structural basis for phenolic-protein interactions (Le Bourvellec and Renard, 2012). Both solution and solid-state NMR can be used for this purpose. Polyphenols can interact with salivary proline rich protein (PRP) fragments, and a stronger interaction was detected by NMR between larger and more complex polyphenols and PRPs (Charlton et al., 2002).

### Size Exclusion Chromatography

Size exclusion chromatography (SEC) is a simple and efficient method to display the binding and size of the water-soluble protein-phenolic complexes (Sun-Waterhouse et al., 2011). However, this technique has limitations related to the interaction of polyphenols with the gel filtration material and the dilution with eluents (Bartolome et al., 2000). Using SEC, Swieca et al. (2013) determined the protein-phenolic interaction in breads enriched with extracts from quinoa leaves (QL).

### High Performance Liquid Chromatography/Reversed Phase-HPLC

HPLC/RP-HPLC can determine phenolic profiles, therefore, this kind technique with/without coupling with MS has been used to monitor the changes in phenolic profiles before and after the interactions of phenolics with other food components (Awad et al., 2002; Soares et al., 2012). Soares et al. (2012) showed that the interactions of tannins, procyanidin trimer or pentagalloylglucose (PGG) with acidic proline rich proteins (PRPs) affected acidic salivary solution, and each tannin interacted differently with each group of proteins.

### SDS-PAGE

SDS-PAGE is probably the most common technique used to separate proteins according to their molecular weight and differential rates of migration through a gel under the influence of an electrical field. Using SDS-PAGE, Veena et al. (2015) found the high molecular weight products formed between *A. racemosus* (shatavari) and milk proteins via interaction.

### Isothermal Titration Calorimetry

Among the techniques for examining binding, ITC is the only one capable of measuring both the magnitude of the binding affinity and the magnitude of the enthalpic and entropic components. It provides a direct and quantitative measurement of the net interaction between phenolic and protein (Pascal et al., 2007) although this technique requires high quantities of samples (Le Bourvellec and Renard, 2012). Using ITC, the binding interactions of green tea flavonoids and milk proteins was examined and the interaction between catechin and  $\beta$ -casein was non-covalent (Yuksel et al., 2010).

### Turbidimetry and Nephelometry

Insoluble protein-phenolic complexes have been mostly studied by protein precipitation. Both turbidimetry and nephelometry are based on light scattering properties of insoluble aggregates (Hamilton, 2014). Turbidimetry measures light transmitted through a suspension of particles and can be used to determine the ability of phenolics to bind proteins (Le Bourvellec and Renard, 2012). Nephelometry also measures light scattered by particles in a suspension and does not require high phenolic concentrations, as this technique is able to neglect the light absorption and has solvated particles' refraction index almost identical with that of the

solution (Le Bourvellec and Renard, 2012). These two techniques do not tell about the number, morphology and structure of aggregates.

## Conclusions

Both covalent (irreversible) and non-covalent interactions (reversible) occur between protein and phenolic compounds. Most published studies focused on non-covalent interactions (reversible) mainly due to the lack of suitable methodologies for quantitating the covalent bonds between molecules. Main factors affect protein-phenolic interactions, and include type of protein, structure of phenolic compounds and environmental factors such as temperature, pH, salt concentration and present of certain reagents/substances. Even though the exact mechanisms of how proteins would influence phenolics remain unknown, many studies have been conducted on the changes in the structural and functional properties of resulting foods. While the fortification of phenolics could improve the health-promoting properties of food products, the interactions of the fortified phenolic compounds with food components especially proteins may have a desired or undesired impact on antioxidant efficiency and protein digestibility. Thus, more in-depth studies are needed on the protein-phenolic interactions in different matrices and under different processing conditions, in order to guide the development of phenolic-enriched functional food products with enhanced nutritional and health benefits.

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# The Potential Role of Polyphenol–Enzyme Interactions on Human Health

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## Introduction

Many reports implicate polyphenols (PPs), some abundant in foods, in inhibition of enzyme-catalysed reactions that could influence human health. Indeed, recent computationally-driven analysis has modelled the PP-protein “interactome” (Lacroix et al., 2018) in which over 350 different PPs were found to interact with over 5500 specific proteins resulting in nearly 12000 interactions. However, a substantial proportion of PP-enzyme inhibition reports involve screening for inhibitory effectiveness with crude extracts and using *in vitro* assays under non-physiological conditions or concentrations. Traditionally, PP-protein interactions have had negative connotations for biochemists who have striven to remove polyphenols during enzyme purification and assays (Pierpoint, 1996). Also, certain PPs (e.g. proanthocyanidins, PACs) in plant feeds have been noted for their anti-nutritional effects due to non-specific inhibition of digestive processes (Butler, 1992). Therefore, it is important to set out certain criteria for judging the relevance of potential PP-enzyme interactions.

1. **Specificity:** The interaction between polyphenol and enzyme must be reasonably specific, and not a non-specific effect on multiple enzymes.
2. **Dose** – The interaction between the polyphenol and enzyme must occur at a dose that can be safely reached through intake of a food, product or pharmacological extract.
3. **Bioavailability** – The polyphenol component must be available in the same location at the target enzymes, e.g. the gut to inhibit digestive enzymes, the bloodstream to influence Angiotensin Converting Enzyme or the extracellular matrix to inhibit metalloproteases.

Using these rather inter-dependent criteria as a guide, I assessed the relevance of potential PP-enzyme interactions and have split the review into those occurring with non-digestive enzymes and then digestive enzymes. I have not considered inhibition of enzymes which could be due to general antioxidant activity of PPs acting on redox-active enzymes (such as (e.g.) peroxidase, catalase, lipoxygenase, tyrosinase and polyphenol oxidase), although these may be important in other areas, especially such as in food and drink. Also, I have not considered the many occasions where PPs have been shown to have interactions with cell receptors and thereby influence intracellular protein kinase cascades implicated in the control of disease progression (Aiyer et al., 2012; Kim et al., 2014) through (say) remodelling of gene expression. As this is a short commentary, I also refer the reader to previous reviews on the definition and structure of PPs (e.g. Del Rio et al., 2013; McDougall, 2017) and their bioavailability and metabolism (Del Rio et al., 2013; D’Archivio et al., 2010; Hu et al., 2017).

## Non-digestive Enzymes

### Acetylcholine Esterase (AChE)

Decreased acetylcholine concentrations appear critical in the development of dementia in neurodegenerative diseases, such as Alzheimer’s disease (Loizzo et al., 2008) and therefore AChE inhibition may be a useful therapeutic step. Crude PP-rich extracts can inhibit AChE *in vitro*, but often at physiologically-unachievable doses. Common dietary flavonols (quercetin and myricetin) gave IC<sub>50</sub> values ~ 15 µM (Xie et al., 2014). However, rosmarinic acid potently inhibited AChE activity *in vitro* [Ki at 40 pM (Gulcin et al., 2016)], which approaches the measured range in rat brain (Li et al., 2007). AChE activity was reduced after supplementation of mice with PP-rich wild blueberry extract (Papandreou et al., 2009) but probably due to reduced gene expression.

### Monoamine Oxidase (MAO)

Inhibition of MAO activity can reduce breakdown of specific amine neurotransmitters (e.g. norepinephrine, serotonin, and dopamine) and MAO inhibitors have been used to treat depression and Parkinson’s disease (Volz and Gleiter, 1998). Flavonoids inhibited MAO *in vitro* with low µM IC<sub>50</sub> values (Nabavi et al., 2017). Anthocyanidins, their 3-glycosides and quercetin were also effective with IC<sub>50</sub> values around 30 µM (Dreiseitel et al., 2009). Although various studies have garnered evidence that serum-available phenolic metabolites can cross blood-brain-barrier models (e.g. Figueira et al., 2017), whether these specific PPs could reach the brain and be effective *in vivo* is debatable. Nevertheless, supplementation with PP-rich black currants was shown to reduce serum MAO activity in healthy adults (Watson et al., 2015).

### Xanthine Oxidase (XO)

XO converts purines to uric acid but also produces superoxide and XO activity has been associated with various disease states (e.g. Pachter et al., 2006). Flavonoids (e.g. chrysin and genistein) gave *in vitro* IC<sub>50</sub> values ~ 1 µM, lower than the known inhibitor, allopurinol (Lin et al., 2015) whereas more common dietary flavonols, kaempferol and quercetin, were 10–25 fold less potent.

However, different studies gave lower IC<sub>50</sub>s and found that quercetin 4'-glucuronide, a known serum-bioavailable metabolite (Mullen et al., 2006), was as effective as quercetin with a  $K_i \sim 0.25 \mu\text{M}$  (Day et al., 2000).

### Angiotensin Converting Enzyme (ACE)

Inhibition of ACE, a dipeptidyl carboxypeptidase commonly found in vascular endothelial cells, prevents conversion of angiotensin I to angiotensin II, a vasoconstrictor that raises blood pressure (Hugel et al., 2016). Synthetic ACE inhibitors can control hypertension but have drawbacks, so natural ACE inhibitors are of interest. Flavonoids inhibit ACE *in vitro* (IC<sub>50</sub>s luteolin 23  $\mu\text{M}$ , quercetin 43  $\mu\text{M}$  and rutin 63  $\mu\text{M}$ ; Guerrero et al., 2012). Different assay formats influence potency as quercetin and quercetin-3-glucuronide had IC<sub>50</sub> values of  $\sim 150$  and 30  $\mu\text{M}$ , respectively, using purified ACE but at  $\sim \text{nM}$  concentrations using cell-based assays (Balasuriya and Rupasinghe, 2012). Grape-seed PAC extracts gave IC<sub>50</sub> values  $< 20 \text{ nM}$  (Godoy et al., 2012) whereas purified epicatechin octamer (EC8) had an IC<sub>50</sub> of  $\sim 40 \text{ nM}$ , compared with EC5 at 2.4  $\mu\text{M}$  (Afonso et al., 2013). Feeding PP-rich blueberries to rats reduced circulating ACE activity (Wiseman et al., 2011) and a human study showed significant effects of Aronia supplementation on ACE levels (Sikora et al., 2014). However, in both cases, the reduction was probably due to reduced gene expression.

### Matrix Metalloproteinases (MMP)

Inhibition of MMP by PPs may reduce metastasis in cancers (Sang et al., 2006), through prevention of extracellular matrix remodelling. Although direct inhibition can be relatively potent, it is more probably likely that PPs *in vivo* cause down-regulation of MMP expression. Intriguingly, Urolithin A, a major colonic metabolite of ellagitannins known to be serum-bioavailable, reduced MMP activity in cultured cancer cells at sub- $\mu\text{M}$  levels (Zhao et al., 2018).

### 3-Hydroxy-3-Methylglutaryl CoA Reductase (HMGCR)

Inhibition of HMGCR by statins controls cholesterol levels through reducing the rate-limiting HMG-CoA to mevalonate step (Willey and Elkind, 2010). However, decreased flow through this pathway may also influence inflammation, nitric oxide synthesis, coagulation and neurological diseases. Epigallocatechin gallate (EGCG) potently inhibited HMGCR *in vitro* ( $K_i \sim \text{nM}$ ; Cuccioloni et al., 2011) due to high-affinity binding at the nicotinamide adenine dinucleotide phosphate (NADPH) co-factor site and bioavailable EGCG levels may compete with NADPH *in vivo*. Molecular docking identified that curcumin also had high affinity for the active site (Islam et al., 2015). Whether HMGCR inhibition by PPs occurs *in vivo* is uncertain as PPs may pre-dominantly influence HMGCR gene expression.

## Enzymes Involved in Digestive Processes

### Protein Digestion

PPs inhibit proteolytic enzymes *in vitro* including pepsin (Martinez-Gonzalez et al., 2017), trypsin (Gonçalves et al., 2007), and chymotrypsin (Wu et al., 2013). Trypsin inhibition by PACs increased with increasing size through both specific and non-specific protein binding (Goncalves et al., 2011a,b). Effects on protein digestion *in vivo* are rarer but studies in pigs noted decreased ileal protein digestibility by grape tannins (Myrie et al., 2008). Recently, a phenolic-rich extract of artichoke, apple and plum also reduced ileal digestibility of protein in mini-pigs (Dufour et al., 2018). Reduced protein digestion could be deemed a negative nutritional outcome but enhanced protein residence in the small intestine may have beneficial effects on appetite and satiety (Fromentin et al., 2012; van Avesaat et al., 2017). However, further *in vivo* studies are required to confirm these findings.

### Lipid Digestion

PPs inhibit pancreatic lipase (PL) *in vitro* (Buchholz and Melzig, 2015) and inhibition of PL through drugs such as Orlistat is clinically proven to reduce fat digestion and uptake (Sjöström et al., 1998). Studies with different sources implicate different PP components: Galloylated catechins (including EGCG) and theaflavins from teas gave  $\mu\text{M}$  IC<sub>50</sub> values (Rahim et al., 2015) and modelling indicated that galloyl groups enhanced potency by direct interactions with active site residues (Clisan et al., 2017). Caffeoyl quinic acids from (e.g.) coffee also act at the active site (Hu et al., 2015), albeit at IC<sub>50</sub> values  $\sim 1 \text{ mM}$ . PACs inhibit PL through non-competitive inhibition (Wang et al., 2014) and ellagitannins from berries (McDougall et al., 2009) act through non-specific astringent effects. Various *in vivo* studies have confirmed that PP intake reduced fat uptake (e.g. boysenberry; Mineo et al., 2015), tea intake (Hsu et al., 2006), galloylated catechins (Ikeda et al., 2005), EGCG (Grove et al., 2012) and theaflavins (Jin et al., 2013) but also apple PACs (Sugiyama et al., 2007). Chronic exposure to PPs could lead to compensatory increases in lipase levels as noted with condensed tannins in rats (Griffiths, 1986). However, inhibition of lipase is only one of many mechanisms whereby polyphenols could influence obesity (e.g. Hsu and Yen, 2007) and this seems a fertile future research area.

## Carbohydrate Digestion

PPs can inhibit both  $\alpha$ -amylase and  $\alpha$ -glucosidase *in vitro*, the main enzymes required for breakdown of starch to glucose *in vivo* (Hanhineva et al., 2010). Inhibitory effects on  $\alpha$ -glucosidase include specific effects on maltase and sucrase activities (Williamson, 2013). Inhibitory potency range encompasses gut-achievable levels ( $\mu$ M to mM) and mechanisms include active site binding (flavonoids/amylase; Lo Piparo et al., 2008; kaempferol/glucosidase; Peng et al., 2016), mixed mode inhibition (tea PPs/amylase; Sun et al., 2016) to less-specific protein aggregation (PACs and amylase; Goncalves et al., 2011a,b). Inhibitory effectiveness approaches levels caused by known inhibitors such as acarbose (e.g. Boath et al., 2012).

Certain PPs also inhibit glucose transporters at the gut lumen which may cause synergistic effects on blood glucose levels (Williamson, 2013). Indeed, PPs and PP-rich foods reduced blood glucose levels after starch and sucrose intake in humans and animals (Coe and Ryan, 2016) but their variable effectiveness may be influenced by dose, trial design and food interactions (Dominguez-Avila et al., 2017). Akin to the lipase situation, chronic exposure to PPs could lead to compensatory enhanced secretion of pancreatic enzymes but recent work has shown that chronic exposure to oleuropein-rich olive leaf extract inhibited sucrase activity and glucose transport in a model gut cell system but also reduced sucrase mRNA expression and the proportion of sucrase present at the cell surface (Pyner et al., 2017).

In a related area, inhibition of gut dipeptidyl-peptidase IV (DDP4) activity by PPs present in PP-rich foods could increase the longevity of incretin hormones [i.e. glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP)] which are intimately involved in glycaemic control and already targeted by pharmacological agents (Lacroix and Li-Chan, 2016).

## Conclusion

Even within the limited scope of this commentary, there are many examples of PP-enzyme inhibition that could have beneficial effects on human health. Given the less limited availability of PPs in the gut, many of these relate to digestive enzymes and this is an area that fully deserves further attention. However, given the continuing interest in the health benefits of PP intake (e.g. Del Rio et al., 2013), it is very likely that other examples of PPs inhibiting novel enzymes will arise, such as the recent report that the flavonol, myricetin, can potently inhibit gastric  $H^+$ ,  $K^+$ -ATPase activity (Miyazaki et al., 2018) and may thereby function as a natural proton pump inhibitor.

Bridging the gap between human and animal studies that confirm beneficial effects on blood glucose levels, lipid digestion and uptake or *in situ* proteolysis and studies that uncover the mechanisms whereby individual PPs can cause these effects, is crucial. Understanding if PP intake can provide acute or chronic benefits is also important to design PP-rich extracts/foods for specific (e.g.) hyperglycaemic effects. Of course, adding to this complexity is the considerable inter-individual response to PP intake noted in recent work (Manach et al., 2017).

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# Thermal Stability of Carotenoids- $\alpha$ -Lactalbumin Complex

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## Overview of Structure-function Relationship of $\alpha$ -Lactalbumin

$\alpha$ -Lactalbumin ( $\alpha$ -LA) is the second most important milk protein, accounting for 20%–25% of the whey proteins and about 3.4% of total milk proteins. According to the description provided by Chrysina et al. (2000),  $\alpha$ -LA molecule consists of a single polypeptide chain of 123 amino acids, with a molecular mass of 14,200 Da and 3D structure homologous to the lysozyme family. The  $\alpha$ -LA tertiary structure has two domains: the larger  $\alpha$ -domain including three major  $\alpha$ -helices of the protein (residues 5–11, 23–24 and 86–98) and two short  $3_{10}$  helices (residues 18–20 and 115–118), and the smaller  $\beta$ -domain with three stranded antiparallel  $\beta$ -sheets (residues 41–44, 47–50 and 55–56), one short  $3_{10}$  helix (residues 77–80) and loops (Al-Hanish et al., 2016). The  $\alpha$ -LA structure is stabilized through four disulfide bonds, and there is no free thiol group available (Gao et al., 2008).

Each  $\alpha$ -LA molecule has a single strong calcium binding site, also called “calcium binding loop”, which is placed in a deep cleft in between  $\alpha$  and  $\beta$  domains (Mohammadi and Moeeni, 2015), and partly contains the  $\alpha$ -helix H3 and  $3_{10}$  helix h2 (N’Negue et al., 2006). The calcium ion is coordinated to the  $\beta$ -carboxyl groups of three aspartic acid residues (Asp<sup>82</sup>, Asp<sup>87</sup> and Asp<sup>88</sup>), two backbone carbonyl oxygens (Lys<sup>79</sup> and Asp<sup>84</sup>), and two water molecules (Acharya et al., 1991; N’Negue et al., 2006). Farkas et al. (2005) explained that calcium binding plays an important role in stabilizing the native state of  $\alpha$ -LA. For instance, the temperature required to induce  $\alpha$ -LA unfolding registered a shift from about 20 °C, corresponding to the apo-form, to nearly 70 °C for the Ca<sup>2+</sup>-bound protein. Stănciuc et al. (2012) studied the effect of pH and temperature on the fluorescence of apo- $\alpha$ -LA, and showed that the protein is sensitive to both environmental factors. The heat-induced denaturation followed a third-state model when unfolding at pH 2.0, and *all-or-none* transitions were registered at pH 4.5 and 7.0. The protein structure appeared more flexible at acidic pH values, favoring the exposure of the hydrophobic residues.

Because the  $\alpha$ -LA molecule has several partially unfolded intermediate states and is known to be present in molten globule form under various conditions, it was extensively used as model for studying the protein folding and unfolding patterns (Chaudhuri and Chattopadhyay, 2014). At low pH values,  $\alpha$ -LA undergoes conformational changes toward the molten globule state, a specific conformation that retains its secondary structure domains but loses its stable tertiary structure. Ryu et al. (2012) studied the pH-induced conformational changes of  $\alpha$ -LA and suggested sequential changes from pre-molten globule to molten globule state at pH 4.75 and 2.75. The first changes were observed in the  $\beta$ -sheet structures, followed by  $\alpha$ -helices alteration at lower pH values.

$\alpha$ -LA has multiple biological and pharmaceutical functions. It has an important *in vivo* role in lactose biosynthesis in mammary gland, as a specificity modifier of galactosyltransferase (Permyakov and Berliner, 2000). In partially disordered states,  $\alpha$ -LA has the ability to interact with membranes (Halskau et al., 2002). The potential of interacting with membranes is of significant relevance, since the complexation of  $\alpha$ -LA with oleic acid may form the HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cells) complex (Svensson et al., 2000, 2003; Brinkmann et al., 2013) which has antimicrobial properties and is tumoricidal against a spectrum of cancer cells while exhibiting far lower toxicity towards differentiated cells.

Milk proteins are widely used as carriers for drugs/bioactive compounds. Several studies indicated the ability of apo  $\alpha$ -LA to interact with different hydrophobic compounds, such as carotenoids (Dumitraşcu et al., 2016; Ursache et al., 2017), retinol (Livney, 2010), vitamin D3 (Delavari et al., 2015), hydrophobic peptides, model lipid membranes, fatty acids (Barbana et al., 2006; Kehoe and Brodtkorb, 2014), and epigallocatechin-3-gallate (Al-Hanish et al., 2016).

## Carotenoids

Carotenoids are intensely yellow to red colored liposoluble pigments synthesized by plants and micro-organisms. The structures of all carotenoids are based on a common 40-carbon linear terpene. Carotenoids are classified into carotenes that are hydrocarbons without oxygen, and xanthophylls that contain one or more oxygen atoms (Bhosale and Bernstein, 2007).

Carotenoids are present in many foods, particularly in fruit, vegetables, and fish (El-Agamey et al., 2004; Yuan et al., 2013). Humans consume a wide variety of carotenoids, with  $\beta$ -carotene, lycopene, lutein, zeaxanthin,  $\beta$ -cryptoxanthin, and  $\alpha$ -carotene accounting for 90% of the circulating carotenoids (Bhosale and Bernstein, 2007). Although other carotenoids, such as violaxanthin and neoxanthin, are also common in the human diet, they do not appear to be absorbed. Because of their antioxidant activities, such as oxygen radical scavenging and singlet oxygen quenching abilities, carotenoids have significant functional properties (Elliott, 2005; Stahl and Sies, 2005).

Among the carotenoids, considerable attention has been paid to the  $\beta$ -carotene, which has a polyene chain with 11 conjugated double bonds and a  $\beta$ -ring at each end of the chain (Knockaert et al., 2012).  $\beta$ -Carotene is one of the most effective vitamin A precursors, and provides additional potential health benefits, as an efficient cancer prevention agent, ulcer inhibitor, life extender, and heart attack inhibitor (von Lintig, 2010; Chen et al., 2014).

Due to their highly conjugated double bonds, carotenoids are rather unstable to oxygen, light, and heat. In their natural forms, carotenoids are insoluble in water and slightly soluble in oil at room temperature (Rodríguez-Huezo et al., 2004). One way to enhance the solubility of carotenoids relies on the formation of complexes with proteins or lipids (Pillbrow et al., 2012). Moreover,

carotenoids microencapsulation by using milk proteins has been proposed as an effective method for increasing their stability and their transportation while facilitating their functionality in delivery systems (Dumitraşcu et al., 2016)

### Fluorescence Spectroscopy-Based Evidences on Carotenoids- $\alpha$ -Lactalbumin Binding and Thermal Stability of the Resultant Complex

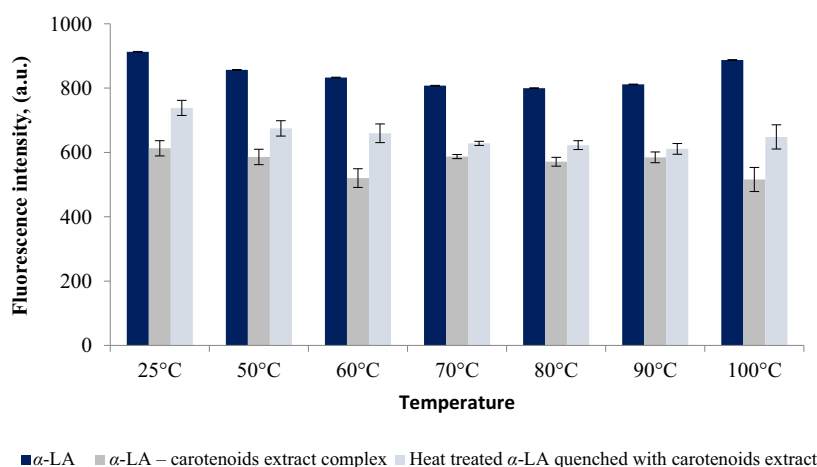
Fluorescence spectroscopy is a spectral technique widely used to characterize proteins and track conformational changes. The natural fluorescence of proteins is mainly attributed to tryptophan (Trp), phenylalanine, and tyrosine residues. The fluorescent properties of Trp are the most widely exploited. The detection of native and unfolded states of proteins is based on the indole side chain of Trp, whose emission spectrum is very sensitive to the polarity of its environment. Therefore, additional molecular events taking place on the same time scale might interfere with the emission properties of Trp (Efink, 1991). Fluorescence intensity of Trp residues can be measured as a function of excitation or emission wavelengths, or as a function of quencher or time. The emission maximum of Trp residues provides important information regarding solvent accessibility. The spectral properties of Trp residues include: (1) if emission maximum ( $\lambda_{\text{max}}$ ) is near 350 nm, residues are considered to be completely exposed to solvent; (2) if  $\lambda_{\text{max}}$  is close to 340 nm, residues are partially exposed to protein surface; (3) if  $\lambda_{\text{max}}$  is in the 315–330 nm range, residues are buried within the protein but interacting with neighboring polar groups; (4) if  $\lambda_{\text{max}}$  is close to 308 nm, residues are fully buried in a non-polar environment.

There are four Trp residues in each  $\alpha$ -LA molecule. As indicated by Chrysina et al. (2000), the Trp residues participate either in defining the aromatic cluster II (Trp<sup>26</sup>, Trp<sup>60</sup> and Trp<sup>104</sup>), or in the aromatic cluster I (Trp<sup>118</sup>). In its native state,  $\alpha$ -LA has intrinsic fluorescence mainly associated to Trp<sup>60</sup> which is exposed to the solvent. When  $\alpha$ -LA is heated alone in the temperature range of 25–70 °C, minor changes appear in Trp fluorescence or  $\lambda_{\text{max}}$  of  $\alpha$ -LA (Figs. 1 and 2). The heat-induced changes in  $\alpha$ -LA at the neutral pH and temperatures up to 70 °C are reversible (McGuffey et al., 2007).

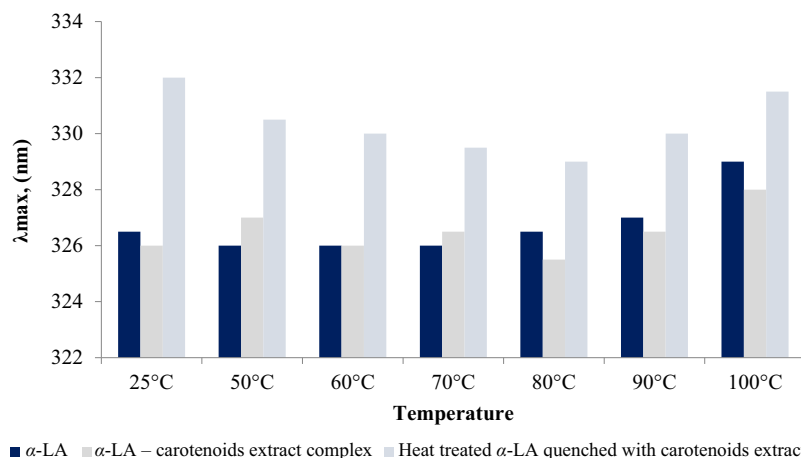
Fluorescence spectroscopy is also frequently applied to study the interaction between proteins and any target compound. Dumitraşcu et al. (2016) studied the mechanism of carotenoids binding through thermal treatment of  $\alpha$ -LA, while Ursache et al. (2017) reported on the thermal stability of the  $\alpha$ -LA-carotenoid complexes obtained at 25 °C. When  $\alpha$ -LA is heated first and then quenched with carotenoids, fluorescence intensity of Trp decrease with increasing concentration, indicating that despite their high hydrophobicity, carotenoids possess significant affinity for  $\alpha$ -LA. This behavior is associated with Trp movement from non-polar toward polar environment. The fluorescence intensity of Trp decreases under both tested conditions, but to a greater extent when the protein was first heated and then quenched with carotenoids. The heat treatment also caused significant differences in  $\lambda_{\text{max}}$  values. When compared with  $\alpha$ -LA and  $\alpha$ -LA-carotenoids complex at 25 °C, the protein quenching with carotenoids resulted in red shifts of at least 6 nm (Fig. 2), indicating a higher amount of exposed Trp for the interaction with polar groups.

Maximum Trp emission in the heated  $\alpha$ -LA subjected to quenching with carotenoids was blue shifted in the temperature range of 25–70 °C, whereas heating the  $\alpha$ -LA and  $\alpha$ -LA-carotenoids complex, in the same temperature range, resulted in no major change in  $\lambda_{\text{max}}$  (Fig. 2). For all the studied experimental set-ups, heating at temperatures over 80 °C caused red shifts of  $\lambda_{\text{max}}$ , indicating the interaction of Trp residues with polar groups (Dumitraşcu et al., 2016).

Stern-Volmer equation is often used to measure whether quenching process is static or dynamic (Dumitraşcu et al., 2016). Quenching experiments can be performed with quenchers such as KI and acrylamide, which allow the detection of the fluorescence of Trp residues with different degrees of exposure. Regardless of the applied heating conditions, quenching titrations with



**Figure 1** Fluorescence intensity of  $\alpha$ -lactalbumin ( $\alpha$ -LA) alone or in the presence of carotenoids added before or after heating. After the results presented by Dumitraşcu et al. (2016) and Ursache et al. (2017).



**Figure 2** Maximum emission of  $\alpha$ -lactalbumin ( $\alpha$ -LA) alone or in the presence of carotenoids added before or after heating. After the results presented by Dumitraşcu et al. (2016) and Ursache et al. (2017).

acrylamide generated higher  $K_{SV}$  values than with KI. The KI quenches the fluorescence of Trp residues located at or near the molecule surface, whereas acrylamide would target both exposed and partially exposed Trp residues (Ursache et al., 2017).

Quenching titrations can be performed with ligands that show the ability of protein to bind different compounds. Quenching experiments were performed on heated  $\alpha$ -LA with carotenoids. Based on the linearity of the Stern-Volmer plots, regardless of carotenoids being added before or after heat treatment of the protein, the quenching is static and the complex formed by carotenoids and  $\alpha$ -LA is in the ground state.

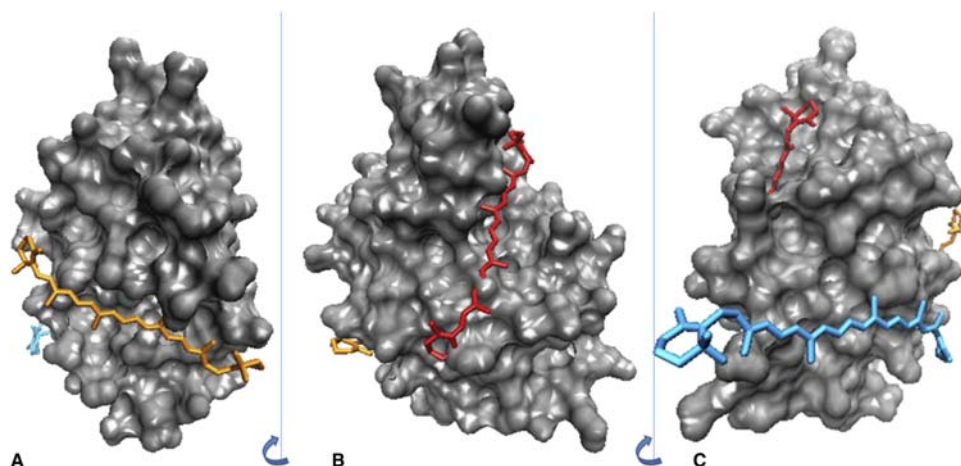
### Molecular Modelling Details on the Interaction Between $\beta$ -Carotene and $\alpha$ -Lactalbumin in Thermal Treatment

Different studies aiming to understand the molecular architecture of proteins alone or in complex with different ligands, relied on the use of various experimental techniques. One way to overcome the limitation of an experimental technique in providing atomic level details on the interactions between different molecules is to complement molecular modelling studies.

$\beta$ -Carotene was found as one of the major compounds in the carotenoid extract from sea buckthorn used by Dumitraşcu et al. (2016) and Ursache et al. (2017) in their investigations on the carotenoids- $\alpha$ -LA complex. The *in silico* investigations were used to examine the particularities of the  $\alpha$ -LA- $\beta$  carotene complex equilibrated at different temperatures as well as the interaction between  $\beta$ -carotene and the preliminarily heated protein. To provide comprehensive insights on the interaction and stability of the  $\alpha$ -LA- $\beta$  carotene complex, molecular docking and molecular dynamics approaches were successfully combined.

When studying the thermally induced structural rearrangements of the unbound  $\alpha$ -LA molecule, Dumitraşcu et al. (2016) showed a significant reduction in the amounts of amino acids involved in defining strands (from 9.8% at 25 °C to 3.3% at 90 °C) and  $3_{10}$  helix (from 7.4% at 25 °C to 2.5% at 90 °C) motifs. Thermal destabilization of the helix was mainly motivated by the dynamics of the hydrogen bonding pattern. The conformation of the aromatic cluster II hosting most of the Trp residues of the molecule (Chrysina et al., 2000) was altered at a high temperature due to significant molecular rearrangements resulting in changes of fluorescent residues exposed to the solvent, and formation of new native-like antiparallel G1 beta bulge, linking Tyr<sup>50</sup>, Gln<sup>54</sup> and Ile<sup>55</sup> residues, and four inverse beta turn motifs involving consecutive residues (Ser<sup>69</sup>-Asn<sup>71</sup>, Ser<sup>70</sup>-Ile<sup>72</sup>, Ile<sup>75</sup>-Cys<sup>77</sup> and Cys<sup>120</sup>-Lys<sup>122</sup>) compacted within 7 Å. In the molecular dynamics set-up proposed by Dumitraşcu et al. (2016), when the protein was heat treated alone, partial folding occurred, as indicated by the total protein surface evolution (6648.6 Å<sup>2</sup> at 25 °C and 6527.86 Å<sup>2</sup> at 90 °C). When the  $\alpha$ -LA molecule was subjected to the thermal treatment in the presence of  $\beta$ -carotene, Ursache et al. (2017) reported the slight relaxation of the protein structure at a high temperature (total protein surface increased up to 6904.8 Å<sup>2</sup> at 90 °C). In the latter case, the molecular structure appeared to be better organized at a high temperature, preserving the  $\alpha$ -helical content with about 80% of the strands specific to the native protein. Superposition of the protein molecules equilibrated at 90 °C using the two different set-ups proposed by Dumitraşcu et al. (2016) and Ursache et al. (2017) resulted in a backbone based root mean square deviation (RMSD) of 3.12 Å.

Regardless of the set-up used in the molecular modeling investigations, the reduction of the affinity was found between protein and  $\beta$ -carotene with increasing temperature. Lower interaction energy and higher interface area between the two components of the complex were reported when thermally treating the complex assembled at room temperature ( $\alpha$ -LA- $\beta$  carotene interaction energy of -175.40 kJ/mol and interface area of 484.7 Å<sup>2</sup> at 90 °C; Ursache et al. (2017)), suggesting better affinity compared to the complex obtained through docking the ligand onto the preliminary heat treated whey protein ( $\alpha$ -LA- $\beta$  carotene interaction energy of -127.19 kJ/mol and interface area of 333.7 Å<sup>2</sup> at 90 °C; Dumitraşcu et al. (2016)). Careful analysis of the forces contributing to the stability of protein-ligand complex showed that London dispersion forces prevail over the short range Coulomb interactions in both cases.



**Figure 3**  $\beta$ -Carotene binding by  $\alpha$ -lactalbumin ( $\alpha$ -LA) equilibrated at 25 °C (A) 60 °C (B) and 90 °C (C). The image was prepared using the Visual Molecular Dynamics (VMD) software (Humphrey et al., 1996).  $\alpha$ -LA is represented in silver using Surf style, while the ligand is represented in orange (25 °C), red (60 °C) and cyan (90 °C) in Licorice style. The amino acids involved in the interaction with the ligand are presented in Table 1.

**Table 1** The amino acid composition of  $\beta$ -carotene binding sites of  $\alpha$ -lactalbumin ( $\alpha$ -LA)

Temperature	Molecular modeling set-up	
	Thermal treatment of $\alpha$ -LA followed by docking with $\beta$ -carotene (Dumitraşcu et al., 2016)	Molecular docking of $\beta$ -carotene on $\alpha$ -LA followed by thermal treatment Ursache et al. (2017)
25 °C	Glu <sup>1</sup> , Gln <sup>2</sup> , Leu <sup>3</sup> , Glu <sup>11</sup> , Asp <sup>84</sup> , Thr <sup>86</sup> , Asp <sup>87</sup> , Met <sup>90</sup>	Glu <sup>1</sup> , Leu <sup>3</sup> , Glu <sup>11</sup> , Leu <sup>12</sup> , Asp <sup>83</sup> , Thr <sup>86</sup> , Asp <sup>87</sup> , Ile <sup>89</sup> , Met <sup>90</sup>
60 °C	Phe <sup>31</sup> , His <sup>32</sup> , Thr <sup>33</sup> , Tyr <sup>36</sup> , Glu <sup>49</sup> , Ile <sup>59</sup> , Tyr <sup>103</sup> , Trp <sup>104</sup> , Leu <sup>105</sup> , Ala <sup>106</sup> , Leu <sup>110</sup>	Glu <sup>1</sup> , Gln <sup>2</sup> , Leu <sup>3</sup> , Thr <sup>4</sup> , Glu <sup>7</sup> , Val <sup>8</sup> , Glu <sup>11</sup> , Leu <sup>12</sup> , Ile <sup>89</sup> , Lys <sup>93</sup>
90 °C	Leu <sup>15</sup> , Lys <sup>16</sup> , Val <sup>21</sup> , Ser <sup>22</sup> , Leu <sup>23</sup> , Glu <sup>25</sup> , Glu <sup>113</sup> , Lys <sup>114</sup>	Glu <sup>11</sup> , Leu <sup>12</sup> , Asp <sup>14</sup> , Leu <sup>15</sup> , Leu <sup>52</sup> , Leu <sup>85</sup> , Thr <sup>86</sup> , Asp <sup>88</sup> , Ile <sup>89</sup> , Met <sup>90</sup>

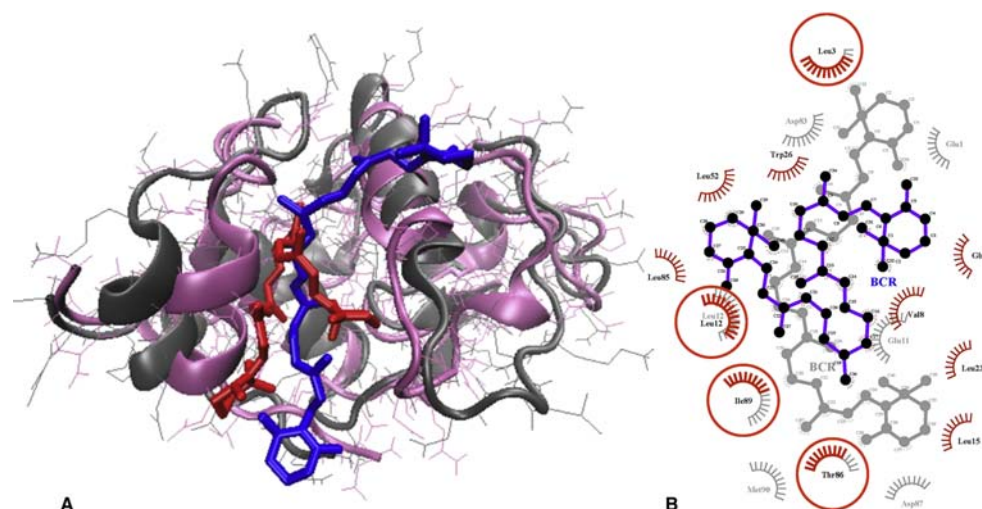
The ability of a protein to recognize and bind ligands is highly influenced by the surface properties. The exposure of different hydrophobic and hydrophilic patches on the protein surface vary with tertiary and secondary structure, and with the environmental conditions. Due to the temperature related expansion of the cavities located on top of protein surface, different binding sites, sharing no common residues, have been reported by Dumitraşcu et al. (2016) through docking the  $\beta$ -carotene molecule to the heat treated  $\alpha$ -LA (Fig. 3 and Table 1). On the other hand, when the complex was thermally treated, it appeared that the amino acids Leu<sup>12</sup> and Ile<sup>89</sup> from  $\alpha$ -LA played a central role in binding the  $\beta$ -carotene in the temperature range of 25–100 °C (Fig. 4). Both residues belong to two different  $\alpha$ -helices, which were the most stable elements of the globular protein structure. Indeed, no significant changes in the organization of the Asp<sup>87</sup>-Lys<sup>98</sup> helix was registered with increasing the temperature from 25 °C to 100 °C. In case of the Lys<sup>5</sup>-Leu<sup>12</sup> helix, the Leu<sup>12</sup> migrated toward a  $\beta$ -turn motif when the  $\alpha$ -LA- $\beta$  carotene complex was heated at 70 °C and 80 °C, because of the changes in the relative orientation of the side chains causing the alteration of the hydrogen bond pattern.

When the complex was heated, the increase of temperature resulted in the increase of the amount of hydrophobic amino acids interacting with the  $\beta$ -carotene molecule. In addition to the hydrophobic amino acids Leu<sup>3</sup>, Leu<sup>12</sup> and Ile<sup>89</sup>, which were in contact with  $\beta$ -carotene molecule at 25 °C, the following residues were found to establish hydrophobic contacts with the ligand at 100 °C: Val<sup>8</sup>, Leu<sup>15</sup>, Leu<sup>23</sup>, Trp<sup>26</sup>, Leu<sup>52</sup> and Leu<sup>85</sup> (Fig. 4). Since the ligand carried no electric charge, in both studied molecular modeling set-ups, the hydrophobic contacts were responsible for stabilizing the  $\alpha$ -LA- $\beta$  carotene complex. The involvement of the electrostatic interactions, which were considered to play a crucial role in rendering specific the protein-ligand binding, was limited to the eventual  $\pi$ - $\pi$  and cation- $\pi$  interactions (Dumitraşcu et al., 2016).

## Conclusions

The computer-assisted techniques used for simulating molecular behavior have been proven to be very efficient in complementing the experimental observations on carotenoids- $\alpha$ -lactalbumin complexes by fluorescence spectroscopy. Thermal treatment at high temperatures affects the stability of the complex. The fundamental knowledge of the atomic-level events responsible for the molecular behavior might allow tracking the origins of the macroscopically observed behaviors, leading to practical solutions for laboratory-scale experiments, or even for efficient technological processing at industrial scale of food ingredients.





**Figure 4** Details on the  $\alpha$ -lactalbumin- $\beta$  carotene docking models at 25 °C and 100 °C. (A) Superposition of the complexes prepared using the Visual Molecular Dynamics (VMD) software.  $\alpha$ -Lactalbumin ( $\alpha$ -LA) is represented in New Ribbons style in *mauve and gray*, whereas the ligand is represented in Licorice style in *blue and red*, at 25 °C and 100 °C, respectively. (B) Hydrophobic contacts established between  $\alpha$ -LA amino acids (represented through *arcs* radiating towards the ligand atoms they are in contact with) and  $\beta$ -carotene molecule at 25 °C and 100 °C. The representation was prepared using LigPlot+ (Laskowski and Swindells, 2011).

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# Component Segregation During Spray Drying of Milk Powder

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## Introduction

### Bulk Composition of Whole Milk

Whole milk, skim milk, and infant formula emulsions are regularly spray dried to powder form at large industrial scale for preservation with increased stability against oxidative rancidity, reduced bulk volume for economy of transportation, and easier processing as ingredient in food products (Vega and Roos, 2006; Schuck, 2002). While the exact composition of bovine whole milk can vary considerably, on average it has a solid content of 12%–13% w/w and, as presented in Table 1, the major components are lactose with an approximate content of 40% w/w in dry matter, fat with 32% w/w, and protein with 28% w/w (neglecting minerals and vitamins). The milk fat is dispersed inside the aqueous continuous phase in form of spherical fat globules of 0.1–10  $\mu\text{m}$  in diameter (Michalski et al., 2002). The globules are stabilized by a surrounding layer of fat globule membranes, which are composed of a complex combination of phospholipids, lipoproteins, other proteins, organic acids, bound water, traces of metals, and enzymes (Singh and Gallier, 2016). About 80% of the total protein content in bovine milk is made of caseins. These phosphoproteins are held together in form of submicron-sized micelles by hydrophobic interaction and calcium ions (Müller-Buschbaum et al., 2007). In studies that investigate milk model emulsions, native casein is often replaced by sodium caseinate as emulsifier. It is produced from precipitated casein by the addition of sodium hydroxide and is soluble in water without developing a micelle structure (Jost, 2002). The remaining protein fraction in milk consists of whey proteins, which are primarily comprised of  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin. Whey protein is also known as milk serum protein due to its greater water solubility than that of casein.

### Powder Surface Composition After Spray Drying

The main components at the surface of spray-dried milk powder usually differ substantially in proportion from the bulk composition of the original milk emulsion because of an overrepresentation of fat at the surface. The surface layer of fat is unwanted, as the chemical surface composition strongly influences the functional powder properties, with surface fat leading to several detrimental effects on the product characteristics (Vignolles et al., 2007). This includes enhanced lipid oxidation during storage and thus faster expiration (Granelli et al., 1996; Hardas et al., 2000; Keogh et al., 2001), reduced reconstitution behavior (Fäldt and Bergenstahl, 1996b; Millqvist-Fureby et al., 2001), and greater stickiness (Kim et al., 2005a; Nijdam and Langrish, 2006). This can cause a deteriorated product quality for the end user, as well as lower production efficiencies due to significant product losses and the requirement of additional processing steps, such as coating with lecithin. Furthermore, an enrichment of protein at the surface of particles from low-fat milk emulsions is sometimes observed. Although being less of a concern for skim and whole milk, in powders that contain particularly high proportions of native casein, the rehydration behavior can also be detrimentally affected by too much surface protein as a result of a network formation between the casein micelles (Schuck et al., 2007; Havea, 2006; Mimouni et al., 2010). It is therefore of great interest to identify the segregation mechanism that induces the surface fat formation or other component segregation processes during spray drying of milk powders. A better understanding will help to find technical solutions to establish more desirable surface compositions. Moreover, knowledge about the chemical surface formation in drying milk droplets is relevant for modeling purposes of industrial applications to predict the functional product properties and to optimize spray dryer designs.

**Table 1** Composition of bovine whole milk (Bylund, 2003)

Species	Concentration (% w/w)
Water	87.5
Lactose	4.8
Fat	3.9
Protein, total	3.4
Casein	2.71
$\alpha_{s1}$ -casein	1.04
$\alpha_{s2}$ -casein	0.27
$\beta$ -casein	1.05
$\kappa$ -casein	0.34
Whey protein	0.65
$\alpha$ -lactalbumin	0.13
$\beta$ -lactoglobulin	0.33
Miscellaneous	0.19
Fat globule membrane proteins	0.04
Minerals, vitamins	0.8

An analysis of the whole manufacturing process of milk powder has indicated that spray drying is the determining step, whereas the following fluidized bed and handling stages have a negligible influence on the surface composition (Kim et al., 2009b). As such, the studies on the surface formation of milk particles reported in literature have mostly focused on the spray drying process itself. It has been attempted to explain the component segregation by mechanisms occurring during the drying stage between droplet generation and completion of the spray drying process (segregation induced by diffusivity, surface activity, and crust formation), or prior to this during disintegration of the feed emulsion into individual droplets (segregation induced by atomization). It is still under debate which of those mechanisms is in fact the predominating driving force, with the respective studies providing either one specific segregation concept as explanation (Table 2) or a combination of two or more (Table 3).

## Chemical Surface Composition of Milk Particles

### Presence of Surface Fat and Effect of Spray Drying Conditions

In general, Tables 2 and 3 show that there is a wide agreement in the observation of a dominant presence of surface fat on convectively dried milk powder, which could not be prevented by variation of the drying parameters or spray dryer type. For milk (model) emulsions with fat contents similar to whole milk, the particles were found to be covered by an almost consistent fat layer that occupied more than 90% of the outmost surface area (Kim et al., 2003; Murrieta-Pazos et al., 2012; Foerster et al., 2016a,b). This corresponds to a fat surface overrepresentation of approximately three times in relation to the feed bulk composition. Skim milk (model) emulsions and milk protein concentrates were observed to also translate into a fat accumulation on the powder surface with 3.5%–45.9% surface fat for fat contents of 0.6%–1.5% in the bulk (Kim et al., 2009a; Nikolova et al., 2014; Kelly et al., 2015; Murrieta-

**Table 2** Summary of studies on component segregation in droplets consisting of milk protein and fat: organized by concepts provided as explanation—part 1: only one concept per study

Concept	System	Surface composition (XPS)	Analysis	Author
Diffusivity	Aqueous solution of sucrose/caseinate in agar gel matrix	20% protein overrepresentation	XPS	Meerdink and Van't Riet (1995)
Diffusivity	Industrial spray dried skim and whole milk	Overrepresentation of fat (whole milk: 98% on surface/29% total, skim milk: 18% on surface/1% total), also enrichment of protein near the surface	XPS, free fat extraction	Kim et al. (2003)
Diffusivity	Single droplet drying of whole and skim milk	Hydrophobic surface due to surface fat	Dissolution behavior	Fu et al. (2011)
Diffusivity	Spray drying of skim milk in pilot scale	Overrepresentation of fat (5.8%–7.4% on surface/0.9% total) and protein (43.0%–49.8% on surface/36.9% total), slight increase in component segregation with longer drying times	XPS	Nikolova et al. (2015a, 2014)
Surface activity	Spray drying of lactose/soybean oil/whey protein or sodium caseinate	Overrepresentation of fat for whey protein, better fat encapsulation with sodium caseinate (whey protein: 45% on surface, caseinate: 3% on surface/30% total)	XPS	Fäldt and Bergenståhl (1996a, 1995)
Surface activity	Spray drying of sucrose/whey protein or sodium caseinate	Overrepresentation of protein (up to 50%–58%/0.5%–1% total)	XPS	Adhikari et al. (2009)
Surface activity	Spray drying of phosphor-caseinate/lactose/milk fat	Overrepresentation of fat (14.5%–18.7% on surface/0.4% total)	XPS	Gaiani et al. (2006)
Surface activity/hydrophobicity	Spray drying of milk protein concentrate with various protein contents	Overrepresentation of fat (3.5%–10.2% on surface/0.6%–1.4% total) and protein (62.7%–97.2% on surface/35.4%–85.8% total)	XPS	Kelly et al. (2015)
Crust formation	Industrial spray dried skim and whole milk	Overrepresentation of fat (whole milk: 93.3% first 5 nm, 58.6% first $\mu\text{m}$ /28.4% total, skim milk: 45.9% first 5 nm, 22.8% first $\mu\text{m}$ /1.5% total) and slight overrepresentation in protein for skim milk (45.1% first 5 nm, 34.4% first $\mu\text{m}$ /38.5% total)	XPS, EDX, free fat extraction	Murrieta-Pazos et al. (2012)

EDX, energy dispersive X-ray analysis; XPS, X-ray photoelectron spectroscopic analysis

**Table 3** Summary of studies on component segregation in droplets consisting of milk protein and fat: organized by concepts provided as explanation—part 2: combinations of concepts

<i>Concept</i>	<i>System</i>	<i>Surface composition (XPS)</i>	<i>Analysis</i>	<i>Author</i>
Diffusivity, surface activity	Spray drying of lactose and fat with casein or whey protein	Overrepresentation of fat for all powders and of protein for many powders, less fat enrichment for casein instead of whey protein and for higher temperatures	XPS	Gaiani et al. (2010)
Diffusivity, surface activity	Spray drying of lactose/rapeseed oil/whey protein	Overrepresentation of fat (55%–65% on surface/30% total)	XPS, free fat extraction	Millqvist-Fureby et al. (2001)
Surface activity, crust formation	Spray drying of whole and skim milk	Overrepresentation of fat (significant) and protein (less distinct)	XPS	Nijdam and Langrish (2005, 2006)
Surface activity, crust formation	Spray drying of walnut oil and skim milk (with Tween 80 or maltodextrin)	Overrepresentation of fat (85.6%–92.5% on surface/50% total)	XPS, free fat extraction	Shamaei et al. (2016)
Diffusivity, surface activity, crust formation	Single droplet and spray drying of milk model emulsions at various fat contents and of milk protein concentrate	Overrepresentation of fat (milk emulsion with e.g., 76%–87% fat on surface/29%–42% total, milk protein concentrate: 17%–27% on surface/1.5% total)	XPS	Chew et al. (2015, 2014)
Surface activity, crust formation, atomization	Spray drying of whole and skim milk (lab and pilot scale), compared with commercial powders	Overrepresentation of fat in form of thin patches on all powders (e.g., commercially dried skim milk: on surface 16.5%/0.7% total, pilot scale: similar, laboratory scale: less surface fat)	XPS, CLSM	Fyfe et al. (2011)
Diffusivity, surface activity, crust formation, atomization	Spray drying of whey protein/sunflower oil/maltodextrin	Overrepresentation of fat (17.7%–34.1% on surface/10% total) and protein (19.8%–42.7% on surface/10% total)	XPS	Xu et al. (2013)
Diffusivity, surface activity, crust formation, atomization	Industrially spray-dried powders of skim and whole milk	Overrepresentation of fat (whole milk: >90% on surface/26% total, skim milk >18% on surface/1% total), slight protein overrepresentation for skim milk (42% on surface/35% total)	XPS	Kim et al. (2009a,b)
Diffusivity, surface activity, atomization	Spray drying of skim milk	High fat overrepresentation and lower protein overrepresentation, surface fat content already high directly after atomization	XPS, flash-freezing of atomized droplets	Wu et al. (2014)
Diffusivity, surface activity, atomization	Spray and single droplet drying of low-fat and regular-fat model milk	High fat overrepresentation and lower protein overrepresentation, surface fat content already high directly after atomization	XPS, CLSM, flash freezing of atomized droplets	Foerster et al. (2016a,b),

CLSM, confocal laser scanning microscopy; XPS, X-ray photoelectron spectroscopic analysis

Pazos et al., 2012; Fyfe et al., 2011). This corresponds to a surface overrepresentation of 6–30 times in comparison with the bulk composition. Nevertheless, in contrast to whole milk particles, it has been shown that the surface of skim milk and milk protein concentrate powders is not dominated by fat because of the low overall fat proportion. The remaining surface area of low-fat milk powders was found to primarily consist of protein (42.0%–62.7% on the very surface for 35.0%–38.5% in the bulk), indicating a slight surface enrichment in protein during drying. Independent from the overall fat content, a distinct lack in lactose

has been consistently reported for all milk powders that were subject of the studies listed in [Tables 2](#) and [3](#), with lactose being sometimes almost completely absent from the outmost surface region ([Gaiani et al., 2006](#); [Nijdam and Langrish, 2006](#)).

To some degree, within the above described range of surface compositions, the exact amount of fat and protein at the particle surface has been reported to be influenced by the drying conditions. [Fyfe et al. \(2011\)](#) studied commercial skim and whole milk powders as well as powders obtained from laboratory- and pilot-scale spray drying. Comparison of the powders showed that there was a difference in surface composition depending on the spray dryer type, albeit always featuring a distinct fat overrepresentation and, for skim milk powders, a moderate protein overrepresentation. Commercial milk powders consisted of more fat and less protein at the surface than the powders produced with laboratory and pilot spray dryers. It remained unclear if this can be ascribed to a larger particle size and higher feed solid content of the industrial drying processes or to the differences in atomization techniques applied. Some studies have indicated that the final particle surface composition will conform more with the bulk composition if the drying rate is increased, for instance by means of a higher drying temperature or a smaller droplet size as influenced by the atomization pressure ([Nikolova et al., 2015a](#); [Gaiani et al., 2010](#); [Kelly et al., 2002](#)).

Furthermore, the properties of the feed emulsion can affect the surface composition. A higher droplet viscosity and earlier crust formation due to an increased feed solid content have been reported to reduce the extent of component segregation during drying of skim milk ([Wu et al., 2014](#); [Kim et al., 2009b](#)). The fat encapsulation efficiency was improved by a stronger reduction in fat globule size during homogenization in studies conducted by [Kim et al. \(2009b\)](#) and [Millqvist-Fureby \(2003\)](#), whereas the surface composition data sets provided by [Xu et al. \(2013\)](#) and [Keogh and O'Kennedy \(1999\)](#) did not demonstrate such a statistically significant relationship for the range of droplet sizes investigated. [Gaiani et al. \(2010\)](#) and [Fäldt and Bergenståhl \(1996a\)](#) observed an influence of the kind of milk protein employed as emulsifier. The use of casein, particularly in the presence of lactose, resulted in less surface fat than the use of whey protein, presumably due to casein's greater surface activity and resistance to denaturation under heat ([Vega and Roos, 2006](#)).

### Analysis Techniques of Component Distribution

X-ray photoelectron spectroscopic (XPS) analysis, also referred to as electron spectroscopy for chemical analysis in this context, is the prevailing method to quantify the chemical surface composition of milk particles. In this sensitive surface analysis technique, the powder samples are irradiated with an X-ray beam of a well-defined energy level under high vacuum, and electrons are emitted if their binding energy is surpassed by the photon energy. Detection of the number of emitted electrons and their respective kinetic energy, which is characteristic for each chemical element, yields the sample composition and the chemical state of the elements. [Fäldt et al. \(1993\)](#) suggested a transformation of the obtained elemental particle surface concentrations, i.e., the relative amounts in carbon, nitrogen, and oxygen, into the surface percentages that are covered by lactose, protein, and fat by means of a matrix linearization based on each component's representative structural formula. This approach has been widely adapted in literature, and the surface compositions presented in [Tables 2](#) and [3](#) were determined in this manner. [Nikolova et al. \(2015b\)](#) discussed the possibility of a distortion of the XPS analysis toward higher carbon contents as a result of organic pollution inside the vacuum chamber and therefore proposed a modified approach where the linearization matrix is derived from experimental measurements of XPS spectra for lactose, protein, and lipid standard samples. Either way, it is generally accepted that component compositions derived from XPS atomic spectra should be considered as semiquantitative estimations only. In addition, it needs to be noted that XPS analysis accounts only for the first few nanometers of the sample surface (about 0–10 nm), with the exact sampling depth being difficult to quantify because of the surface curvature of the milk particles. As such, it only describes the outmost surface layer of the analyzed powders, which does not allow a conclusion about the overall amount of free surface fat, especially for high-fat milk powders.

The total amount of free surface fat, often expressed by the encapsulation efficiency of a powder sample, is frequently quantified by surface fat extraction. In this method, the mass of extracted fat is determined by measuring the sample weight before and after extraction with a nonpolar, organic solvent, such as hexane or petroleum ether. In terms of powder concentration, duration of the extraction process and intensity of the contact between powder and solvent during mixing, no standard procedure has been established yet and the techniques reported in literature vary considerably among each other, as summarized by [Vega and Roos \(2006\)](#). The amount of extracted fat further depends on the particle size and porosity ([Twomey et al., 2000](#); [Vignolles et al., 2007](#)). A significant amount of extractable fat is believed to not originate from the particle surfaces, but from inner free fat that is extracted from the inside of the particles through pores and cracks, in particular at relatively intense mixing and long exposure times to the solvent ([Buma, 1971](#); [Buchheim, 1982](#)). For this reason, the results from fat extraction of milk powder samples might most often represent the amount of free fat instead of the amount of free surface fat ([Kim et al., 2005b](#)). As a consequence, free (surface) fat extraction might provide a more suitable technique than XPS to quantify the total amount of surface fat, but is typically limited to a qualitative comparison among samples analyzed with the same extraction technique, rather than giving robust absolute values.

Other, less-frequently employed analysis methods include confocal laser scanning microscopy (CLSM) for the protein and fat distribution inside the whole particle ([Taneja et al., 2013](#)), transmission electron microscopy (TEM) for protein–lipid or protein–protein interactions ([Mckenna et al., 1999](#)), and energy dispersive X-ray (EDX) analysis of the atomic surface composition at a sampling depth of about 1  $\mu\text{m}$  ([Murrieta-Pazos et al., 2012](#)). As these analysis techniques describe the particle composition at different distances from the extreme surface, often various combinations are employed to obtain a comprehensive insight into the component distribution, such as XPS measurements together with free fat extraction and CLSM investigation ([Vignolles et al., 2009](#)) or XPS and EDX analysis ([Murrieta-Pazos et al., 2012](#)).

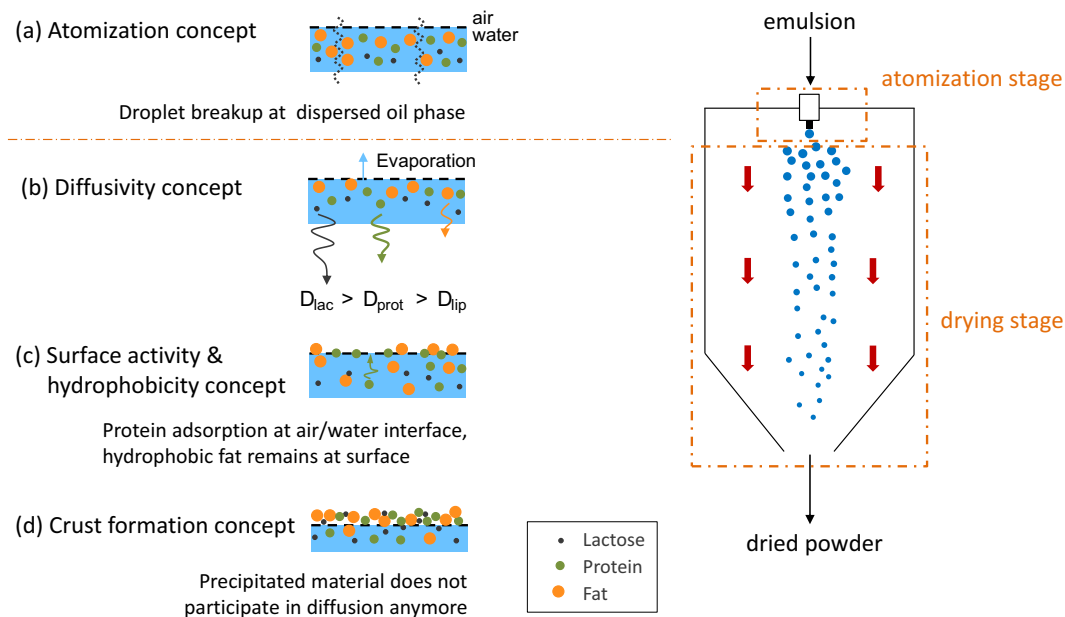
## Potential Segregation Mechanisms

### Diffusivity

As illustrated in **Fig. 1B**, Meerdink and van't Riet suggested that the component segregation could occur due to differences in the components' diffusivities (Meerdink, 1994; Meerdink and Van't Riet, 1995), a concept that has been adopted in a series of subsequent studies (Kim et al., 2003; Fu et al., 2011; Chew et al., 2014; Nikolova et al., 2015a). As water evaporates from the droplet surface, radial gradients in the component concentrations are formed and cause the components to diffuse toward the inside of the droplet. Because the diffusivity of a species increases with decreasing hydrodynamic radius, the outer droplet region might become depleted in the smaller, faster diffusing components, such as lactose. As a first approximation of the binary diffusion coefficient of a species in liquid medium, there is an inverse proportionality between the hydrodynamic radius of the species and its diffusivity according to the Stokes–Einstein equation (Cussler, 2009). In homogenized, bovine whole milk, the radius is in the range of 100–10,000 nm for lipid globules (Leman et al., 1989; Michalski et al., 2002), 50–300 nm for casein micelles (Dewan et al., 1974; Müller-Buschbaum et al., 2007), and 0.1–1 nm for lactose (Bylund, 2003). Thus, the diffusivity of lactose is two to five orders of magnitude greater than the diffusivity of the fat globules. The longer the drying process takes (for instance the lower the drying temperature or the higher the initial water content), the stronger might the diffusivity effect influence the component segregation (Kentish et al., 2005; Nikolova et al., 2015a; Vehring et al., 2007).

### Protein Surface Activity and Fat Hydrophobicity

Furthermore, the surface composition of milk powder might be influenced by the components' interaction with the air–water interface (**Fig. 1C**). Milk protein's surface activity has mostly been used as explanation of the protein surface overrepresentation observed in several studies. Milk proteins and particularly casein, due to its relatively flexible molecule structure, feature a high degree of surface activity as a result of an amphiphilic nature (Leman et al., 1989). Thus, their adsorption at the air–water interface has been widely reported, with the movement to the interface being primarily controlled by diffusion, although other factors such as convection as a result of temperature and pressure gradients can also have an influence (Graham and Phillips, 1979; Landström et al., 1999). Fäldt and Bergenstahl observed a migration of milk protein to the surface of drying emulsion droplets consisting of soybean oil, lactose, and either whey protein or sodium caseinate (Fäldt and Bergenstahl, 1996a, 1995) and explained this by the protein's surface activity. This concept has been adopted in other studies (Gaiani et al., 2006; Adhikari et al., 2009). Additionally, as the adsorbed protein is bound to the air–water interface, this impacts the gradient in protein concentration inside the droplet and counteracts any concentration driven diffusion processes away from the surface (compare to section **Diffusivity**). Similarly, the hydrophobic nature of milk fat might attenuate diffusion away from the droplet surface toward the inside of the droplet for fat globules that have already accumulated at the air/water interface (Fu et al., 2011). Furthermore, it has even been suggested that the hydrophobicity causes fat globules to migrate from the droplet center toward the surface (Kelly et al., 2015).



**Figure 1** Explanations proposed in literature for the component segregation in drying milk droplets: Schematics of (A) atomization concept, (B) diffusivity concept, (C) protein surface activity and fat hydrophobicity concept, (D) crust formation concept.



### Crust Formation Concept

It has additionally been proposed that the final particle surface composition might be subject to the crust formation and the solubility of the dissolved protein and lactose solutes (Fig. 1D), as first discussed by Charlesworth and Marshall (1960) and later adopted by others on dairy droplets (Kim et al., 2009b; Wang and Langrish, 2009). These reports argued that the precipitated solutes, once part of the solidified crust, do not participate in the diffusion process anymore. Assuming a decreasing water concentration from the droplet center to the surface due to evaporation, saturation concentrations, and consequent precipitation are first reached near the droplet surface. A lower solubility of one component could thus lead to an enrichment near the surface in comparison with the other components.

Furthermore, it has been suggested that on advanced solidification of the drying droplet the fat phase might move through the crust's network of cracks and pores as driven by overpressure imposed by internal vacuoles and capillary forces (Nijdam and Langrish, 2005). As a result of mechanical stress during drying or atomization, the fat globule membranes might become ruptured to some extent and the lipid phase could hence spread freely inside the drying particle and on its surface at the elevated temperatures of the spray drying process, thus forming a consistent fat surface film (Xu et al., 2013).

### Atomization Concept

As Tables 2 and 3 illustrate, the component segregation in drying milk droplets has primarily been presumed to take place during the drying stage of the spray drying process, with different combinations of the three concepts described above being most often presented as potential driving forces.

However, a smaller number of reports have considered the possibility that an atomization-induced segregation mechanism might take place prior to the drying stage (Fig. 1A). In a study by Fyfe et al. (2011), a pilot-scale spray dryer resulted in a considerably greater fat overrepresentation on skim milk particles' surfaces in comparison with the same powders produced with a laboratory-scale spray dryer, despite both instruments employing the same atomizer type and drying air temperature profile. It was speculated whether this observation can be ascribed to an influence of the different pressure and shear levels during atomization on the disintegration process of the feed emulsion film. Xu et al. (2013) investigated powders that were produced with a bench-top spray dryer from emulsions consisting of sunflower oil, maltodextrin, and whey protein. An increasing fat content on the particle surface with larger fat globule size of the emulsions after homogenization was found. This was explained by liquid fat leakage and consequent spreading of a thin fat film at the particle surface caused by rupture of the fat globule membranes, which become less stable with larger globule sizes. The rupture was supposed to take place either during atomization as a result of shearing or during the drying stage as a result of differential stress on the membranes as they shrink at constant fat globule volume. Kim et al. (2009b) reported respective surface fat contents of more than 90% and 18% on whole milk and skim milk particles that had been dried at a drying air inlet temperature of 205 °C. Because of the intense heat exposure and the consequent fast solidification and immobilization of the components during the drying stage, it was concluded that the surface fat must have already been formed, at least in part, during the preceding atomization stage.

As it is difficult to draw reliable conclusions about the impact of the atomization step from the powder characteristics after completed spray drying, Wu et al. (2014) and Foerster et al. (2016a) compared the surface composition of atomized droplets immediately after atomization, as obtained by cryogenic flash freezing, with the one of the corresponding spray-dried particles. Their work showed that the surface fat on the skim and whole milk particles was caused by the atomization stage for the range of emulsions and spray dryers investigated. After atomization the milk droplets featured surface fat contents of 9%–13% and 83%–92% v/v fat for feed emulsions of 0.5% and 44.2% v/v fat (dry matter basis), respectively, and the surface fat contents remained relatively unchanged throughout the following drying stage (Foerster et al., 2016a). Foerster et al. (2016a), along the argumentation of Kim et al. (2009b), proposed that the atomization-induced fat accumulation on freshly generated milk droplets might originate from the impact of the dispersed fat phase on the disintegration behavior of the emulsion feed. A perforation mechanism was provided as explanation where the milk emulsions preferably disintegrate along the fat globules, causing the fat globules to cover the surface as soon as individual droplets have been formed. This hypothesis was based on earlier photographic investigations of the break-up behavior of emulsions as a function of oil content (Dombrowski and Fraser, 1954; Zakarlan and King, 1982).

### Relevant Mathematical Modeling Approaches

Mathematical simulation of the component distribution in drying milk droplets promises further insight into the segregation mechanism and a useful tool for the theoretical prediction of powder properties at certain drying conditions. A number of modeling approaches on the convective drying of droplets are available in literature, such as models that incorporate the concept of the characteristic drying curve (Keey, 1991; Langrish and Kockel, 2001; Harvie et al., 2002; Tran et al., 2016) or the lumped reaction engineering approach (Chen and Putranto, 2013; Lin and Chen, 2005). The former approach divides the drying process into a perfect shrinkage stage (shrinkage proportional to volume reduction as governed by a constant evaporation rate) and a second stage defined by a falling evaporation rate, whereas the latter approach utilizes empirical correlations between the overall droplet moisture content and the water vapor pressure at the surface as well as the droplet diameter. Although in their simplest form these approaches only incorporate a global mass balance without computation of concentration gradients, some basic models allowing



spatiotemporal resolution of one or more species concentrations emerged from these in the 1970s and 1980s and were further refined in recent times. The majority of models discussed in the following section was developed for description of food material droplets during spray drying and was validated against the drying kinetics obtained from single droplet drying experiments. Their accuracy is usually limited by the difficulty of finding reliable formulations for the influence of the forming crust on the droplet shrinkage rate, on the effective diffusivities of water and the components due to a rising viscosity and mass transport resistance of the solidified material, and on the partial vapor pressure at the surface and thus on the evaporation rate (Kentish et al., 2005).

### Approaches With a Spatiotemporal Water Mass Balance

The earliest spatiotemporal approaches modeled water concentration profiles in drying skim milk droplets (Cheong et al., 1986; Ferrari et al., 1989; Sano and Kee, 1982; Wijnhuizen et al., 1979). An evaporation term was set as outer boundary condition and Fick's second law of diffusion was incorporated along the droplet radius for the water mass balance. The challenge to describe the effective diffusivity and evaporation rate more accurately was addressed in ensuing models. A work by Seydel et al. (2004) described the formation of hollow salt particles by simulating the radius-dependent change in particle density, which was unique in its attempt to account for component precipitation by means of a population balance that was solved independently from the mass and energy balances. Handscomb et al. (2009) formulated a model that aimed to predict shrinkage and shell thickening rates by balancing capillary contraction forces with the structural strength. The complexity of the crust properties and their effect on evaporation and shrinkage was further highlighted in a study by Vehring et al. (2007) on the drying of a protein-in-water droplet. Using the Peclet number, the model predicted the drying time until commencement of precipitation to stop the simulation at that point, because from then on a sufficient accuracy in simulating the declining evaporation rate was considered to be not feasible. Particularly, the effective diffusion coefficient of moisture through a solidified porous crust medium is difficult to predict with reasonable precision from theoretical equations (Perré, 2014).

### Approaches With Spatiotemporal Binary Mass Balances

Adhikari and coworkers have been pioneering the simulation of binary component migration in drying droplets to work toward the prediction of the surface composition (compare to Table 4), presenting a model on fruit juice samples (Adhikari et al., 2003, 2004, 2007). Lumping together the juice components into one averaged component, a pseudo-binary system was formulated. The model was based on a discretization of the spatial coordinate, which was divided into equally long increments. Each of the increments represented a spherical disk with homogeneous composition under radial symmetry assumption. Diffusive fluxes were calculated from the differences in concentration and length between the centers of adjacent disks according to Fick's first law of diffusion. The increasing evaporation resistance was incorporated via estimation of the moisture-dependent water activity in the surface disk and perfect shrinkage was assumed. The differential equations were solved with the method of lines (Iserles, 2009). After the simulated drying kinetics had been compared with suspended single droplet drying experiments for validation, the model was applied on spray drying conditions to predict the particle stickiness from the glass transition temperature.

Gopireddy and Gutheil (2013) utilized a description of the diffusion resistance imposed by the solid surface layer and water activity correlations to account for the retardation caused by solidification. Solving a set of differential diffusion and energy equations with a finite difference approach, in this way the concentration profiles of mannitol or polymer solutions during the early stages of single droplet drying were simulated.

### Approaches With Spatiotemporal Multicomponent Mass Balances

To date, only a few models can be found in literature that allow theoretical prediction of the concentration profiles of more than one component (see Table 4). A computation of the component distribution requires a spatiotemporal resolution of the mass balances, either as a set of simple ordinary differential equations by spatial discretization or as a set of partial differential equations. A global energy balance is typically sufficient under the assumption of a uniform temperature profile. This has been validated by dimensionless analysis (Biot numbers well below unity) for spray drying applications and even for significantly larger droplets in single droplet drying configurations (Chen and Peng, 2005; Patel et al., 2005). Furthermore, radial symmetry is generally assumed.

A spatiotemporal multicomponent model by Wang and coworkers was applied to resemble the spray drying process of an aqueous lactose/caseinate solution by variation of the Reynolds number and drying air temperature over time (Wang and Langrish, 2009; Wang et al., 2013). Spatial discretization of the droplet radius comparable to the above described work by Adhikari and coworkers, under the assumption of perfect shrinkage, allowed a simple incorporation of precipitation by comparison between the saturation concentration of each component and the calculated concentration in each disk at each time step. Also the protein surface activity was incorporated into the mass balance of the surface disk. Therefore, the model stands out in respect to its applicability on the component segregation during convective drying of milk droplets. The component diffusivities were expressed as temperature-dependent functions, but lacked an influence of the water concentration. The water evaporation rate was governed by an additional term in the water mass balance of the surface disk as a function of local water activity, which was estimated from the components' individual moisture-dependent water activity correlations. As available isotherms describing how the presence of solids affect the water activity have been either generated from liquid phase or fully formed solids, however, using either one to describe the entire drying process may not provide a precise description of the evaporation rate. The model was validated via

**Table 4** Modeling approaches that can be applied for prediction of the surface composition of binary or multicomponent droplets during drying: overview of included segregation mechanisms, consideration of the influence of solidification and experimental validation

Components	Incorporated segregation concepts					Influence of crust formation on			Validation	Author
	Diffusivity	Surface activity	Hydrophobicity	Precipitation	Atomization	Diffusivity	Evaporation	Shrinkage		
Lumped fruit juice solids	n/a	n/a	n/a	no	no	no	yes	no	Drying rate and temperature during SDD	<a href="#">Adhikari et al. (2007)</a>
Mannitol	n/a	n/a	n/a	yes <sup>a</sup>	no	yes <sup>a</sup>	yes <sup>a</sup>	yes <sup>a</sup>	Drying rate and temperature of pure water during SDD	<a href="#">Gopireddy and Gutheil (2013)</a>
Lactose, caseinate	yes	yes	n/a	yes	no	no	yes	no	Surface composition after SD and drying rate during SDD	<a href="#">Wang et al. (2013)</a>
Sucrose, caseinate	yes	yes	n/a	yes	no	yes	yes	no	Experiments used to fit simulation parameters	<a href="#">Meerdink and Van't Riet (1995)</a>
Sucrose, whey protein	yes	yes	n/a	no	no	no	no	no	–	<a href="#">Porowska et al. (2015)</a>
Lactose, milk protein, fat	yes	no	no	no	no	yes	no	no	Surface composition after SD	<a href="#">Chen et al. (2011)</a>
Lactose, milk protein, fat	yes	yes	no	no	yes	yes	yes	yes	Surface composition during SDD	<a href="#">Putranto et al. (2017)</a>

SD, spray drying; SDD, single droplet drying.

<sup>a</sup>only early drying stages.

comparison of the simulated surface composition with particles obtained from spray drying experiments. A surface overrepresentation of protein, which was decreasing with higher drying temperature, was predicted. From a sensitivity study it was concluded that the difference in component diffusivities and the protein surface activity were the major driving forces for component segregation, while the components' solubility imposed a subordinate influence.

Three other models were less comprehensive, but nevertheless interesting in their unique approach. Meerdink and Van't Riet (1995) described the component segregation during drying of small sucrose/sodium caseinate solution slabs. The model contained Maxwell–Stefan partial differential equations with concentration-dependent diffusion coefficients. Since measuring and approximation tools for Maxwell–Stefan multicomponent diffusion coefficients in concentrated aqueous solutions are lacking, the parameters of the diffusivity equations had to be fitted so that the simulated concentration profiles agreed with the experimental results. In the modeling work by Porowska et al. (2015) on drying whey protein/sucrose droplets, the phenomena taking place during crust formation were largely disregarded. The focus was laid on the surface activity of the whey protein, and it was supposed that the adsorption process would halt upon commencing shell formation. The protein surface activity was described with the aid of experimental determination of the dynamic surface tension as a function of protein bulk content and time. Also noteworthy, despite not being a spatially resolved model, Chen and coworkers presented an analytical continuum approach to simulate the solid formation in the outermost layer of a drying water droplet with dissolved lactose, fat and milk protein (Chen et al., 2011, 2013; Xiao and Chen, 2014). Under the assumption that the concentration only changes in a thin surface region, an explicit equation for the surface composition was obtained. Protein surface activity was not covered by this model. The Stokes–Einstein equation was introduced in an extended form to account for the concentration dependency of the effective component diffusivities.

Putranto et al. (2017) utilized the same modified Stokes–Einstein equation for a model on drying low-fat milk droplets. It accounted for the protein's surface activity as a boundary condition. Spatiotemporal balances for energy, momentum and mass of water, lactose, protein, and fat formed a set of partial differential equations that was solved with the method of lines. A realistic description of the influence of solidification on the water diffusivity, evaporation rate, and particle shrinkage was aspired by incorporation of semiempirical drying and shrinkage kinetics, which were obtained from suspended single droplet drying experiments and expressed via the reaction engineering approach. As such, the model required some experimental work to obviate the need for a perfect shrinkage assumption and for approximations of the effective water diffusivity. A new single droplet drying technique was employed for result validation, which allowed tracking of the changes in chemical surface composition over time. The experimentally and theoretically obtained surface concentrations agreed reasonably well, but better agreement might have been impeded as solute precipitation and the influence of the fat's hydrophobic nature on the component distribution had not been implemented. From the experimental study also the initial surface composition of the droplets was known, which was used as initial condition for the component mass balances of the simulation. The study highlighted the influence of the atomization stage on the component distribution in drying milk droplets. Accordingly, it was concluded that the use of experimentally observed initial surface compositions after droplet generation are critical for an accurate simulation of the component distribution in spray-dried milk particles.

## Concluding Remarks

The surface composition of milk powder imposes a critical influence on its functional properties. It is widely accepted that a strong overrepresentation of fat, in comparison with the bulk composition, emerges on the surface during spray drying at both industrial- and laboratory scale with detrimental impact on the powder quality. However, the surface formation process is far from being thoroughly understood. Four different processes might potentially affect the component segregation in drying milk droplets; the components' diffusivities, the components' interaction with the air/water interface (protein's surface activity and the lipid phase's hydrophobicity), the crust formation/precipitation process, and the atomization process. As the degree of influence of each of these mechanisms is still under debate, further experimental work is required to test and validate the different concepts. While the subordinate enrichment of protein is most often perceived to take place during drying due to its lower diffusivity than lactose and primarily due to its surface activity, the atomization stage seems to have a critical impact on the formation of surface fat. Some promising modeling approaches have been published in recent times that are, in principle, capable of simulating the component distribution of drying milk droplets. These should be further developed to account for all of the four potential segregation mechanisms for a better understanding of the surface formation and a more accurate prediction of the powder properties.

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# Impact of Antioxidants on Oxidized Proteins and Lipids in Processed Meat

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## Glossary

**Oxidative stress** Describes an imbalance between the pro-oxidant action of reactive oxygen species (ROS) and a biological system's ability (endogenous antioxidant defenses) to readily detoxify the reactive intermediates or to repair the resulting damage in molecules of biological significance (lipids, proteins, DNA, etc.).

**Antioxidants** Are molecules that inhibit the oxidation of other molecules. They may neutralize free radicals or ROS by accepting or donating electron(s), or hindering the formation of ROS by, for instance, acting at low concentrations as radical scavenger, hydrogen donor, electron donor, metal chelator and peroxide decomposer, with the resulting oxidized molecule not able to propagate the chain reaction to other biomolecules (thus terminating the chain reactions).

## Nomenclature

3-NT 3-nitrotyrosine  
4-HNE 4-hydroxy-nonenal  
APOPs advanced protein oxidation products DNA: deoxyribonucleic acid  
CAT catalase  
DLHMB DL-2-hydroxy-(4-methylthio) butanoic acid  
GPx glutathione peroxidase  
GR glutathione reductase  
GSH glutathione  
GSSG oxidized glutathione  
HMSeBA 2-hydroxy-4-methylselenobutanoic acid  
MDA malondialdehyde  
Met methionine  
NO nitric oxide  
NO<sub>2</sub><sup>-</sup> nitrite  
OH• hydroxyl radical  
PUFA polyunsaturated fatty acids  
RNS reactive nitrogen species  
ROS reactive oxygen species  
Se selenium  
SeCys selenocysteine  
SOD superoxide dismutase  
SeMet selenomethionine  
T-AOC total antioxidant capacity

## Introduction: Oxidation as a Threat to Meat Quality and Safety

The application of antioxidants in the meat industry is a common practice. Yet, the consequences of muscle lipid and protein oxidation are traceable throughout the food chain, from farm to fork (Estévez, 2015). Hence, a better understanding of the mechanisms implicated in redox reactions is required in order to put forward efficient and safe antioxidant solutions.

Redox reactions are fundamental in all biological systems as assorted functions and biochemical processes (i.e. the energetic metabolism) depend on the oxidative degradation of fuel molecules, although as a result, the formation of reactive oxygen substances (ROS) is unavoidable (Kohen and Nyska, 2002). Under physiological conditions, the balance between pro-oxidants and the endogenous antioxidant defences guarantees the homeostasis and molecules with relevant biological functions are protected against ROS. However, a disproportionate imbalance towards pro-oxidants leads to uncontrolled oxidative damage to cellular components, known as oxidative stress (Kohen and Nyska, 2002). The impact of ROS is now recognized to be as harmful



as that exerted by reactive nitrogen species (RNS) (which interact cooperatively to cause oxidation including nitrosation in muscle lipids and proteins). While oxidative stress occurs in live animals under diverse circumstances (subject to pathological or heat stress, intake of oxidized feed, etc.), a pro-oxidative environment takes place in *post-mortem* muscles with oxidative reactions being considerably enhanced upon animal slaughter owing to the collapse of the endogenous antioxidant defences (Bekhit et al., 2013). Provided with insufficient antioxidant resources such as reducing compounds (tocopherols, sulphur compounds, etc.) and antioxidant enzymes (catalase, glutathione reductase, etc.), meat lipid and proteins would then confront oxidative threats during handling, processing, storage and culinary preparation, prior to consumption (Estévez, 2015). Oxidative and nitrosative reactions are known to take place during the three digestion phases (gastric, intestinal and colonic), with the nature and intensity of these reactions depending on external (diet composition and food combination) and internal (microflora, physiological responses, etc.) factors (Van-Hecke et al., 2017).

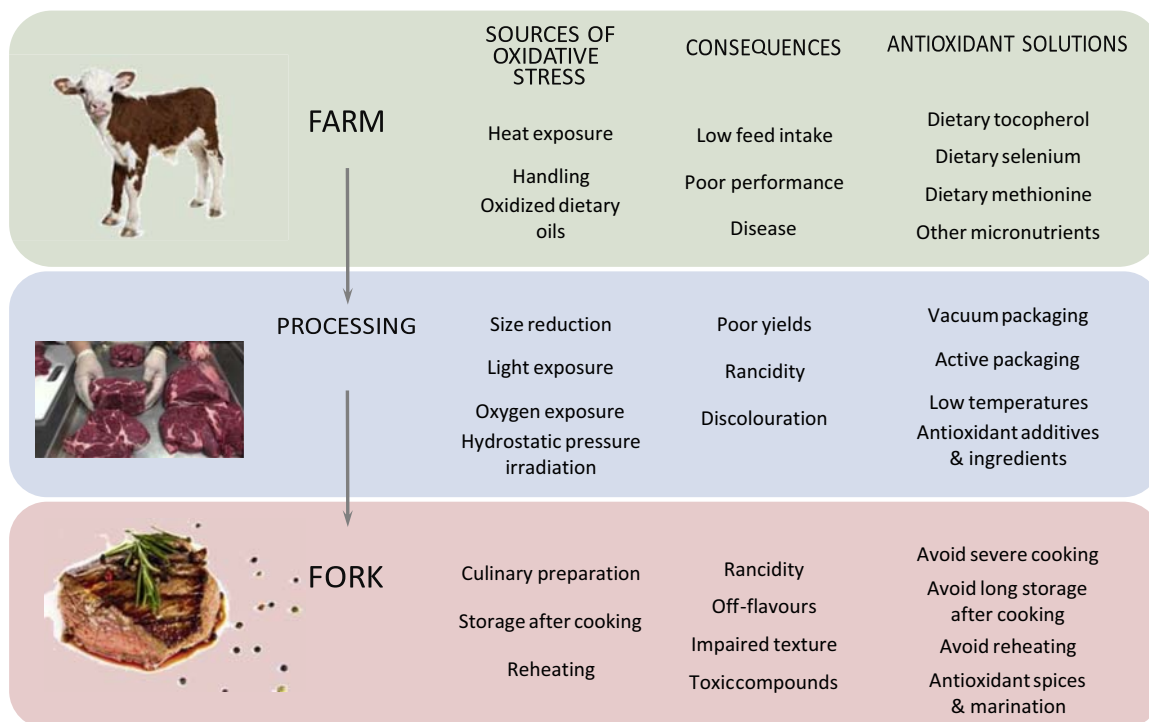
The adverse effects of meat oxidation vary with factors such as the process between farm and fork, the target (whether lipids and/or proteins are oxidized), and the severity the damage to meat components (Soladoye et al., 2015). If oxidative stress is manifested in live animals, compromised animal welfare, low production rates and poor meat quality would be possible. For meat products, oxidation-induced impairments of their sensory properties include (i) the onset of unpleasant flavours and odours (e.g. rancid, fishy), (ii) increase of hardness due to protein oxidation, and (iii) discolouration caused by co-oxidation of pigments (Bekhit et al., 2013; Soladoye et al., 2015). Oxidation of muscle proteins may also affect their functionalities causing reduced water-holding ability, fat emulsification and formation of gels (Utrera and Estévez, 2012). As a result, the nutritional value of meat is negatively impacted owing to losses of antioxidant vitamins, essential fatty acids (especially polyunsaturated fatty acids) and essential amino acids (i.e. lysine, tryptophan, etc.). Moreover, the repeated intake of oxidized lipid and protein products poses a threat to consumer's health (Estévez and Luna, 2017). Harmful species such as malondialdehyde (MDA), 4-hydroxy-nonenal (4-HNE), protein carbonyls and carboxylic acids, advanced protein oxidation products (AOPs) and dityrosines, may be formed during processing, or subsequently produced during digestion and exposure to the intestinal and luminal oxidative stress, inflammation and various other pathological conditions (Estévez and Luna, 2017; Estévez et al., 2017). Specific tryptophan and tyrosine oxidation products (kynurenines and dityrosines, respectively) have recently been identified as red or white meat discriminating metabolites in colonic digests (Rombouts et al., 2017). Recent scientific evidences support the implication of lipid oxidation products and N-nitroso-compounds in the formation of DNA adducts upon the consumption of processed red meat (Hemeryck and Vanhaecke, 2016). Besides their local impacts in the gut, certain low-molecular weight oxidation products may be absorbed and distributed through the bloodstream to impair the biological functions of specific organs such as the liver, kidney, pancreas and brain, as indicated by some animal studies (Estévez et al., 2017).

### Animal Handling and Feed Manipulation

The oxidative stress suffered by animals is a significant cause of the onset/development of assorted pathological conditions that eventually affect animal welfare and production rates (Fellenberg and Speisky, 2006). Likewise, many infections, physiological and metabolic disorders cause impaired redox status and then oxidative stress (Lykkesfeldt and Svendsen, 2007). Lykkesfeldt and Svendsen (2007) indicated that, at least, theoretically, oxidative stress can easily be prevented through the use of antioxidants although such a therapy remains controversial. From a technological perspective, the impact of oxidative stress on meat quality (Estévez, 2015) implies the necessity of applying antioxidant solutions at the farm to support the improvement of production rates and animal welfare, especially the animals such as pigs and domestic birds that are frequently exposed to oxidized feeds and heat (Zhang et al., 2011).

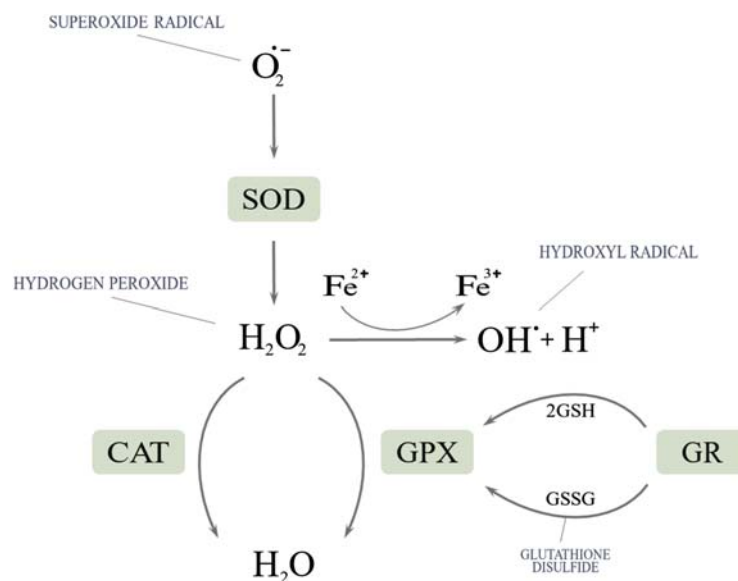
Further, the antioxidant status of livestock can be optimized by diet, and through which the oxidative stability and quality of corresponding animal meat would be improved. In practice, an efficient antioxidant strategy can be built on two essential pillars: i) monitor fatty acid composition, avoid high PUFA rates and oxidized feeds, and ii) supplement with  $\alpha$ -tocopherol, organic selenium, sulphur amino acids and arguably, ascorbate, phytochemicals and other microelements (zinc, copper, magnesium, etc.). Interestingly, dietary antioxidants have been found to alleviate negative influence of external oxidative stress on pigs and broilers (e.g. those heat-induced) (Ismail et al., 2013). The supplementation with  $\alpha$ -tocopherol (100–200 mg tocopherol/kg feed), a key component of endogenous antioxidant defences of skeletal muscle and a main contributor to the oxidative stability of biological membranes, is a common practice to improve chicken, pork and beef quality (Chan and Decker, 1996; Sales and Koukolová, 2014). The positive effects of  $\alpha$ -tocopherol supplementation have been profusely documented, including the promotion of higher oxidative stability in fresh meat during storage (Buckley et al., 1995), protection against discoloration (Sales and Koukolová, 2014), and reduction of the oxidation of PUFAs, cholesterol and proteins in processed meat products (Gobert et al., 2010; Botoglou et al., 2012). It is worth mentioning that low levels of oxidation are usually associated with reduced rancidity, improved sensory properties and consumer acceptability (Sohaib et al., 2016; Albertí et al., 2017). These positive effects have been achieved through the supplementation with  $\alpha$ -tocopherol acetate or via feeding animals with natural materials naturally rich in tocopherols such as pasture (Descalzo and Sancho, 2008), acorns (Ventanas et al., 2007), chestnuts (Pugliese et al., 2009) and olive leaf (Botoglou et al., 2012) (Fig. 1).

Dietary selenium (Se) supplementation has also been found to increase the oxidative stability of meat plausibly by promoting the concentration of glutathione peroxidase (GPx) in muscle. GPx is known to work cooperatively with catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD) and glutathione (GSH) to neutralize radical and non-radical pro-oxidant species in



**Figure 1** Sources and consequences of oxidative stress as well as potential antioxidant solutions at three key stages of the meat production and supply chain.

meat systems (Fig. 2). Se may be supplemented as inorganic Se (mainly as selenite or selenate) or organic Se (in the form of selenomethionine, SeMet). The latter may be used in the form of Se-yeast or SeMet preparations (Surai and Finislin, 2016). SeMet, a naturally occurring amino acid, is widely recognized to be more efficient than inorganic Se in terms of prompting bioavailability and antioxidant protection (Surai and Finislin, 2016). SeMet is converted, via *in vivo* transsulfuration, to selenocysteine (SeCys), and then to selenide that is critical for the synthesis of selenoproteins such as GPx. A hydroxyl analog of SeMet, 2-hydroxy-4-methylselenobutanoic acid (HMSeBA) has been recently proposed to be a highly efficient and bioavailable form of SeMet (Jlali et al., 2014). The benefits of supplementation of organic Se to the quality of poultry meat and chicken products are well documented (as reviewed by recently Estévez, 2015), although the effects of such a supplementation on mammals remain unclear.



**Figure 2** Cooperation of glutathione peroxidase (GPx) with catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD) and glutathione (GSH) to neutralize radical and non-radical pro-oxidant species in meat systems.

Recent studies on pork yielded contradictory results: some revealed certain positive effects of Se supplementation on oxidative stability, color and water holding capacity (Li et al., 2011; Calvo et al., 2016; Batorska et al., 2017), whilst others did not find any beneficial effect (Lisiak et al., 2014) or even reported some adverse effects at doses of 0.5 mg inorganic selenium/kg feed (Schwarz et al., 2017). Scarce studies on beef quality agree on the protective effect of Se supplementation on myoglobin oxidation and beef color stability (O'Grady et al., 2001). Give the location of selenoproteins such as GPx in the cytoplasm, the effective protection of selenoproteins on myoglobin and other sarcoplasmatic and myofibrillar proteins against ROS seems a more plausible hypothesis than that related to other endogenous antioxidant defences such as tocopherols. In addition to the supplementation with SeMet, adjusting the concentrations of sulphur amino acids in livestock feeds using DL-Met or its hydroxyl equivalent (DL-2-hydroxy-(4-methylthio) butanoic acid, DLHMB) is also recommended as an alternative effective approach for antioxidant protection. Supplementing DL-Met above recommendations by the National Research Council (NRC) (>0.38%) would lead to high antioxidant protection of broiler and pig muscles thus improving meat quality traits such as color and cooking losses (Castellano et al., 2015; Zhai et al., 2016).

Phytochemicals are used in animal nutrition, which aligns with in the global trend against antibiotic growth promoters and towards natural products for animal welfare and production. Phytochemicals refer to assorted materials from the plant kingdom (e.g. spices, herbs, fruits, nuts, fresh leaves, essential oils, plant extracts, etc.). They are natural sources of phytochemicals (phenolic acids, polyphenols, terpenes, vitamins, etc.) and have displayed abilities to improve the growth rate, nutrient digestibility and gut health of livestock. Providing farm animals with this '5-vegg-a-day' Mediterranean-like diet is proven to display immunomodulation and antimicrobial activities, technological benefits at the farm (gas production), and even impact sensory properties on animal products (e.g. astaxanthin on meat color) (Estévez, 2015). Hong et al. (2012) reported that feeding 125 ppm of oregano essential oil, anise and citrus peel powder leads to more tender breast muscles and juicier thigh muscles compared to the control group. Supuka et al. (2015) used the agrimony extract (*Agrimonia eupatoria* L., 0.2%) and its combination with a sage extract (*A. eupatoria* L. + *Salvia officinalis* L., 0.1% + 0.1%) for water supplementation during the fattening period of broiler chickens, to lower ( $P < 0.05$ ) SOD activity in blood and the amount of thiobarbituric acid reactive substances in thigh meat during 8-day chilling storage. Cherian et al. (2013) significantly enhanced muscle lipid stability in broilers fed with 2% and 4% sweet wormwood (*Artemisia annua* L.). Sun et al. (2014) found that lycopene supplementation (20, 40, and 80 mg/kg) significantly increased fertilization rates in hens with increased SOD, total antioxidant capacity, and reduced glutathione to oxidized glutathione ratio GSH/GSSG in serum and liver and significantly decreased total cholesterol and increased high density lipoprotein cholesterol and triiodothyroxine.

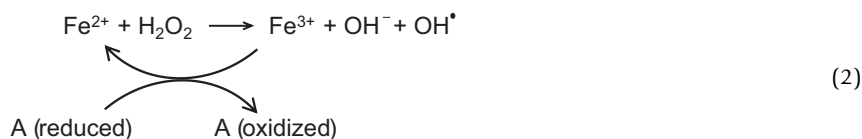
### Food Formulation: Classic Antioxidant Additives

After slaughter and upon the resolution of the *rigor mortis*, the biochemical environment in *post-mortem* meat is genuinely full of pro-oxidants owing to the collapse of physiologically controlled cell functions causing formation of ROS, release of hydrolases, and occurrence of myoglobin, non-heme iron and other promoters of oxidative reactions (Estévez, 2011). The reactions between such reactive and susceptible species (e.g. PUFA, cholesterol, certain amino acid residues in sarcoplasmatic/myofibrillar proteins and vitamins), are facilitated during the operations intended to transform raw meat into processed muscle foods (Soladoye et al., 2015). Undesirable factors like light exposure, oxygen exposure, irradiation, size reduction and heat treatment would expedite the oxidation of muscle lipids and proteins. In this scenario, only limited amounts of endogenous antioxidant defenses are available in the meat system to counteract the oxidative threat, namely, membrane tocopherols, enzymes (like SOD, CAT and GPx), thiol-based antioxidant molecules (glutathione, taurine, isotaurine, etc.) and other water-soluble molecules with antioxidant properties such as ascorbate, carotenoids, etc. Therefore, the use of antioxidant additives during meat processing becomes a common practice to improve antioxidant protection.

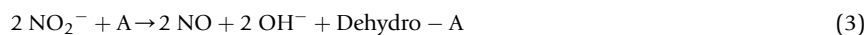
Non-meat ingredients and additives such as nitrite, ascorbate, phosphates, non-muscle proteins and peptides, and assorted seasonings have been profusely reviewed (Honikel, 2008; Weiss et al., 2010; Varvara et al., 2016; Xiong, 2017). It is worth mentioning that some of the chemical additives typically used in the formulation of meat products are redox-active compounds, thus, their involvement in oxidative reactions is possible. The antioxidant action of nitrite may be based on a number of mechanisms including its combination with molecular oxygen and metal chelation (Honikel, 2008). However, nitrite ( $\text{NO}_2^-$ ), displays pro-oxidant actions owing to its ability to abstract an electron from a sensitive molecule and hence, initiate oxidative reactions [Reaction 1].



Ascorbate (A) is known as an efficient scavenger of radical species (Buettner and Jurkiewicz, 1996), and also exhibits pro-oxidant properties by reducing transition metals such as iron and perpetuating the formation of hydroxyl radicals ( $\text{OH}^\bullet$ ) through the Fenton reaction [Reaction 2].



Nevertheless, the combination of nitrite and ascorbate additives usually leads to an overall positive outcome as the formation of reaction products such as nitric oxide (NO) [Reaction 3] and eventually lipid radicals (LOO•) scavenging, through which lipid oxidation is inhibited [Reaction 4].



Although the abilities of nitrite and ascorbate to control lipid oxidation are previously recognized, recent studies have re-examined their effects on protein oxidation during meat processing. Villaverde et al. (2014a) reported that nitrite alone had a negligible effect on protein oxidation but induced the formation of a specific marker of nitrosative stress, namely 3-nitrotyrosine (3-NT), and that ascorbate was efficient in inhibiting the formation of 3NT via a dose-dependent anti-nitrosative effect to enable the antioxidant action of nitrite. The same authors found pro-oxidant actions of nitrite on meat proteins during sausage fermentation and abilities of ascorbate to counteract the oxidative and nitrosative damage of nitroso compounds on meat proteins (Villaverde et al., 2014b). Berardo et al. (2016) reached a similar conclusion after studying the effect of nitrite and ascorbate on dry-fermented sausages. Further, the antioxidant effects of phosphates have been reported, which were linked to their chelating abilities, inhibition of pro-oxidant action of transition metals, and controlling lipid oxidation and warmed-over flavours (Cheng and Ockerman, 2007).

### Food Formulation: Novel Antioxidant Alternatives

Currently, there are increasing consumer interest in the so-called 'natural' ingredients, especially plant materials, their extracts, essential oils or phytochemicals as natural antioxidant alternatives to conventional additives in meat products (Kumar et al., 2015; Fernandes et al., 2016). Ben Slima et al. (2017) recently proposed the partial replacement of nitrite curing agent (i.e. down to 50 mg/kg) with probiotic *Lactobacillus plantarum* TN8 in beef sausages, leading to decreased pathogenic bacteria counts, low oxidation rates and human health hazards. Vossen et al. (2012) used dog rose as a functional ingredient in frankfurters (without adding ascorbate and nitrite), and found a positive antioxidant effect on lipids and proteins. Fermented rooibos (*Aspalathus linearis*) could retard lipid oxidation in nitrite-free ostrich salami Cullere et al. (2013). A 2% grape pomace improved the oxidative and color stability of nitrite-reduced cooked beef sausages (Riazi et al., 2016), and acid whey and mustard seed suppressed lipid oxidation of nitrite-free organic fermented sausages (Karwowska et al., 2015). It is worth noting that some of the currently used approaches are not reducing the overall concentration of nitrate/nitrite in the product but replacing nitrate/nitrite with certain vegetable sources naturally containing nitrate/nitrite (Ko et al., 2017). Caution must be taken in using certain plant phenolics alone or in combination with nitrite/ascorbate, as unexpected pro-oxidant effect may appear. Lin et al. (2017) observed an acceleration of lipolysis and lipid oxidation in pepperoni sausages treated with green tea extract (0.05%) and sodium nitrite (0.009%).

Additional antioxidant protection is increasingly demanded for ready-to-eat muscle foods. Extensive technological processing applied to these products e.g. pre-heating and cold storage-reheating would considerably promote oxidative reactions, leading to final products with elevated amounts of potentially harmful compounds (MDA, protein carbonyls, oxidized cholesterol, etc.) and impaired sensory properties (Utrera et al., 2012; Feng and Ahn, 2016; Akcan et al., 2017a; Ferreira et al., 2017). In these products, addition of antioxidant additives/ingredients of natural origin (plant materials, spices, essential oils, fruits, etc.) is preferred with/without the need of additional packaging strategy for oxygen exclusion. However, Ferreira et al. (2017) observed that 200 mg/kg gallic acid equivalents of acorn (*Quercus ilex* L.) extract was effective in inhibiting basal lipid and protein oxidation in ready-to-eat chicken patties independently of the packaging system (normal vs. reduced oxygen level). Hawberry (*Crataegus monogyna* Jacq.) extracts (200 and 800 gallic acid equivalents) were also found efficient against lipid oxidation in ready-to-eat pork patties (Akcan et al., 2017b). Moreover, non-muscle proteins and hydrolysates have been introduced to provide antioxidant protection as well as additional technological, sensory and nutritional benefits to processed meat products (Lafarga and Hayes, 2017; Sohaib et al., 2017). Plasma and dairy protein hydrolysates have been found efficient in enhancing the oxidative stability and other quality properties of processed meat products (Seo et al., 2016; Kumar et al., 2016). Bacteriocin BacTN635 exhibited protective effects against lipid and protein oxidation in refrigerated turkey sausages (Smaoui et al., 2017).

It is worthy to stress the scientific and technological interest in using muscle foods as vehicles to deliver dietary antioxidant compounds to humans. Antioxidants can be used from farm (the livestock) to the fork (final consumer) including the intermediate stages of food processing and storage. Such an antioxidant approach may not be limited to antioxidant properties but other health benefits associated with these bioactive compounds such as antimicrobial (phytochemicals), antihypertensive (peptides), odorants (terpenes from essential oils), anti-inflammatory, etc. However, the effectiveness of plant-derived antioxidants as well as their safety (despite the fact of having a 'natural' origin) need to be thoroughly examined before being used in meat processing plants. Although the antioxidant benefits of the assorted plant materials and derived antioxidants are reported in the scientific literature (reviewed by Karre et al., 2013; Falowo et al., 2014; Shah et al., 2014), the adverse (pro-oxidant) effects of phytochemicals on foods and human health have also been documented (Halliwell, 1994; Cando et al., 2014; Van-Hecke et al., 2016).

The dose and means of delivery seem critical for ensuring the effectiveness and safety of plant-derived antioxidants. These antioxidants comprise phenolic acids (hydroxybenzoic and hydroxycinnamic), phenolic diterpenes (i.e. rosmanol, carnosol, carnosic acid), flavonoids (flavones, flavonols, flavanols and flavanones), condensed tannins (i.e. proanthocyanidins) and volatile terpenes (i.e. thymol, carvacrol) (Brewer, 2011). They are sourced from plumb, grapeseed, cranberry, pomegranate, bearberry, pine bark, oregano and rosemary (Karre et al., 2013; Riaz et al., 2016; Ghaderi-Ghahfarokhi et al., 2016; Fernandes et al., 2017). Table 1 summarises some recent and significant applications of antioxidants including those from plant kingdom in processed meat products. Both antioxidant and pro-oxidant actions have been reported (Utrera and Estévez, 2013; Cao et al., 2016). It is worth noting that certain compounds are able to prevent lipid oxidation but may not be effective in inhibiting protein oxidation (Estévez, 2011). The impact of plant phenolics on the oxidative stability of meat proteins is determined by the interactions between the active moieties of phytochemicals (hydroxyl or quinone groups) and the amino acid residues. These interactions are dependent on the amount and chemical state of the phenolic compound as well as the size, conformity, overall charge and oxidative status of the protein (Estévez, 2011).

### Innovative Means of Antioxidant incorporation in Processed Meat Products

While direct addition of antioxidant preparations in the form of a powder or extract to comminuted meat products is simple and feasible (Fernandes et al., 2018), marination, spraying and injection with antioxidants have also been tested for whole meat products (Rimini et al., 2014). Bioactive compounds and antioxidant additives have been incorporated in microcapsules (Sickler et al., 2013), edible films (Akcan et al., 2017a), polyethylene terephthalate (PET) trays (Contini et al., 2014) and chitosan nanoparticles (Hu et al., 2015). Regarding marination, Rimini et al. (2014) evaluated the effects of a mixture of thyme and orange oils (EO) on the quality characteristics and oxidative stability of chicken meat (breast and wing). These authors noticed that both fresh and frozen EO breasts and EO wings were less susceptible to lipid oxidation compared to the control breasts and wings. Incorporation of cinnamon oil into chitosan nanoparticles that are included in low density polyethylene films could retard lipid oxidation in pork meat, indicating the synergistic antioxidant effects of cinnamon oil and chitosan (Hu et al., 2015). Regarding the antioxidant-releasing packaging materials, one of the main benefits, as compared to the direct addition of antioxidants to meat products, is that active materials may act as a source of antioxidants to be released to the meat products at controlled rates during storage (Mastromatteo et al., 2010). The antioxidant films containing 3% eugenol improved the efficacy of vacuum packaging and stabilized raw beef patties against oxidation (Park et al., 2012). Contini et al. (2014) also showed that antioxidant active packaging with citrus extract was effective in reducing the lipid oxidation of cooked turkey meat during storage along with maintaining its sensory characteristics (particularly tenderness) and overall acceptability. Akcan et al. (2017a) reported that the lipid oxidation in cooked meatballs during frozen storage at  $-18^{\circ}\text{C}$  for 60 days was effectively retarded by the incorporation of whey protein isolate-based edible films containing natural antioxidant extracts from laurel (*Laurus nobilis* L.) or sage (*S. officinalis*) into the edible films. Baldin et al. (2016) assessed the addition of microencapsulated jabuticaba extract (as the natural antioxidant source) to fresh sausage and noticed that the extract at 2% and 4% led to reduced lipid oxidation of fresh pork sausages during 15 days of storage.

**Table 1** Recent and significant applications of alternative antioxidant sources including those from plant kingdom in processed meat products

Antioxidant source	Dose	Application method	Meat system	References
Rosemary extract	150 ppm carnosol/kg fat	Direct addition	Frozen chicken nuggets	Rocío Teruel et al. (2015)
Lactic acid bacteria isolate	$10^7$ cfu/kg	Inoculation	Experimental sausage	Chen et al. (2015)
Thyme	2%	Edible films	Cooked cured ham	Ruiz-Navajas et al. (2015)
Mustard seed	1%	Direct addition	Nitrite-free fermented sausage	Karwowska et al. (2015)
<i>Myrciaria cauliflora</i>	2%–4%	Microencapsulation	Pork sausage	Baldin et al. (2016)
Thyme essential oil	0.1%	Nanoencapsulation	Beef burgers	Ghaderi-Ghahfarokhi et al. (2016)
Grape pomace	2%	Direct addition	Nitrite-reduced meat emulsions	Riaz et al. (2016)
Blackcurrant extract	1%	Direct addition	Vacuum packed sausages	Nowak et al. (2016)
<i>Rosa canina</i> L.	500 mg GAE/100 g	Brine injection	Boiled ham	Armenteros et al. (2016)
Camel milk casein hydrolysate	0.03%–0.09%	Direct addition	Goat meat emulsion	Kumar et al. (2016)
Chestnut leaves extract	2000 ppm	Microencapsulation	Dry-cured sausage	Munekata et al. (2017)
<i>Laurus nobilis</i> & <i>Salvia officinalis</i>	2%–4%	Edible films	Cooked meatballs	Akcan et al. (2017a)
Hawberry	200–800 ppm GAE	Direct addition	Ready-to-eat patties	Akcan et al. (2017b)
Cinnamon essential oil	0.1%	Nanoencapsulation	Beef burgers	Ghaderi-Ghahfarokhi et al. (2017)
<i>Lactobacillus plantarum</i> TN8	7 log CFU/g	Inoculation	Nitrite-reduced beef sausages	Ben Slima et al. (2017)
Acorn extract	200 ppm GAE	Direct addition	Ready-to-eat patties	Ferreira et al. (2017)
Bacteriocin BacTN635	500–1000 AU/g	Direct addition	Refrigerated turkey meat sausage	Smaoui et al. (2017)
Borage extract	0.5%–10%	Sprayed	Lamb leg chops	Bellés et al. (2017)
Lotus rhizome powder	1%–3%	Direct addition	Cooked pork sausages	Ham et al. (2017)
Tea	1%–3%	Biocomposite film	Minced beef meat	Ashrafi et al., (2016)
<i>Origanum vulgare</i>	5–8 mg/kg	Direct addition	Sheep sausage	Fernandes et al. (2018)



Ghanderi-Ghanhfarokhi et al. (2017) also observed that encapsulated cinnamon essential oil effectively retarded lipid oxidation and improved the consumer acceptability of beef patties.

## Conclusions

Antioxidant solutions are commonly applied to control lipid and protein oxidation at every stage from farm to fork. While some antioxidant strategies are well-established such as the use of dietary tocopherols and formulation with antioxidant additives, novel solutions to the oxidation of proteins and lipids in processed meat are requested by consumers who demand safer and effective strategies. Accordingly, more integrative antioxidant solutions should aim to protect the livestock, the muscle food and the consumer, to minimize the negative consequences on human health of intake of oxidation products. The combination of phytochemical antioxidants with novel packaging strategies would guarantee the quality and safety of processed meat products.

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# Plant Antioxidant Extracts: Effect on Lipid or Protein Oxidation in Seafood Products

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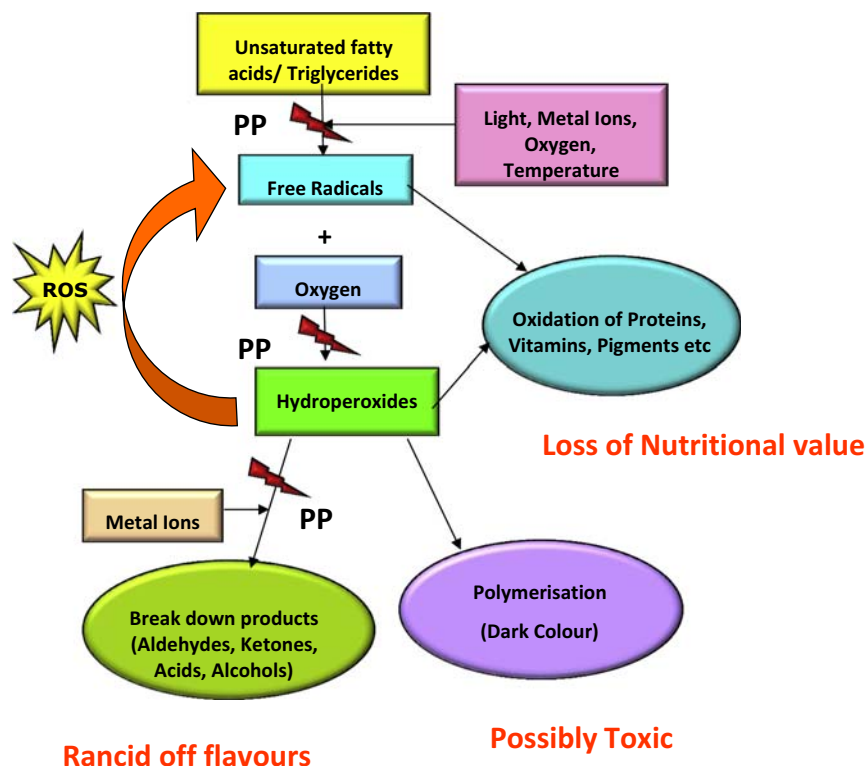
## Introduction

Seafood is recognized as a healthy component of human diet due to its high eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contents (Wang et al., 2006). A number of health benefits have been associated with EPA/DHA intake and fish consumption such as reduced risk of coronary heart disease (CHD), certain cancers, rheumatoid arthritis and depression, as well as improved visual and cognitive development (Ruxton et al., 2004). However, fatty fish is extremely susceptible to oxidation due to the presence of polyunsaturated fatty acids. Lipid oxidation will lead to the development of unpleasant odor, rancid taste and discoloration (Frankel, 2005). Secondary oxidation products resulted from lipid oxidation can further initiate reactions to modify proteins, amino acids and vitamins causing loss of protein functionality and nutritional quality (Soladoye et al., 2015). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butyl hydroquinone (TBHQ) have been widely used in the 19th century to reduce/prevent quality loss due to lipid oxidation in seafood (Boyd et al., 1993). But the use of BHA and BHT in foods was later found harmful and have been restricted because of their carcinogenesis potential (Saito et al., 2003). In addition to carcinogenicity, BHA and BHT have been reported to be cytotoxic (Saito et al., 2003) and have adverse effects on the kidney, liver as well as lung tissues (Siman and Eriksson, 1996). Hence, the use of antioxidants from natural sources such as plant phenolics is encouraged.

Plant phenolics are secondary metabolites containing one or more phenolic ring, which are produced by plants as a part of defense response to ultraviolet radiation or aggression by pathogens (Beckman, 2000; Parr and Blowell, 2000). Studies showed that long-term consumption of diets rich in plant polyphenols could allow protection against some cancers, diabetes, cardiovascular diseases, neurodegenerative diseases and osteoporosis (Arts and Hollman, 2005). Plant phenolics include phenolic acids, flavonoids, lignans, stilbenes and tannins (Arts and Hollman, 2005). Plant phenols are good antioxidants, and their modes of antioxidative action depend on the chemical structure of individual phenolic compounds (Bendary et al., 2013). The mechanisms by which phenolic antioxidants retard lipid oxidation include (1) scavenging reactive oxygen species that initiate peroxidation, (2) quenching  $\cdot\text{O}_2^-$  and preventing the formation of peroxides, and (3) preventing the generation of the reactive oxygen molecule and decomposition of lipid peroxides by metal chelation (Fig. 1). Various factors are reported to affect the phenolic composition and also the antioxidant activity of plant extracts such as genotype, climatic conditions, growing environment, processing, post-harvest storage and solvent used for phenolic extraction (Howard et al., 2003; Kevers et al., 2011). Many purified phenolic compounds and plant phenolic extracts have demonstrated their abilities to retard oxidation of lipids and proteins in different kinds of seafood. This chapter reviews in detail plant phenolic extracts and their roles in retarding lipid or protein oxidation in seafood products.

## Lipid and Protein Oxidation in Seafood

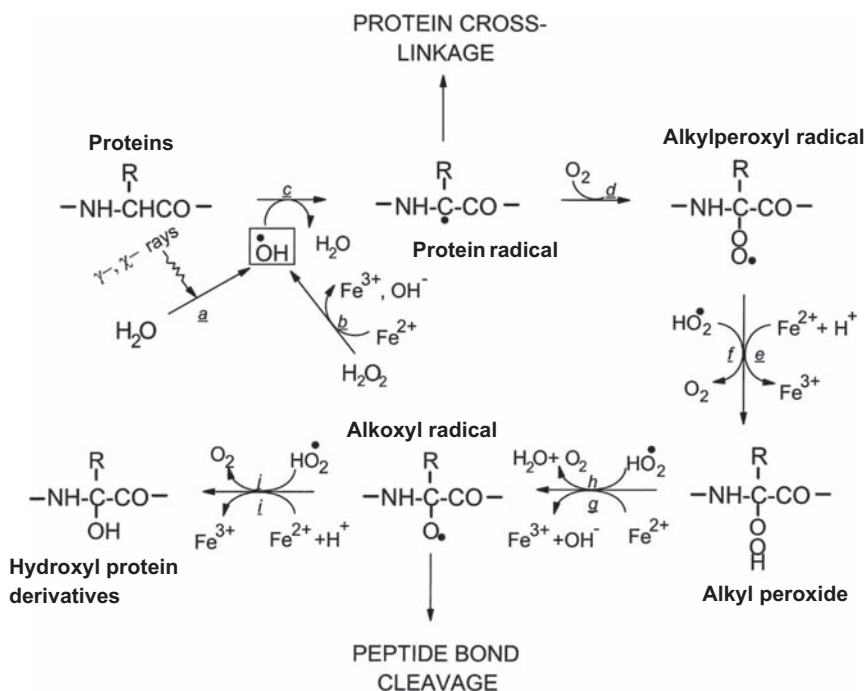
Lipids and proteins fractions in fish meat are easily prone to oxidative deterioration due to rapid depletion of endogenous antioxidants after the death of fish especially during post-mortem handling and storage (Jacobsen et al., 2008). The mechanism of lipid oxidation involves three stages: initiation, propagation and termination (Frankel, 1985). During initiation, lipid or alkyl radicals ( $\text{L}\cdot$ ) are formed in the presence of catalysts (such as oxygen, light, heat and metal ions) and irradiation. These radicals then react with oxygen to form peroxy radicals ( $\text{LOO}\cdot$ ), which abstract hydrogen atom from other lipids to form hydroperoxides leading to the generation of a new lipid radical ( $\text{L}\cdot$ ) and the cycle continues. During the termination step, the interaction of alkyl and peroxy radicals leads to the formation of non-radical products such as aldehydes, alkanes and conjugated dienes (Frankel, 1985). The rate and extent of lipid oxidation is influenced by factors such as fatty acid composition, concentrations of pro-oxidants and antioxidant(s), endogenous ferrous iron, enzymes, pH, temperature and oxygen available (Underland, 2001). Iron and oxygen play influencing roles in the lipid oxidation of fish muscle. Iron in fish muscle may occur in associated forms (e.g. heme pigments, non-heme iron complexed to proteins (ferritin) or as low molecular weight metabolites (Richards and Hultin, 2002). Reducing compounds occurring in the seafood tissue such as ascorbate can convert the inactive ferric iron to the active ferrous iron. There are also enzyme systems that use reducing equivalents from NAD(P)H to reduce ferric iron. Iron is also part of the active site of specific enzymes like lipoxygenase (which may participate in lipid oxidation) (Banerjee, 2006). Thus, the iron derived from the above-mentioned sources can activate molecular oxygen and promote lipid oxidation (Richards and Hultin, 2002). Reducing the pH also enhance lipid oxidation in fish muscle, due to increased autooxidation of Hb at a decreased pH and subsequent release of iron (Tsuruga et al., 1998).



**Figure 1** Schematic Diagram of Lipid Oxidation and the Antioxidant Mechanisms of Plant Phenolics (PP).

Protein oxidation is defined as covalent modification of proteins either by reactive oxygen species (ROS) or via the interaction with secondary lipid oxidation products (Shacter, 2000). Oxidation of proteins is believed to proceed via a free radical chain reaction similar to that of lipid oxidation, although more complex pathways and a wider range of oxidation products may be involved in lipid oxidation (Davies and Dean, 2003). Protein oxidation begins with the abstraction of hydrogen atoms from a protein (PH) via ROS to form a protein carbon-centered radical ( $P^{\bullet}$ ). This protein radical, in the presence of oxygen, is converted into an alkylperoxyl radical ( $POO^{\bullet}$ ) and ultimately to an alkylperoxide ( $POOH$ ) by obtaining a hydrogen atom from another susceptible molecule (Fig. 2). Subsequently, alkoxy radical ( $PO^{\bullet}$ ) and its hydroxyl derivative ( $POH$ ) are produced when they react with ROS (such as  $HO_2^{\bullet}$ ) or reduced forms of transition metals ( $Fe^{2+}$  or  $Cu^{+1}$ ). Finally, carbonyl groups and acyl radicals are formed when these alkoxy residues undergo  $\beta$ -scission or fragmentation reaction via cleavage of peptide bonds (Lund et al., 2011). Moreover, protein oxidation also occurs when lipid oxidation products (such as hydroperoxides, aldehydes, reducing sugars and ketone) interact with nitrogen or sulfur centers of reactive amino acid residues of protein (Viljanen, 2005) (Fig. 3). Protein fragmentation can occur as a result of a free radical attack of glutamyl, aspartyl and prolyl side chains (Hawkins and Davies, 2001). Oxidative modifications of proteins and amino acids can lead to the formation of cross-linked protein derivatives, alteration of side chains amino acids and oxidative cleavage of peptide chains, and as a result, cause changes in protein functionalities (e.g. solubility, water holding capacity, viscosity, and emulsification) and muscle quality (Xiong, 2000).

A number of simplified model systems are available for studying the efficacy of antioxidant molecules. The most common model systems used to study the antioxidant activity of plant phenolics or extract against lipid/protein oxidation in seafood products are bulk fish oil, fish oil-in-water emulsions, and fish muscle systems. Bulk fish oils are homogeneous systems in which the affinities of antioxidants towards the air-oil interfaces determine their effectiveness against lipid oxidation with polar antioxidants being especially efficient in this system (Frankel, 1998). Emulsions may involve oil-in-water emulsions or water-in-oil emulsions. The nonpolar antioxidants are more efficient than polar antioxidants in oil-in-water emulsion because they locate more efficiently at the oil-water interface (Cuvelier et al., 2000). The behaviors of phenolic antioxidants in emulsions are influenced by several factors including pH, emulsifier, and the interaction of phenolic antioxidants with emulsifier and iron (Sorensen et al., 2008). Fish muscle or fish mince is a complex system containing lipids, proteins, antioxidants (e.g. endogenous  $\alpha$ -tocopherol and ascorbic acid) and pro-oxidants (heme proteins such as hemoglobin and myoglobin, free and bound iron). The lipid oxidation in fish muscle/mince is complex and various factors such as fatty acid content, concentrations of pro-oxidants and antioxidants, and harvest, post-harvest and processing conditions, all influence the oxidation process (Underland, 2001).



**Figure 2** Radical Mediated Protein Oxidation Mechanism. Adopted from Berlett, B.S., Stadtman, E.R., 1997. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* 272, 20313–20316.

## Plant Extracts in the Prevention of Lipids and Protein Oxidation in Seafood Products

### Extracts From Herbs

Herbs have historically been used in cooking, and can not only enhance flavor but also impart health-promoting properties including antioxidant properties (Harborne and Williams, 2000). They are rich in phenolic compounds.

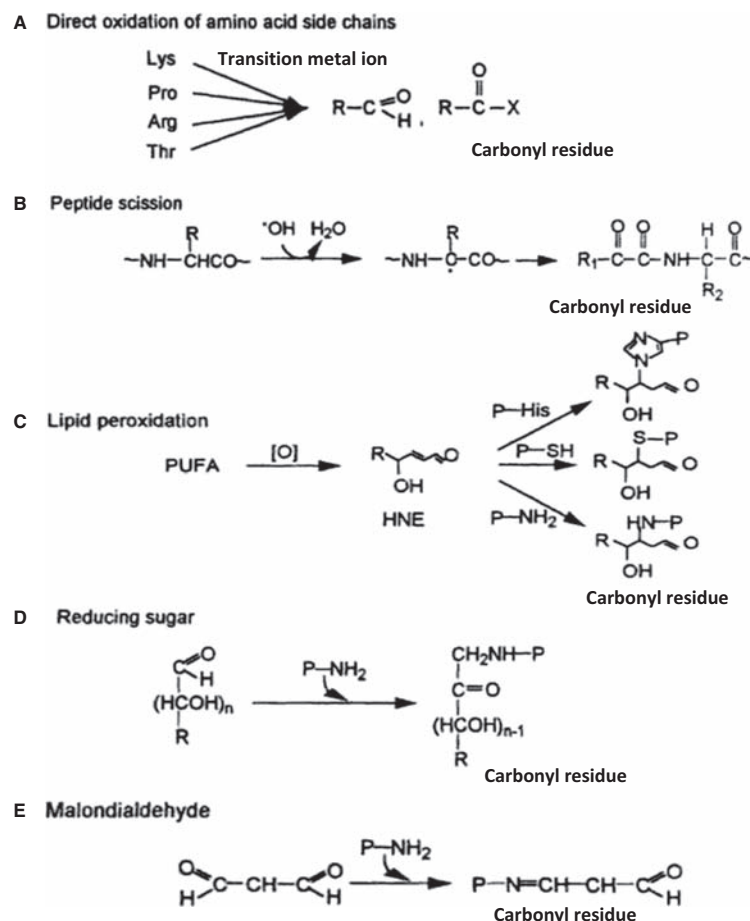
#### Rosemary Extract

Among the plant antioxidant extracts, rosemary extract is one of the most common extracts used in food industry. The antioxidant effect of rosemary extract is attributed to its diterpene phenolics, carnosic acid, and derived oxidation products carnosol, rosmanol and galdosol (Frankel et al., 1996; Wang et al., 2011). Fish oil supplemented with 0.2 mg/g carnosic acid was better than tocopherol in retarding lipid oxidation in fish oil (Wang et al., 2011). Rosemary extract at different concentrations was reported to prevent lipid oxidation efficiently in mackerel oil (Tsimidou et al., 1995), Menhaden oil (Bhale et al., 2007) and Cod and Pollack liver oil (O'Sullivan et al., 2005). A strong synergistic effect was also reported between  $\alpha$ -tocopherol and rosemary extract in preventing lipid oxidation in sardine oil model system at 30 °C and in frozen Bonito mince. The mechanism underlying this synergistic effect was proposed as the regeneration of  $\alpha$ -tocopherol by the rosemary constituents (Wada and Fang, 1992).

Frankel et al. (1996) found that rosemary extract and its constituent's carnosic acid and carnosol efficiently retarded the lipid oxidation in bulk fish oils, but failed to prevent lipid oxidation when tested in fish oil-in-water emulsions. The reason behind this was proposed as the orientation of hydrophilic carnosic acid and carnosol at the air-oil interface thereby protecting the bulk oil from oxidation. A reduction of protection offered by rosemary extract and its constituents in oil-in-water emulsion might be due to the partitioning of antioxidants into the aqueous phase (Frankel et al., 1996; Erdmann et al., 2015). Synergist effects between fish protein and rosemary extracts in the prevention of lipid oxidation were also reported in fish muscle emulsions, which were likely attributed to the partitioning behavior of antioxidants to the fish muscle and the protective effect of fish proteins in inhibiting carnosic acid oxidation (Medina et al., 2003).

Rosemary extracts have shown excellent antioxidant properties in the refrigerated and frozen storage of rainbow trout and salmon muscle (Akhthar et al., 1998; Tironi et al., 2009), horse mackerel and Mediterranean hake (Vareltzis et al., 1997), Cooked fish fake (Boyed et al., 1993), Sardine mince (Serdarolu and Felekolu, 2005) and coated fried Escollar fish fillets (Sarabi et al., 2017). Though being effective in safeguarding the omega-3 fatty acid and red color of salmon muscle, rosemary extracts could not prevent protein alterations (Tironi et al., 2009). The antioxidant activity of these extracts was greater than  $\alpha$ -tocopherol in modified atmospheric packaging (MAP) and vacuum packed Gilt head bream fillets and Sardines, respectively (Giménez et al., 2004; Ozogul et al., 2010). After marinated with rosemary extracts, deep water pink shrimp had a longer shelf life under chilled storage (extended to >75 days) (Cadun et al., 2008). Pressure-induced seafood gels are more prone to oxidation. Rosemary extract was found to





**Figure 3** Mechanisms Involved in the Formation of Protein Carbonyls (A) Metal Catalyzed Oxidation of Basic Amino Acid Side Chains. (B) Peptide Backbone Cleavage or Peptide Scission. (C) Binding to Lipid Peroxidation Products Such as 4-Hydroxy-2-Nonenal (HNE) (D) Reaction with Reducing Sugars. (E) Binding to Lipid Peroxidation Products Such as Malondialdehyde. Adopted from Xiong, Y.L., 2000. Protein oxidation and implications for muscle food quality. In: Decker, E., Faustman, C., Lopez-Bote, C.J. (eds), *Antioxidants in Muscle Foods: Nutritional Strategies to Improve Quality*. John Wiley and Sons, New York, pp. 85–111.

prevent the high pressure/thermal induced lipid oxidation in gels made from the flesh of mackerel with fortified rosemary extract and omega-3 unsaturated fatty acids (Pérez-Mateos et al., 2002). Active packaging materials incorporated with rosemary extracts were found highly efficient in retarding lipid oxidation of smoked rainbow trout (Emir Çoban and Pelin-Can, 2013). The overall mechanisms related to the antioxidant activity of rosemary phenolics may be metal chelation and ability to donate hydrogen to free radicals by the  $-OH$  groups in the aromatic rings (Brewer, 2011).

### Extracts of Oregano and Other Herbs

Culinary herbs such as oregano, thyme and parsley impart antioxidant activities in seafood products. The primary antioxidant constituents of these herbs are carvacrol and thymol (Milos et al., 2000). Tsimidou et al. (1995) compared the antioxidant activity of oregano with red chilies, bay leaf and rosemary extracts in mackerel oil stored at 40 °C in the dark. They found that Oregano at 0.5% was as effective as BHA or 0.5% rosemary extract in retarding lipid oxidation in mackerel oil. Similarly, Jimenez-Alvarez et al. (2008) evaluated the antioxidant effect of oregano, parsley, and olive mill wastewaters on bulk oils and oil-in-water emulsions enriched with 5% tuna oil. The olive mill wastewater was found highly effective in retarding lipid oxidation in both bulk fish oil and an oil-in-water emulsion. Hydroxytyrosol and caffeic acid are the main antioxidant phenolics present in olive mill wastewater and parsley respectively (Shan et al., 2005; Visioli et al., 1999).

In the chilled storage of anchovy and Chilean jack mackerel, incorporation of aqueous extracts of oregano, thyme, and rosemary leaves into ice showed a significant decrease in lipid oxidation (Quitral et al., 2009; Bensid et al., 2014). Oregano and thyme oil added to a coating medium exhibited a protective effect against lipid oxidation in ready-to-eat squid rings (Sanjuas-Rey et al., 2012). Oregano essential oil added at 0.8% to salted Sea bream fillets functioned synergistically with MAP to counteract the increased thiobarbituric acid reactive substances (TBARS) (Goulas and Kontominas, 2007). A combination of oxygen absorber



and oregano essential at 0.4% could reduce lipid oxidation significantly in rainbow trout fillets stored at 4 °C (Mexis et al., 2009). Similarly, 0.1% thyme essential oil and dried thyme showed a synergistic effect with MAP on swordfish fillets (Kykkidou et al., 2009) and vacuum packed Tuna flesh, respectively, in terms of protecting lipids against oxidation (Selmi and Sadok, 2008). The main antioxidant constituents in thyme are thymol and carvacrol. The ethyl acetate extracts of *Majorana syriaca*, which is commonly used as a flavoring agent in the Middle East, were able to prevent, in a dose-dependent manner, both the lipid oxidation and microbial growth in the chilled storage of yellowfin tuna mince (Al-Bandak et al., 2009). Thymol and carvacrol were reported to be the constituents responsible for the observed antioxidant activity (Al-Bandak and Oreopoulou, 2007).

It is worth mentioning the incorporation of plant phenolic extracts to seafood-derived edible/biodegradable packaging films was found to be effective in reducing lipid oxidation. Fish skin gelatin films incorporated with the essential oil from basil had a higher antioxidant activity than plai and lemon (Tongnuanchan et al., 2014). Ahamad et al. (2012) also reported that wrapping of Seabass slices with gelatin film incorporated with 25% (w/w) lemongrass essential oil (LEO) significantly enhanced the prevention from lipid oxidation.

## Spice Extracts

### Clove and Cinnamon Extracts

Aroma extracts from dried clove buds were as effective as  $\alpha$ -tocopherol in the prevention of cod liver oil oxidation (Lee and Shibamoto, 2001). Though preventing lipid oxidation of silver carp fillets under chilled storage, clove bud extracts were less pronounced in controlling protein oxidation (Shi et al., 2014). The greatest protection against lipid oxidation in gutted and beheaded anchovy was achieved when they were stored in ice containing clove (0.02% w/v) (Bensid et al., 2014). Similarly, gelatin and chitosan-based edible coatings incorporated with cinnamon essential oil at 2% protected trout fillets efficiently against lipid oxidation during refrigerated storage (Andevani and Rezaei, 2011; Ojagh et al., 2010). Cinnamaldehyde and eugenol are the primary compounds in cinnamon responsible for this bioactivity.

### Ginger and Garlic Extracts

Application of fresh ginger paste or garlic extract before hot smoking was found to inhibit substantially lipid oxidation and extend the shelf life of catfish (*Clarias gariepinus*) (Kumolu-Johnson and Ndimele, 2011) and sardines (Guiñares et al., 2013). Similarly, a dried or fresh form of garlic and ginger reduced TBARS formation during the chilled storage of liquid smoked silver carp (Frank et al., 2014). A combination of 1% essential oil and 20% aqueous extract of ginger could inhibit the oxidation of fish oil-in-water emulsion. Emir Çoban (2013) found that addition of 1% ginger oil to fish finger significantly reduced the TBARS formation during the refrigerated storage. Ginger essential oil was found more efficient than garlic or rosemary essential oils in reducing TBARS formation during the refrigerated storage of fish sausages (El-Sherif and El-Ghafour, 2016). The primary compounds occurring in the ginger extracts were sesquiterpenoids including zingiberene, copaene and monoterpenoids such as camphane and geranial (Kumari et al., 2014). Alliin, allicin and allyl cysteine occurring in garlic are the compounds responsible for the detected antioxidant effects (Chung, 2006).

## Other Spices

Other spices are also known for their antioxidant and antibacterial activities in seafood products. The polyphenolic compounds derived from some common spices include curcumin (turmeric), capsaicin (chili) and kaempferol (cumin and fennel) (Shan et al., 2005; Hinneburg et al., 2006). Ramanathan and Das (1993) found that dried spices were more effective antioxidants than fresh spices for cooked groundfish. Among the fresh spices, ginger and turmeric were more potent than garlic and onion in suppressing lipid oxidation. Among dried spices, cloves showed the highest antioxidant activity, followed by cinnamon, cumin, black pepper, fennel and fenugreek. Kiin-Kabari et al. (2011) found that the extracts from three African spices, *Piper guinensis* (uziza), *Xylopiia aethiopicum* (okada) and *Myrustica monodora* (ehuru), were effective in preventing lipid oxidation in smoked-dried catfish stored for six weeks, with *P. guinensis* (uziza) being the most efficient antioxidant. Javanese turmeric and red ginger essential oils enriched cassava starch-based edible coating was found to delay significantly lipid oxidation in frozen patin fillets (Utami et al., 2016).

The extracts from Galangal, a spice used in Thai cuisine, were found to prevent hemoglobin-mediated lipid oxidation of washed mince at pH 6.5 (Buaniaw et al., 2010). Washing the sea bass fillet having a low preformed lipid hydroperoxide (LHP) content with a galangal extract solution (0.2% w/v GAE) could effectively inhibit the TBARS formation throughout the storage, although this approach was inefficient to retard lipid oxidation in longtail tuna (*Thunnus tonggol*) with a significant amount of preformed LHPs. Preventing the formation of deoxyhemoglobin (a potent pro-oxidant) was likely the principal mechanism for this phenomenon (Buaniaw et al., 2010).

## Extracts From Teas

Green tea extracts (GTEs) are available in the market and well believed to prevent lipid oxidation in seafood products. GTEs were reported to exhibit a higher antioxidant activity in cod and pollack liver oil compared to other plant extracts such as Black clove oil,

white clove oil, mustard and oregano (O'Sullivan et al., 2005). However, GTE could exhibit a pro-oxidant effect in refined, bleached and deodorized seal blubber oil and menhaden oil due to the pro-oxidant effect of their chlorophyll constituents, whereas, dechlorophyllized GTE had an excellent antioxidant activity in both oils (Wanasundra and Shahidi, 1998). The primary antioxidant constituents in tea are catechins. Among the four catechins, epicatechin gallate was the most effective followed by epigallocatechin gallate, epigallocatechin and epicatechin. The epicatechin gallate seemed more effective than TBHQ (Wanasundara and Shahidi, 1996). Zhu et al. (2009) indicated that the green and black tea extracts and their constituents could trap peroxidation-derived reactive carbonyl substances such as acrolein and reduce the peroxidation of seal blubber oil at high temperatures. GTEs were also found to protect against oxidation in the fish muscle and mince during refrigerated and frozen storage (Tang et al., 2001, Alghazeer et al., 2008). Glazing the fresh bonito fillets with GTEs with different degree of fermentation could be an efficient way to protect fillets against lipid and protein oxidation with green tea and pouching tea at 5% being more effective than black tea (Lin and Lin, 2005). Seto et al. (2005) found that among the hot water extracts of five Taiwanese tea (Longjing-type green tea, Shy Jih Chuen oolong tea, Tungting oolong tea, Pouchong tea and black tea), the extracts of Tungting oolong tea and Longjing-type green tea were highly effective in suppressing pro-oxidation of meat and skin extracts of blue sprat. The detected antioxidant activity correlated well with the content of catechins (especially epigallocatechin gallate), but did not correlate with the content of total polyphenols (Seto et al., 2005).

### Extracts From Vegetables and Their By-products

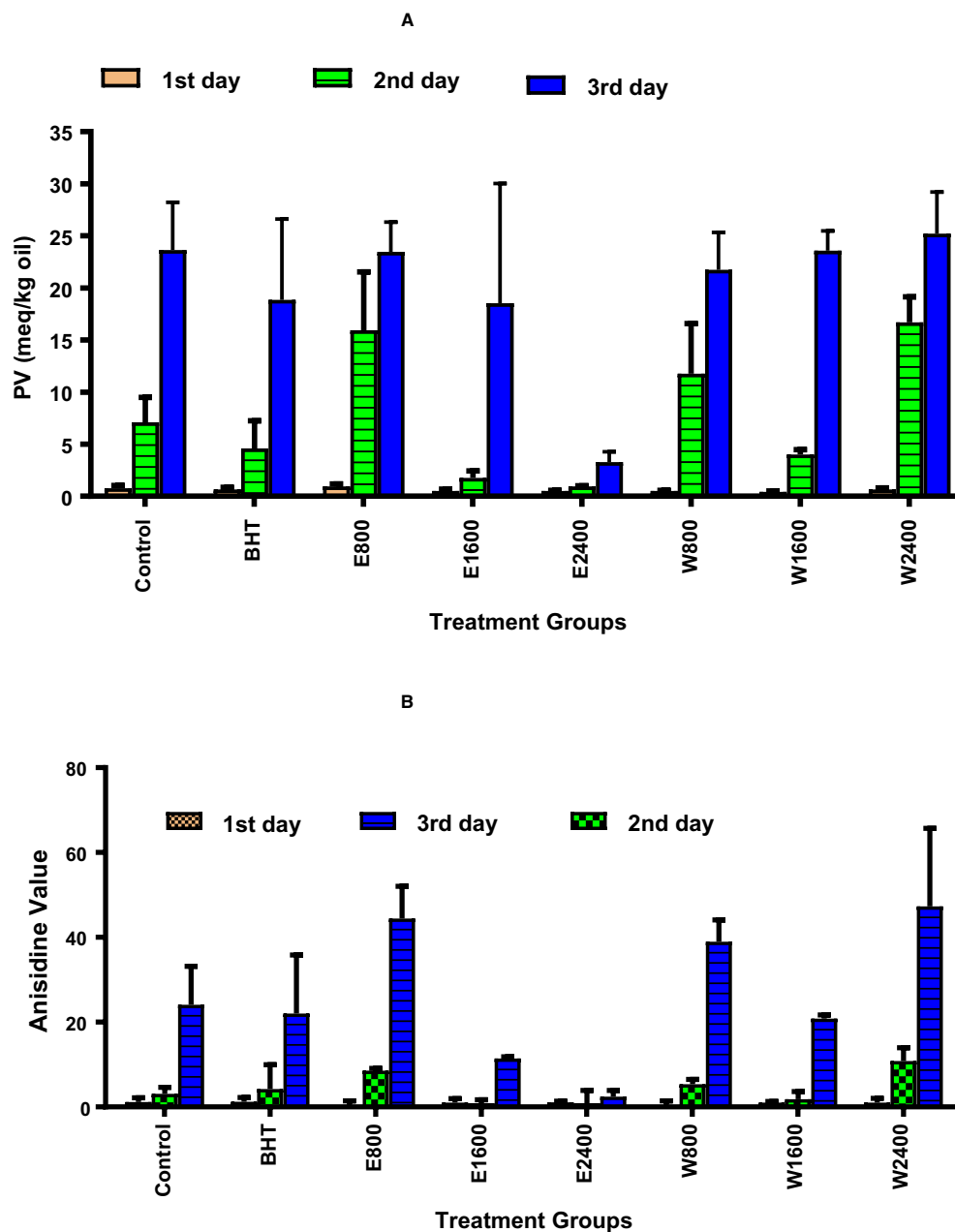
Vegetables are rich in polyphenolic compounds, and their consumption is desirable because of their health benefits and high potential in prevention of many chronic diseases. Pezeshk et al. (2011, 2013) studied the effect of extracts of shallot or a combination of shallot and turmeric extracts on the refrigerated vacuum packed rainbow trout. Immersion of live fish before gutting or gutted fish in 1.5% shallot extract had the same effect in delaying oxidative deterioration under the refrigerated storage (Pezeshk et al., 2011). The combination of shallot and turmeric extracts led to a greater protection against lipid oxidation of vacuum packaged rainbow trout (Pezeshk et al., 2013). Moreover, Farvin et al. (2012) found that potato peel extract can be a useful source of natural antioxidants and could retard lipid oxidation in omega 3 PUFA-containing system, irrespective of the presence of oxidation inducers or activators such as heat, iron or hemoglobin (Farvin et al., 2012; Habeebullah et al., 2010). The authors evaluated the antioxidative effect of both the ethanolic and water extracts of two varieties of potato peel in bulk fish rapeseed oil and oil-in-water emulsions. The study found that the phenolic composition and antioxidant activity varied with potato species and extraction medium. The peel extract from "Sava" variety potato had a higher phenolic content than that of "Bintje", and ethanol was more efficient than water in extracting polyphenolic compounds. The ethanolic extracts of "Sava" showed a higher antioxidant efficacy in bulk fish-rapeseed oil and oil-in-water emulsion where the oxidation was likely induced by heat and  $\text{Fe}^{2+}$ , respectively. Water extracts showed no antioxidant activity in bulk oil (Fig. 4) but a pro-oxidative effect in the oil-in-water emulsion, which might result from the low total phenolic content and absence of certain phenolic acids such as caffeic, salicylic and coumaric acids in the water extract (Habeebullah et al., 2010). The authors also found that the ethanolic extracts of potato peels provided the highest protection against lipid and protein oxidation in horse mackerel mince. The causes proposed for the higher antioxidant activity of the ethanolic extracts of potato peel extracts were radical scavenging activity, metal chelating activity, reducing properties and tocopherol sparing properties of different phenolic compounds occurring in the extracts (Habeebullah et al., 2010).

### Fruit and Fruit By-product Extracts

#### Grape By-products

The primary antioxidant components in grape seed extracts are flavonoids and stilbenes like resveratrol (Hao et al., 2009). Extracts from grape by-products could inhibit lipid oxidation in a variety of fish lipid-containing systems. Pazos et al. (2005a) studied the phenolic fractions containing flavanol monomers, oligomers (procyanidins) and glycosylated flavonols from grape seed extracts, and found that in terms of retarding lipid oxidation, monomers were more effective in bulk fish oil whilst the oligomers were more efficient in oil-in-water emulsions and fish muscle. The authors concluded that an optimal combination of procyanidins, degree of polymerization and percentage of galloylation were the critical factors that determine the efficacy of grape phenolics in different systems. A grape phenolic extract was found to prevent the lipid oxidation in frozen mackerel and horse mackerel mince through preserving the endogenous antioxidants such as  $\alpha$ -tocopherol, ubiquinone-10 and total glutathione (Pazos et al., 2005a, 2005b). Glazing with a grape seed extract before a frozen storage could inhibit the lipid oxidation in bonito fillets (Yerlikaya and Gokoglu, 2010). Spraying the extract containing grape procyanidins and hydroxytyrosol on the horse mackerel fillets before freezing seemed more effective in lowering lipid oxidation than glazing (Pazos et al., 2006). Washing the fillets with water before spraying the phenols-rich extract could synergistically improve the effectiveness of these phenolic compounds, especially for the grape procyanidins, possibly due to a better distribution of procyanidins onto the fillet surface by the residual water in the fillet (Pazos et al., 2006). Crude grape dietary fiber often contain natural antioxidants such as procyanidins, catechins and other flavonoids (Saura-Calixto, 1998), and addition of white and red grape fiber (at 2 or 4%) could inhibit oxidation in the frozen horse mackerel mince (Sanchez-Alonso et al., 2007; Sanchez-Alonso and Borderias, 2008).

Besides flavonoids, grapes and grape-derived product extracts also contain stilbenes. Stilbenes include resveratrol (3,5,4'-trihydroxystilbene), pterostilbene (a dimethylated derivative of resveratrol), piceid (a resveratrol glucoside) and viniferins (resveratrol oligomers) (Fremont, 2000). Medina et al. (2010) have evaluated the antioxidant activity of



**Figure 4** Antioxidant activity of potato peel in bulk oil where oxidation is induced by heat. (A) Peroxide value and (B) anisidine value. The prefix “E” represents ethanol extract and “W” represents water extract. The numbers indicates the concentration in ppm. Adopted from Habeebullah, S.F.K., Nielsen, N.S., Jacobsen, C., 2010. Antioxidant activity of potato peel extracts in a fish-rape seed oil mixture and in oil-in-water emulsions. *J. Am. Oil Chem. Soc.* 87, 1319–1332.

Resveratrol, and its acylated and glycosylated derivatives in a bulk fish oil, oil-in-water emulsion and fish muscle system, and found that resveratrol was effective in retarding lipid oxidation in all the systems studied. Resveratrol and piceid (glycosylated resveratrol) showed high antioxidant activity in an oil-in-water emulsion. Lipophilization or glycosylation of resveratrol did not improve its antioxidant activity in either emulsions or bulk fish oil, mainly due to their localization at the interior of oil droplet or in the aqueous phase rather than the oil-in-water interface.

#### Apple Peel Extracts

Polyphenols obtained from frozen and dried apple peels were reported to stabilize omega-3 polyunsaturated fatty acid-rich fish oil (Sekhon-Loodu et al., 2013). The major constituents of these extracts were flavanols, quercetin-3-O-glycosides and phenolics such as phloridzin, phloretin, cyanidin-3-O-galactoside, chlorogenic acid and epicatechin. Compared to the initial crude apple peel extracts, fractionated polyphenols showed greater inhibition of lipid oxidation compared to  $\alpha$ -tocopherol and BHT

(Sekhon-Loodu et al., 2013). An apple skin extract prepared from “Northern spy” cultivar could inhibit lipid oxidation in the aqueous eicosapentaenoic acid (EPA) emulsions and bulk fish oil. This extract could reduce the oxidation induced by heat, UV light and peroxy radicals, and interestingly, removal of sugars and organic acids from the crude ethanol extract of apple skin enhanced the antioxidant properties (which were comparable to  $\alpha$ -tocopherol and BHT) in both the emulsion and bulk fish oil systems (Rupasinghe et al., 2009).

### Berries and Associated Products

Berry extracts were found to protect omega-3 containing lipids. Luther et al. (2007) reported that among Chardonnay and black raspberry seed extracts, black raspberry seed flour extract was more powerful in suppressing lipid oxidation and rancidity development in fish oil under accelerated oxidative conditions. A garden strawberry leaf extract at 5% was reported to lower lipid oxidation in fish oils (Raudonite et al., 2011). In terms of antioxidant efficacy, proanthocyanidins showed less antioxidant activity against the hemoglobin-induced oxidation in washed cod muscle compared to other polyphenolics in cranberry extract such as phenolic acids, anthocyanins and flavanols (Lee et al., 2006). The decreasing order of the effectiveness of preventing TBARS formation in washed cod muscle was phenolic acids > flavanols > anthocyanins > proanthocyanidins. The component that was efficient in inhibiting Hb-mediated lipid oxidation has tentatively been identified as quercetin (Lee et al., 2006). In another study, a flavanol-rich fraction from cranberry juice powder also showed excellent protection on cod mince against hemoglobin-induced oxidation (Sanchez-Alonso et al., 2007). Presoaking the fillets in a quince (*Cydonia oblonga* Miller) polyphenolic extract efficiently inhibited lipid oxidation in mackerel fillets during refrigerated storage. The protective effect of the quince extract was attributed to the radical scavenging activity of procyanidin B dimer and hydroxycinnamic acids in the extracts (Fattouch et al., 2008).

### Seaweed Extracts

Seaweeds are gaining increasing attention as a potential antioxidant source. Polyphenolic compounds such as phenolic acids, catechins, flavonoids and phlorotannins have been identified from different species of seaweeds (Yoshie-Stark et al., 2003; Koivikko et al., 2005). Recently, marine macroalgal extracts have been extensively studied for their usefulness as antioxidants in fish muscle or fish oil enriched food products. Antioxidant activity of several brown and red algal species was reported to be responsible for their protection on fish oil (Athukorala et al., 2003; Siriwardhana et al., 2004). The antioxidant capacity of seaweed varies with species, location of collection, concentrations of individual phenolic compounds, pigments, polysaccharides and extractant medium. Kindleysides et al. (2012) evaluated the antioxidative effect of lipid extracts from two brown (*Ecklonia radiata* and *Macrocystis pyrifera*) and two red species (*Champia* sp. and *Porphyra* sp.) in an elevated temperature (60 °C) storage of Hoki (*Macruronus novaezelandiae*) oil. They found that the brown seaweed extracts outperformed the red seaweed extracts, and the most effective seaweed extract in the prevention of Hoki liver oil oxidation was *E. radiata*, followed by *Champia* sp., *M. pyrifera* and *Porphyra* sp. extracts.

Different seaweed extracts offered protection against oxidation of fish oil emulsions (Farvin and Jacobsen, 2015; Hermund et al., 2015). The absolute ethanol and 50% ethanol extracts of red seaweed *Polysiphonia fucoides* had higher antioxidant activities than those of *Fucus serratus* in 5% oil-in-water emulsions with and without added iron (Farvin and Jacobsen, 2015). Although a higher amount of phenolic compounds were extracted with absolute ethanol compared to 50% ethanol, the latter exhibited higher antioxidant properties in emulsions (which were comparable to that of BHT). Besides phenolic compounds, protein and polysaccharides were present in the 50% ethanolic extracts. The absolute ethanolic extracts of both seaweed species showed a pro-oxidant effect. Babakhani et al. (2016) observed a similar effect for the ethanolic extract of the red seaweed *P. fucoides* in retarding the lipid and protein oxidation in mackerel muscle mince. The extract, although protecting the loss of  $\alpha$ -tocopherol and tryptophan residues and prevented carbonyl formation, had minimal effect on protein oxidation. Hermund et al. (2015) found that the ethyl acetate fraction of Icelandic *F. vesiculosus* was the most efficient in preventing lipid oxidation in fish oil enriched milk emulsions and mayonnaise. Water, ethanolic and acetone extracts of Icelandic *F. vesiculosus* were reported to prevent fish oil-enriched mayonnaise from oxidation (Honold et al., 2016) and skin care emulsions (Poyato et al., 2017). Radical scavenging and metal chelating activities of the phenolics and carotenoids present in these extracts would be responsible for the prevention from oxidation. Phlorotannin-enriched solvent extracts of *Fucus vesiculosus* was reported to protect against Hb-mediated lipid oxidation for the washed cod muscle (Wang et al., 2010) and Granola bars fortified with fish oil in water emulsion (Hermund et al., 2016).

### Other Plant Extracts

Antioxidant activities of other plant extracts against oxidation in seafood have also been investigated. Carob seed peel was found effective against lipid and protein oxidation in minced horse mackerel (Albertos et al., 2015). Crude brown lead seed with chlorophyll showed protective effect on minced mackerel in a dose-dependent manner (Benjakul et al., 2013). An ethanolic extract of Kiam wood at 0.1%, (w/w) retarded the hemoglobin-induced lipid oxidation in washed seabass mince and also in menhaden oil (Maqsood and Benjakul, 2013). The defatted soy flour extract was very effective at 5% level in preventing the oxidation of menhaden oil and over 60% of DHA and EPA retained when the oil was heated at 150 °C for 30 min (Yue and Xu, 2010). Similarly, a cactus pear fruit (*Opuntia ficus-indica*) extract was reported to inhibit the lipid oxidation in fish oil and

fish oil-in-water emulsion in a dose-dependent manner (Siriwardhana and Jeon, 2004). A flax-seed extract inhibited the secondary oxidation products and hence rancidity in frozen whole mackerel (*Scomber scombrus*) (Stodolnik et al., 2005). Further, food packaging films incorporated with phenolic extracts from barley husk could increase the oxidative stability of packed salmon flesh (de Abreu et al., 2010). Lyophilised water extracts of borage leaves at a concentration of 340 ppm could be as effective as that of BHT at 200 ppm in preventing dry fermented sausage enriched with n-3 PUFA from lipid oxidation (de Ciriano et al., 2009).

## Conclusion

Plant extracts are extensively used as antioxidant sources to protect the biologically important long-chain polyunsaturated fatty acids in seafood products against oxidation. The antioxidative/prooxidative nature of the plant extracts is complex. The concentrations of antioxidant molecules in the obtained extracts vary with factors such as genotype, environmental conditions, plant parts, extraction medium employed. Further, detailed studies are needed to characterize the co-existing constituents present in the extracts before one makes concluding statements on the beneficial effect(s) of particular extracts. Some substances co-extracted, such as alkaloids from the potato peels and kainic acid (a potential neurotoxin compounds) from some seaweeds, may cause health-related problems to the consumers.

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# Protein–Lipid–Phenolic Interactions During Soybean and Flaxseed Protein Isolation

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## Overview

The nature of protein-phenolic interactions in plant-based food systems (e.g., soybean, flaxseed and sorghum seeds) can be classified into two groups as reversible or irreversible interactions (Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2013, 2017d). Reversible protein-phenolic interactions may form insoluble complexes in solutions through non-covalent interactions such as hydrogen bonds, Van der Waals interactions and hydrophobic interactions. Irreversible protein-phenolic interactions may lead to a new molecular structure resulting in increased solubility of proteins and phenolic compounds in solution through their interactions via covalent bonds, non-disulfide linkages, and cross-linking (Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2013, 2017d). The functionality of protein-phenolic complexes and their nutraceutical properties are determined by the stability, structure, amino acid composition and ratio, distribution and ratio of positive to negative charge of the proteins, as well as by the complexity of the phenolic compounds (e.g., the number of phenolic rings and presence of different functional groups) (Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2013, 2014, 2017d). Interactions of phenolics and proteins with lipids present in foods may also occur (Jakobek, 2015).

The stability and conformation of proteins in terms of their primary, secondary, tertiary and quaternary structures may affect the affinity of phenolic compounds for conjugation with proteins and therefore influence the nutraceutical properties of resultant complexes. Moreover, the presence of one or more aromatic rings and hydroxyl groups in the chemical structure of phenolic compounds can enhance their binding affinity for different sites of the protein molecular structure (Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2013, 2014, 2016a, 2017a, 2017d).

Protein-lipid-phenolic interactions in food materials may occur naturally or develop during food processing, handling and storage (Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2013, 2014, 2016a,b, 2017a, 2017d). Other interactions may occur *in vivo*, for instance in the gastrointestinal tract. The main types of interactions responsible for the stability of protein-phenolic complexes are hydrogen bonding and hydrophobic interactions, while the interactions involving lipids are mostly based on hydrophobic interactions (Alu'datt, 2006; Le Bourvellec and Renard, 2012; Ozdal et al., 2013; Jakobek, 2015; Alu'datt et al., 2013, 2016b, 2017d).

## Phenolic Composition and Properties of Flaxseed and Soybean

Phenolics are secondary metabolites produced by plants and found in numerous plant-based foods such as those that contain flaxseed and soybean. Phenolics have extremely diverse chemical structures (e.g., one or more phenolic groups and various functional groups), which contribute to their biological activities (Shahidi and Ambigaipalan, 2015; Alu'datt et al., 2017b). Phenolics can be formed from their precursors, phenylalanine and tyrosine amino acids, through shikimate and acetate pathways (Alu'datt, 2006; Bravo, 1998; Alu'datt et al., 2017b, 2017d). The major features of phenolics in plant foods are their contribution towards color and flavor, as well as their biological and pharmaceutical properties (Alu'datt, 2006; Alu'datt et al., 2013, 2014, 2017b, 2017d).

Flaxseed (*Linum usitatissimum*) has a wide range of food and non-food applications (Alu'datt, 2006; Alu'datt et al., 2016b, 2017b, 2017d), as this plant material is a rich source of health-promoting compounds, both nutrients and non-nutrients, including unsaturated fatty acids (e.g., omega-3 fatty acids), phenolics, proteins, lignin and dietary fiber (Cunneane and Thompson, 1995; Alu'datt et al., 2013, 2014, 2017b).

One main class of health-promoting constituents of flaxseeds are phenylpropanoids, including *o*-coumaric, *p*-coumaric, *p*-hydroxybenzoic, ferulic, sinapic and vanillic acids, which occur in either free forms or bound with carbohydrates (Alu'datt, 2006; Alu'datt et al., 2013, 2016b, 2017b, 2017d). Phenylpropanoids play a key role in the biosynthesis of naturally-occurring phenolics. The main bound phenolic-carbohydrate complexes are *p*-coumaric acid glycosides and ferulic acid glucosides (Johnsson et al., 2000; Alu'datt, 2006; Alu'datt et al., 2017b, 2017d). The total content of esterified, free and bound phenolic compounds in dehulled defatted flaxseed meal was 81.0, 73.9 and 7.2 mg/100 g, respectively (Dabrowski and Sosulski, 1984).

Phenolic compounds may be responsible for objectionable off-flavor and undesirable sensory properties such as brown color and bitter taste in flaxseed and derived food products (Arai et al., 1966; Alu'datt, 2006; Alu'datt et al., 2017b, 2017d). The total content of phenolic acids in flaxseeds was reported to be in range of 355–442 mg/100 g (Varga and Diosady, 1994; Alu'datt,

2006; Alu'datt et al., 2017b) and 800–1000 mg/100 g (Oomah et al., 1995; Alu'datt et al., 2017b), out of which the insoluble bound phenolic compounds accounted for 26%–29% (Varga and Diosady, 1994) and 48%–66% in the esterified form (Oomah et al., 1995). Flaxseed is considered as a relatively good source of lignans compared to other plant foods and flaxseed lignan is composed of two cinnamic acid units (Pszczola, 2002). The main lignan present in flaxseed is secoisolariciresinol diglucoside (SDG), which has demonstrated several roles in supporting human health, for instance, anti-carcinogenic, cardioprotective, phyto-estrogenic and antioxidant effects (Westcott and Muir, 2000; Clifford, 2000; Anon, 2001; Alu'datt, 2006; Shahidi and Ambigaipalan, 2015; Alu'datt et al., 2017b). The content of SDG in flaxseed ranges from 1% to 3% in defatted meals (Bakke and Klosterman, 1956; Thompson, 1994; Johnsson et al., 2000; Alu'datt et al., 2017b). Matairesinol is another type of lignin, which is present in flaxseed albeit in a relatively low amount compared to SDG (Mazur and Adlercreutz, 1998; Alu'datt et al., 2017b). Other lignans found in flaxseed in low amounts include isolariciresinol, SDG isomer and pinorensinol diglucoside (Bambagiotti-Alberti et al., 1994; Meagher et al., 1999; Qiu et al., 1999; Alu'datt et al., 2017b).

The three major cultivars of soybean are *Glycine max*, *Glycine ussuriensis* and *Glycine gracilis*. Asian countries have the highest dietary consumption of soybean in the world. Soybean has been used, since ancient times, as both human food and animal feed due to its high contents of oil and proteins compared to other plant foods (Alu'datt et al., 2016b, 2017b). In addition, the high contents of bioactive compounds such as phenolics, dietary fiber, functional peptides and vitamins in soybean lead to the role of soybean as a functional food and an important source of nutraceutical ingredients in the food and healthcare industries (Alu'datt, 2006; Alu'datt et al., 2013, 2016b, 2017b, 2017d).

Numerous studies have reported the roles of bioactive plant-derived compounds in promoting human health due to diverse biological activities such as estrogenic, anti-carcinogenic, anti-mutagenic, antioxidant and prebiotic activities (Alu'datt, 2006; Alu'datt et al., 2013, 2016b, 2017b, 2017d; Cardona et al., 2013). The presence of phenolic compounds in soybean, in the free and bound forms together with other food constituents, can cause objectionable and undesirable sensory properties affecting the flavor and color of soybean and its products (Alu'datt, 2006; Alu'datt et al., 2017b, 2017d). The main phenolics present in soybean, namely, isoflavones, are rather complex. Several health benefits of isoflavones have been reported (Messina et al., 1994; Alu'datt et al., 2017b), although they can also contribute bitterness and astringency to soy food products. The main isoflavones present in soybean are genistein, daidzein, glycitein as well as their conjugate derivatives, e.g., aglycones, glucosides, acetylglucosides and malonylglucosides (Wang and Murphy, 1994; Alu'datt et al., 2017b). Such derivatives include malonyl-daidzein, malonyl-glycitein, malonyl-genistein, acetyl-daidzein, acetyl-glycitein and acetyl-genistein (Gyorgy et al., 1964; Ingham et al., 1981; Kudou et al., 1991; Barnes et al., 1994; Hoeck et al., 2000; Hosny and Rosazza, 2002; Alu'datt et al., 2017b). The isoflavone content in soy ranges between 47.2 and 420 mg/100 g (Wang and Murphy, 1994, 1996; Simonne et al., 2000). The major isoforms of isoflavones present in soy foods contain glucosides that are hydrolyzed by colonic bacteria *in vivo*, leading to an increased solubility of the aglycone isoflavone (Barnes et al., 1994). Isoflavones and other phenolics of soybean have a strong natural affinity to attach to proteins (Alu'datt, 2006; Alu'datt et al., 2013, 2014, 2016b, 2017e).

For soybean flour, the contents of free phenolics and free phenolic acids were 73.6 mg/100 g and 0.23 mg/g, respectively (Dabrowski and Sosulski, 1984; Alu'datt et al., 2017b). For defatted soy flour, the major identified free phenolic compounds are syringic acid (Arai et al., 1966; Alu'datt et al., 2017b), ferulic acid, syringic acid and vanillic acid (Maga and Lorenz, 1974; Alu'datt et al., 2017b), and *o*- and *p*-coumaric acids and ferulic acids (How and Morr, 1982; Alu'datt et al., 2017b).

## Nature of Protein–Phenolic Interactions in Flaxseed, Soybean and Derived Food Systems

In recent years, plant phenolics and other bioactive compounds have spurred tremendous interest in the food and health industries. The properties of phenolic compounds have been extensively applied in the food and drug industries mainly because of their antioxidant properties (Alu'datt, 2006; Alu'datt et al., 2013, 2017d). The chemical structures of phenolic compounds allow an affinity towards proteins via conjugation and precipitation (Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2013, 2017d). Phenolic compounds can bind to proteins and decrease their solubility (Alu'datt, 2006; Le Bourvellec and Renard, 2012; Ozdal et al., 2013; Alu'datt et al., 2014, 2017d). Reduced protein solubility can decrease the digestibility and biological utilization of proteins (Myer and Gorbet, 1985). Phenolic compounds have been found to bind to proteins in fruit juice, wine and beer, causing haze formation and turbidity (Siebert, 1999; Alu'datt, 2006; Alu'datt et al., 2017d). The enzymatic and non-enzymatic oxidation of phenolic compounds (Haslam, 1989; Alu'datt, 2006; Alu'datt et al., 2017d) may promote their interaction with some amino acids and decrease the activity of digestive enzymes such as trypsin and lipases resulting in decreased digestibility (Milic et al., 1968; Alu'datt, 2006; Alu'datt et al., 2017d). Several studies have reported tannin-protein interactions, which are influenced by many factors such as temperature, pH, structural complexity of the phenolic compound, and the size and amino acid composition of the protein (Serafini et al., 1997; Alu'datt, 2006; Alu'datt et al., 2017d).

## Formation, Characteristics and Importance of Protein–Phenolic Interactions

The conjugation of bovine serum albumin (BSA) with ferulic acid, caffeic acid and (+)-catechin has been reported, which was suggested to affect BSA antioxidant activity (Hagerman, 1989). Haslam et al. (1999) reported that the hydroxyl groups in phenolics led to an affinity, via conjugation, for the amino and carbonyl groups of proteins through hydrogen bonding. In addition, lysine and

cysteine have a strong affinity for oxidized phenolics through covalent linkages, which contributes to the conjugation of sunflower proteins with chlorogenic acid (Sastry and Rao, 1990; Bianco et al., 1997; Siebert, 1999). Non-polar or hydrophobic interactions may also occur through the aromatic ring of phenolics and the non-polar hydrocarbons of the protein molecules (Bartolome et al., 2000; Alu'datt, 2006; Alu'datt et al., 2013, 2017d). Interactions between proteins and phenolics fall into two main types, namely, reversible and irreversible interactions (Haslam et al., 1999; Alu'datt et al., 2013, 2017d; Ozdal et al., 2013).

The main requirement for irreversible protein-phenolic interactions is the presence of oxygen and polyphenol oxidases (PPO) (Mole and Waterman, 1986; Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2017d), which can cause darkening of some products, for instance the browning of cut fruits and vegetables. The main requirements for reversible protein-phenolic interactions involve weak, non-covalent interactions such as hydrophobic interactions, hydrogen bonds and solvation which cause the formation of protein-phenolic complexes (Haslam et al., 1999; Alu'datt, 2006; Alu'datt et al., 2013, 2017d; Ozdal et al., 2013).

The chemical structure of proteins allows them to conjugate with other compounds, including phenolics, lipids, carbohydrates and other proteins (Alu'datt, 2006; Alu'datt et al., 2013, 2014, 2017d; Ozdal et al., 2013). Phenolic compounds can also conjugate with glycosylated proteins (Haslam et al., 1999), and precipitate proteins at a pH close to their isoelectric points (Siebert, 1999). Alu'datt et al. (2013 and 2017d) proposed mechanisms explaining the affinity of phenolics to interact with proteins, lipids and carbohydrates in soybean and flaxseed.

Several factors determine the strength and affinity between proteins and phenolics, including molecular size, number of phenolic rings, protein flexibility and conformation, and number of hydrophilic moieties (Murray et al., 1994; Haslam et al., 1999; Alu'datt, 2006; Alu'datt et al., 2013, 2014, 2017d). Tannins are a major constituents of certain plants, including some common consumer plant-based foods and beverages (Murray et al., 1994; Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2017d). Tannins are classified into two groups, namely the hydrolysable tannins, which are esters (usually with D-glucose) of gallic acid and its derivatives, and the condensed or non-hydrolysable tannins (e.g., proanthocyanidins), which are oligomers of the flavan-3-ol skeleton, e.g., epicatechin (Bravo, 1998; Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2017d). Tannins have a wide range of biological effects including inhibition of some digestive enzymes and non-hemic iron absorption, alteration of food taste by inducing astringency, and reduction of food nutritional value by interfering with nutrient digestion and absorption (Haslam, 1989; Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2017d). Protein-phenolic interactions involving tannins have been found in sorghum, a staple and nutritionally significant food in several countries. It has been shown that the presence of condensed tannins in sorghum decreased the *in vitro* and *in vivo* protein digestibility via inhibition of digestive enzymes (Nguz and Huyghebaert, 1998; Nyamambi et al., 2000; Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2017d). Likewise, the ability of phenolic compounds to precipitate kafirin and prolamin proteins of sorghum has been studied extensively. Sorghum condensed tannins can bind irreversibly to kafirin, which plays an important role in decreasing protein digestibility in sorghum with a high tannin content causing haze formation (Emmambux and Taylor, 2003).

Soy glycinin and soy trypsin inhibitors can interact with chlorogenic, caffeic acid, cinnamic and gallic acids. Soy flavonoid compounds, including flavone, apigenin, kaempferol, quercetin and myricetin, can also interact with soybean proteins. Such derivatizations can reduce the nutritional value of soybean proteins due to the decreased availability of lysine, cysteine and tryptophan residues (Wang and Murphy, 1994; Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2017d). Tannins, in general, can interact with proteins and form both soluble and insoluble complexes, which is largely responsible for the anti-nutritional effects observed in animals, particularly non-ruminants fed tannin-rich diets (Martin-Tanguy et al., 1977; Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2017d). The formation of tannin-protein complexes depends on the size, conformation and charge of protein molecules, and the size, length and flexibility of tannins (Shahidi and Naczki, 1995; Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2017d). Globular proteins, such as ribonuclease, lysozyme and cytochrome C, tend to have a low binding affinity towards tannins, while proteins with open conformational structures such as gelatin have a high affinity for binding with tannins (Hagerman and Butler, 1981; Alu'datt, 2006; Alu'datt et al., 2017d). These complexes with tannin can further interact with essential amino acids and other nutrients, causing decreased bioavailability (Ohlson and Anjou, 1979; Alu'datt, 2006; Ozdal et al., 2013; Alu'datt et al., 2017d). The precipitation of tannin-protein complexes is pH sensitive (Hagerman and Butler, 1978). Naczki et al. (1996) reported that the solubility of such complexes reached the lowest level at pH 0.3–3.1. In such complexes, BSA, fetuin, collagen and pepsin could precipitate at a pH range of 3.0–5.0. Tannins may need to possess at least three flavanol subunits to be effective as protein precipitating agents. Dimers are less effective precipitating agents with simple flavanols unable to precipitate proteins (Artz et al., 1987).

The interactions of simple phenolic compounds with proteins have been investigated in model systems. Smyk and Drabent (1989) reported that BSA-phenolic complexes were favored under neutral and basic pH conditions. Zadernowski (1987) reported that BSA binds via an ester bond to sinapine. Condensed tannins extracted from canola hull samples were found to precipitate 3.0–59.0 mg of BSA per gram of hulls (Leung et al., 1979). Mehansho et al. (1987) reported that the mammalian response to the interaction of salivary proline-rich proteins with dietary tannins could lead to an astringent taste associated with the interactions between polyphenolic compounds in food and salivary proline-rich proteins (Luck et al., 1994; Baxter et al., 1997).

The affinity of tannins towards proteins depends on the chemical and structural properties of the proteins and tannins as well as the properties of their surrounding milieu (e.g., temperature, pH and ionic strength), all of which determine the involvement of hydrophobic interactions, hydrogen bonds and other types of interactions (Oh et al., 1980; Hagerman and Butler, 1980; McManus et al., 1985; Hagerman, 1989; Alu'datt, 2006; Alu'datt et al., 2017d).

One of the main consequences of protein-phenolic interactions is the generation of dark color and unpleasant taste(s) (like bitterness and astringency) in some plant protein-based foods, with the involvement of various mechanisms including hydrogen



bonding, ionic bonding, electrostatic interactions, hydrophobic interactions and covalent linkages (Alu'datt, 2006; Alu'datt et al., 2013, 2014, 2016a,b, 2017d). For example, thimasidic acid can conjugate with proteins at pH 7.0 or 8.5 and room temperature through hydrophobic interactions (Rubino et al., 1996).

The ability of proteins to interact with phenolics through hydrogen bonding is due to a combination of factors including the presence of the hydroxyl groups in the main structure of phenolics and the carbonyl group in the peptide linkage of proteins. These interactions may also occur in the oxidation stage of quinines which can interact with the reactive functional groups on the amino acids of proteins (Loomis and Battaile, 1966; Alu'datt, 2006; Alu'datt et al., 2017d). Phenolic-protein complexes may also form via hydrophobic interactions (Shahidi and Naczki, 1995; Shahidi and Ambigaipalan, 2015). To our knowledge, protein-phenolic interactions do not occur via ionic interactions under normal physiological pH conditions (Butler et al., 1984).

The characteristics of phenolics such as relatively low boiling points and flavor-imparting/altering ability have led to a broad range of applications in the food flavor industry (Alu'datt, 2006; Alu'datt et al., 2013, 2017b, 2017d). Recently, many studies have reported the interactions of phenolic flavor compounds with lipids, salts, carbohydrates, proteins and other phenolics (Alu'datt, 2006; Alu'datt et al., 2013, 2014, 2016a,b, 2017b, 2017d). Siebert et al. (1996a,b) investigated the biological and pharmacological effects of these interactions. As observed in citrus, the chemical nature of flavonoids may affect the volatility of flavor compounds. The volatility of ethyl benzoate and 2,3-diethylpyrazine in citrus decreased in the presence of flavonoids, with no effect on the volatility of limonene (King and Solms, 1981, 1982).

One of the effects of protein-phenolic interactions on plant food proteins is to alter the nutritional properties of proteins and their products by affecting their digestibility and absorption (Bravo and Saura-Calixto, 1998; Alu'datt, 2006; Alu'datt et al., 2013, 2017d). Plant phenolics have the affinity for digestive enzymes to conjugate in the human gastrointestinal system, causing reduced digestibility of proteins, carbohydrates and lipids (Quesada et al., 1996; Alu'datt, 2006; Jakobek, 2015; Alu'datt et al., 2013, 2014, 2016a,b, 2017d). Many studies reported that the inhibitory activity of phenolics towards amylolytic enzymes, by which the digestibility of dietary carbohydrates would be reduced, with a beneficial reduction of the postprandial glycemic response (Thompson et al., 1984; Alu'datt, 2006; Jakobek, 2015; Alu'datt et al., 2013, 2016a,b, 2017d).

A few studies reported the effect of phenolic interactions with proteins on the functional and bioactive properties of food products (Alu'datt, 2006; Alu'datt et al., 2013, 2014, 2017d). Martin-Carron et al. (1997) found that the presence of phenolics and condensed tannins increased fecal lipid excretion in an animal model. Consumption of food and food products with grape tannins, tannic acid and tea catechins was further shown to decrease the blood levels of high density and low density lipoproteins (Muramatsu et al., 1986; Martin-Carron et al., 1997).

## **Extraction and Analysis of Free, Bound and Total Phenolics in Flaxseed and Soybean**

The free and bound phenolics in proteins and byproducts of flaxseed and soybean can be determined according to the Folin-Ciocalteu colorimetric method, as described by Hoff and Singleton (1977) and modified by Alu'datt (2006) and Alu'datt et al. (2013 and 2016b). Several organic solvents and water have been reported to extract free and bound phenolics from plant materials (Shahidi and Naczki, 2004; Alu'datt, 2006; Alu'datt et al., 2017c, 2017d). Shahidi and Naczki (2004) reported that the best solvent for extracting free phenolics from plants is methanol. Free phenolics were extracted from protein isolates of flaxseed and soybean at both room temperature and 60 °C (Alu'datt, 2006; Alu'datt et al., 2013, 2016b). Bound phenolics were extracted from protein isolates of flaxseed and soybean by acid or basic hydrolysis, followed by methanol extraction at room temperature (Alu'datt, 2006; Alu'datt et al., 2013, 2016b). Various analytical techniques have been used to characterize the profiles of extracted free phenolics from different plant materials, including high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), gas chromatography (GC), nuclear magnetic resonance (NMR), capillary electrophoresis (CE) and capillary zone electrophoresis (CZE) (Montedoro et al., 1992a,b; Pirisi et al., 2000; Alu'datt, 2006; Alhakmani et al., 2013; Wong-Paz et al., 2015; Liu et al., 2016; Alu'datt et al., 2013, 2016b). Protein-phenolic and protein–lipid–phenolic complexes in flaxseed and soybean can be identified and characterized using HPLC and mass spectrometry (Alu'datt, 2006).

## **Distribution of Free and Bound Phenolics in Proteins Isolated From Soybean and Flaxseed**

The total content of free and bound phenolics in protein isolates from defatted soybean (3.42 mg/g) was found to be higher than in the protein isolates from full-fat soybean (1.96 mg/g) (Alu'datt, 2006; Alu'datt et al., 2013). The content of bound phenolics in protein isolates from full-fat and defatted flaxseed (0.70–1.91 mg/g) was higher than in protein isolates from full fat and defatted soybean (0.37–0.39 mg/g) (Alu'datt, 2006; Alu'datt et al., 2013). These authors further showed that the profiles of free phenolics in soybean and flaxseed protein isolates were different from those of bound phenolics. The antioxidant activity of free phenolics was higher than that of bound phenolics from isolated proteins of soybean and flaxseed (Alu'datt, 2006; Alu'datt et al., 2013). Protein–phenolic and protein–lipid–phenolic interactions were present in soybean and flaxseed, respectively.

Alu'datt (2006) and Alu'datt et al. (2013) reported that the major free phenolics identified in the protein isolates from soybean were hydroxybenzoic acid, gallic acid, sinapic acid, syringic acid, *p*-coumaric acid, ferulic acid, quercetin, hesperidin and caffeic acid.



The major bound phenolic compounds in soybean protein isolates were syringic acid, hesperidin and *p*-coumaric acid. [How and Morr \(1982\)](#) identified free phenolics in soy protein isolate as *p*-coumaric, *o*-coumaric, syringic, ferulic and genistic acids.

In flaxseed protein isolates, the free phenolics identified were syringic, hydroxybenzoic, protocatechuic, *p*-coumaric, gallic, ferulic and caffeic acids, while the bound phenolics were hydroxybenzoic, gallic, caffeic, syringic, *p*-coumaric and ferulic acids ([Alu'datt, 2006](#); [Alu'datt et al., 2013](#)). The bound phenolic contents of protein isolates from full-fat and defatted flaxseed were positively correlated with the antioxidant activity values. In comparison, the bound phenolic contents of protein isolates from full-fat or defatted soybean were weakly positively correlated with the antioxidant activity. A weak positive correlation was also noted between the free phenolic content and antioxidant activity for the protein isolates from full-fat flaxseed. The free phenolic contents in protein isolates from defatted flaxseed, full-fat soybean and defatted soybean were positively correlated with corresponding antioxidant activities ([Alu'datt et al., 2013](#)).

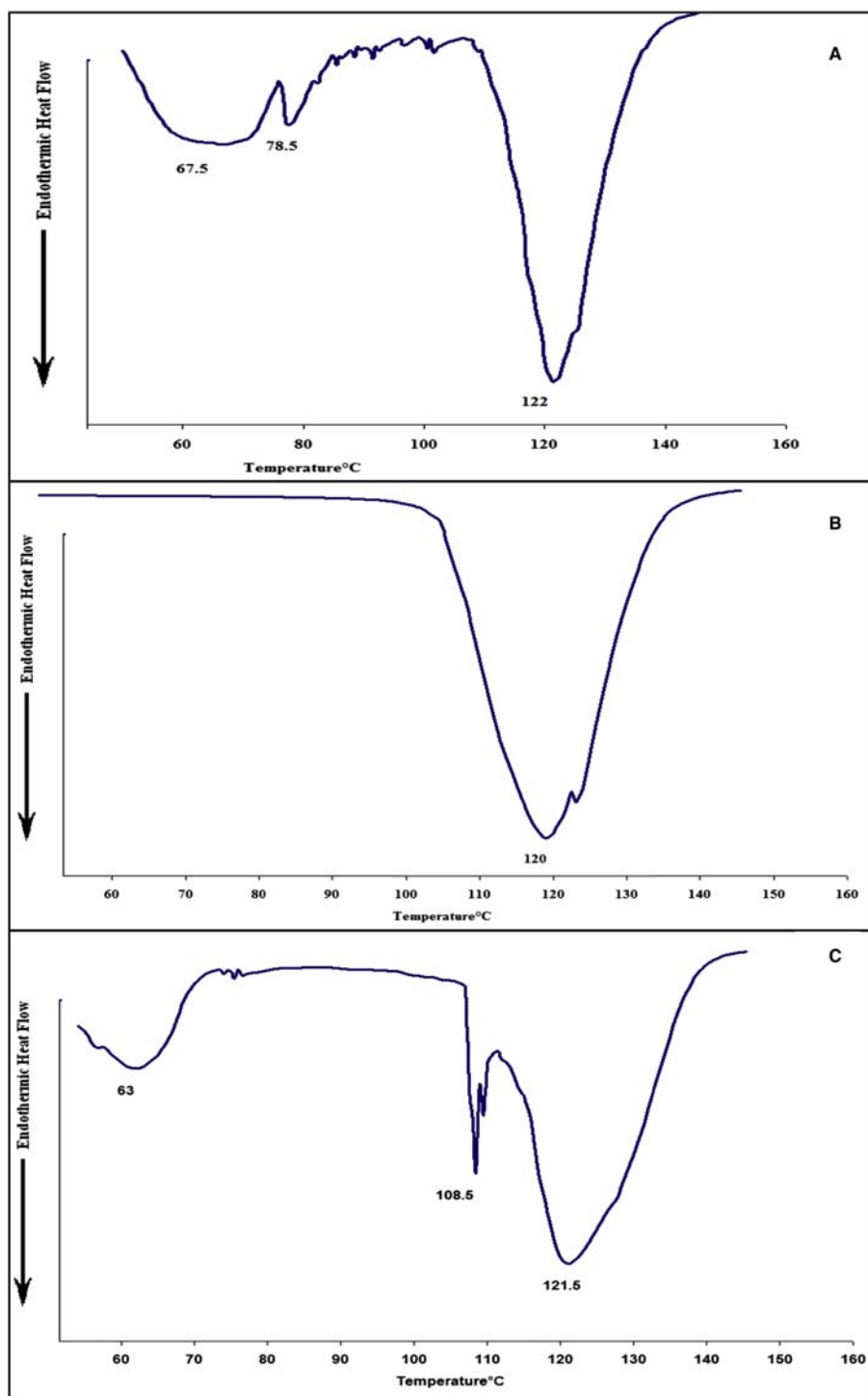
[Alu'datt et al. \(2016b\)](#) reported that the content of bound phenolics in residues obtained after protein isolation from flaxseed was higher (56%–62%) than the content of free phenolics in flaxseed. In contrast, the free phenolic content in residues obtained after protein isolation from soybean was higher than the content of bound phenolics. In the supernatant obtained after protein precipitation from soybean and flaxseed, the free phenolic content was higher than that of bound phenolics ([Alu'datt et al., 2016b](#)). The main free phenolics found in the residue and supernatant obtained after isolation of soybean proteins were ferulic acid, quercetin, hesperidin, *p*-coumaric acid gallic acid, caffeic acid, *p*-hydroxybenzoic acid, sinapic acid and syringic acid ([Alu'datt et al., 2016b](#)). These researchers found that there were no bound phenolics in supernatants obtained after isolation of soybean proteins. The main bound phenolics identified in residues obtained after isolation of soybean proteins were ferulic acid, *p*-hydroxybenzoic acid, sinapic acid, *p*-coumaric acid and syringic acid ([Alu'datt et al., 2016b](#)). The main free phenolics identified in the flaxseed supernatants and residues obtained after isolation proteins were *p*-coumaric acid, syringic acid, gallic acid, caffeic acid, hydroxybenzoic acid, protocatechuic acid, ferulic acid and sinapic acid ([Alu'datt et al., 2016b](#)). The main bound phenolics in flaxseed supernatants and residues obtained after isolation proteins were sinapic acid, ferulic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid and caffeic acid ([Alu'datt et al., 2016b](#)). Protein-lipid-phenolic interactions in flaxseed were thought to occur through sinapic acid ([Alu'datt et al., 2013, 2016b](#)).

### Effect of the Removal of Phenolics on Antioxidant Activity, Water Holding Capacity, Rheological and Thermal Properties of Isolated Proteins From Soybean and Flaxseed

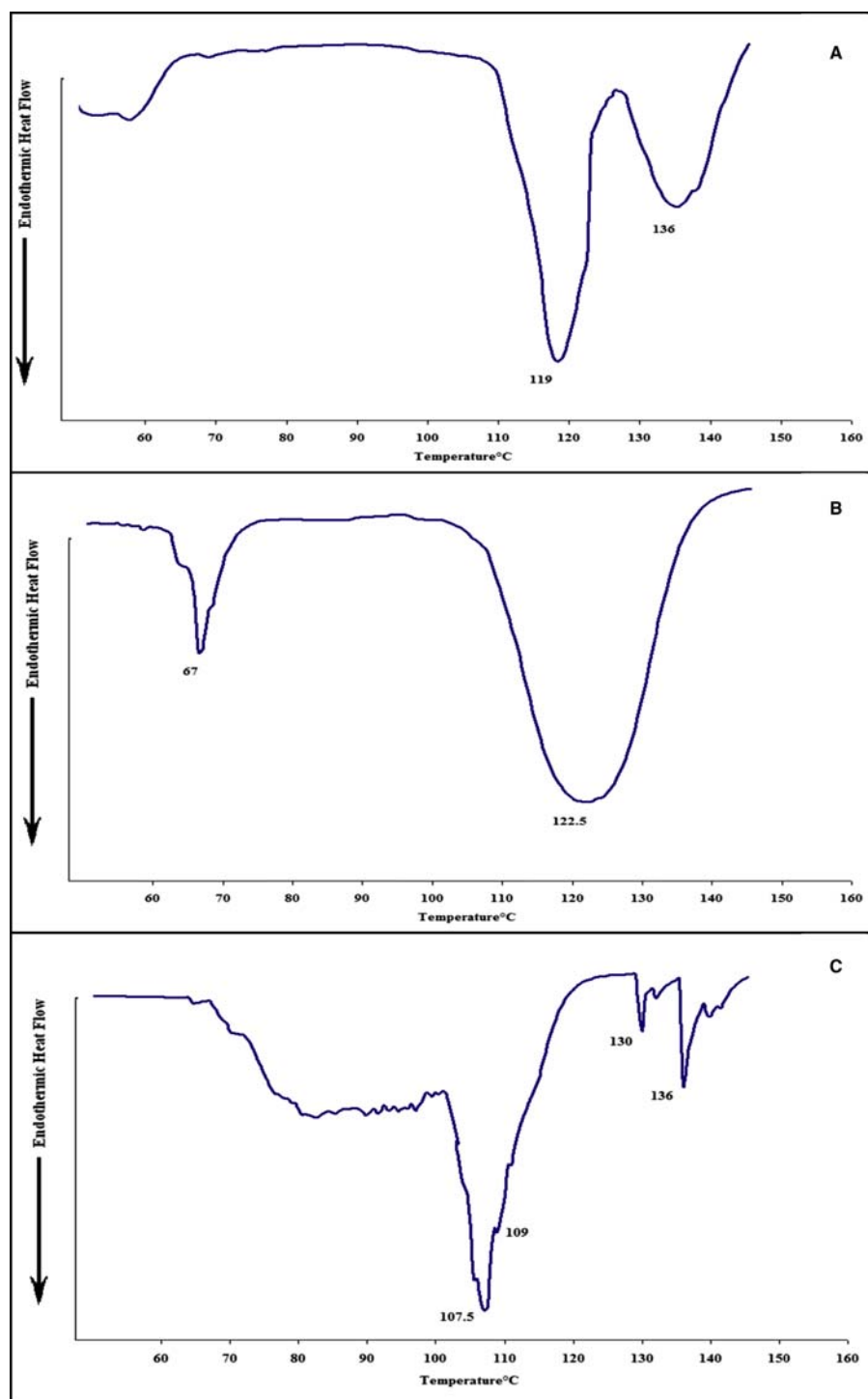
The effects of phenolic removal on isolated proteins from defatted and full-fat soybean and flaxseed were demonstrated by differential scanning calorimetry (DSC) ([Figs. 1–4](#); [Alu'datt, 2006](#); [Alu'datt et al., 2014](#)). The removal of free and bound phenolics from protein isolates of defatted soybean lowered the thermal stability of glycinin ([Fig. 1](#)). However, the sole removal of free phenolics from protein isolate from full-fat soybean led to a higher thermal stability of glycinin compared to the removal of both free and bound phenolics ([Fig. 2](#); [Alu'datt, 2006](#); [Alu'datt et al., 2014](#)).

In defatted soybean, the removal of both free and bound phenolics from isolated proteins also led to lower gel viscosity, greater elasticity and lower water holding capacity (WHC), compared to the initial isolated proteins without any phenolics removed ([Alu'datt, 2006](#); [Alu'datt et al., 2014](#)). In full-fat soybean, the removal of free phenolics caused an increase in gel viscosity, WHC, and thermal stability of glycinin compared to the removal of both free and bound phenolics ([Alu'datt, 2006](#); [Alu'datt et al., 2014](#)). In flaxseed, the removal of both free and bound phenolics from isolated proteins from either full-fat or defatted flaxseed caused a decrease in WHC, viscoelastic properties and thermal stability compared to the removal of free phenolics only ([Alu'datt, 2006](#); [Alu'datt et al., 2014](#)). For protein isolates from full-fat flaxseed, defatted flaxseed, full-fat soybean and defatted soybean, the effect of phenolic removal on WHC was as follows: 70.0, 63.0, 77.0 and 99 for protein isolates after removal of free phenolics, respectively, and 86.0, 93.0, 99.0 and 88.0 for protein isolates after removal of both free and bound phenolics, respectively, as compared to 59, 98, 74.0 and 98.0 for protein isolates before removal of phenolics. The aforementioned effects can be ascribed to the ability of phenolics to interact with proteins and form different types of complexes with high to low polarity ([Papadopoulou and Frazier, 2004](#); [Alu'datt, 2006](#); [Alu'datt et al., 2013, 2014, 2016a, 2016b, 2017d](#)), which will influence their bioavailability. The bioavailability of phenolics and their complexes with proteins depends on many factors, including phenolic chemical structure, co-existing food constituents and their composition, and genetic factors ([Scalbert and Williamson, 2000](#); [Alu'datt, 2006](#); [Alu'datt et al., 2017b, 2017d](#)). The presence of protein-phenolic complexes also influences the physiological properties and bioavailability of certain macro- and micro-nutrients ([Serafini et al., 1997](#); [Wollgast and Anklam, 2000a,b](#); [Alu'datt, 2006](#); [Jakobek, 2015](#); [Alu'datt et al., 2013, 2017d](#)).

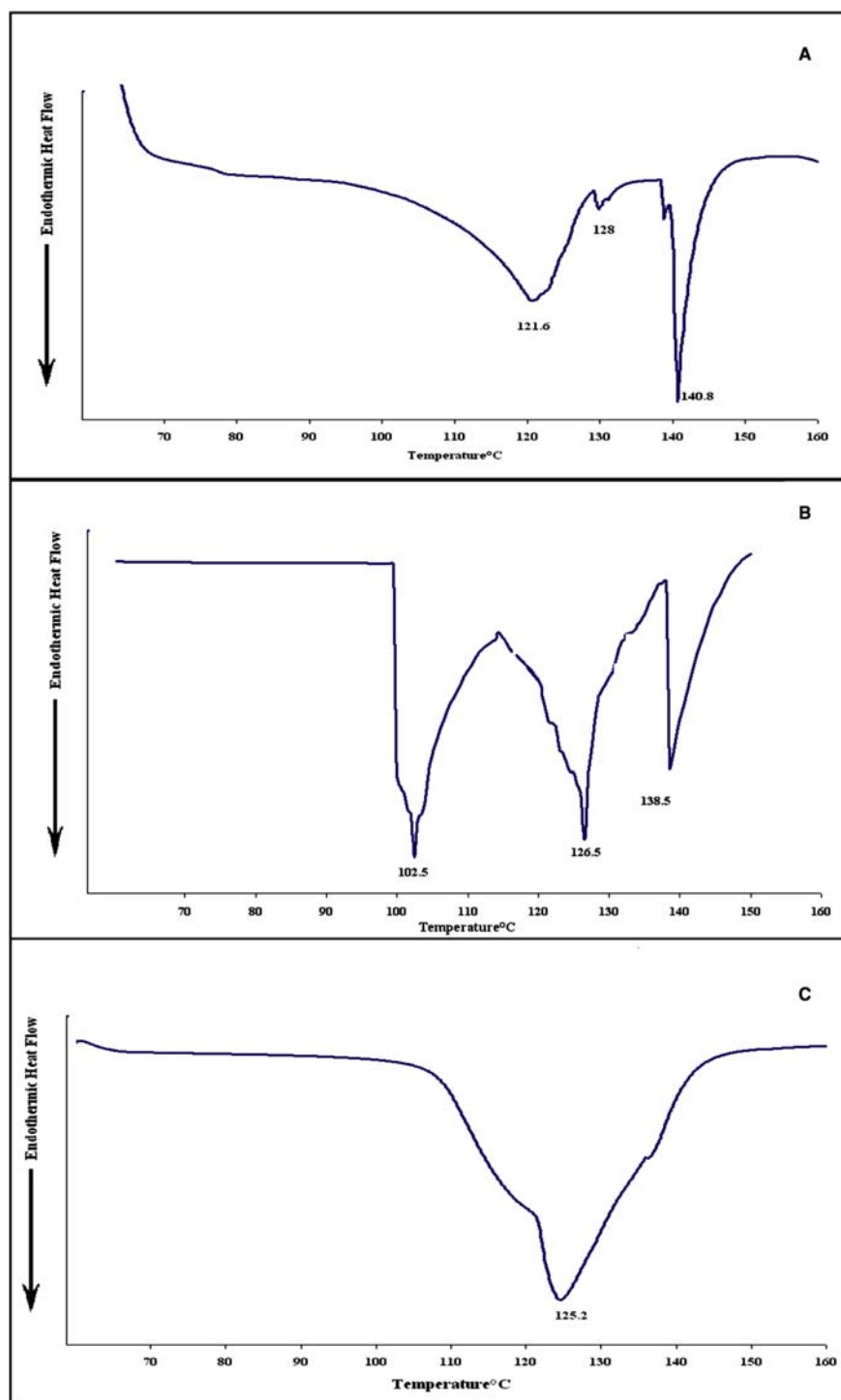
[Fig. 5](#) shows the antioxidant activity of free and bound phenolics from isolated proteins from full-fat and defatted flaxseed and soybean meals ([Alu'datt, 2006](#); [Alu'datt et al., 2013](#)). The antioxidant capacity of protein-phenolic complexes in different plants such as soybean and flaxseed was found to be different from that of free phenolics ([Riedl and Hagerman, 2001](#); [Alu'datt, 2006](#); [Alu'datt et al., 2013, 2016b, 2017d](#)). [Alu'datt \(2006\)](#) and [Alu'datt et al. \(2016b\)](#) reported substantial antioxidant activities derived from the bound phenolics in residue and supernatant obtained after protein isolation from full-fat flaxseed and soybean, while minimal antioxidant activity was found for the bound phenolics in residues and supernatants obtained after protein isolation from defatted flaxseed and soybean ([Alu'datt, 2006](#); [Alu'datt et al., 2016b](#)). The contents of bound phenolics in residues and supernatants obtained after protein isolation from flaxseed and soybean showed strong positive correlations with corresponding antioxidant activity of phenolics. In comparison, the contents of free phenolics from supernatant and residue obtained after protein



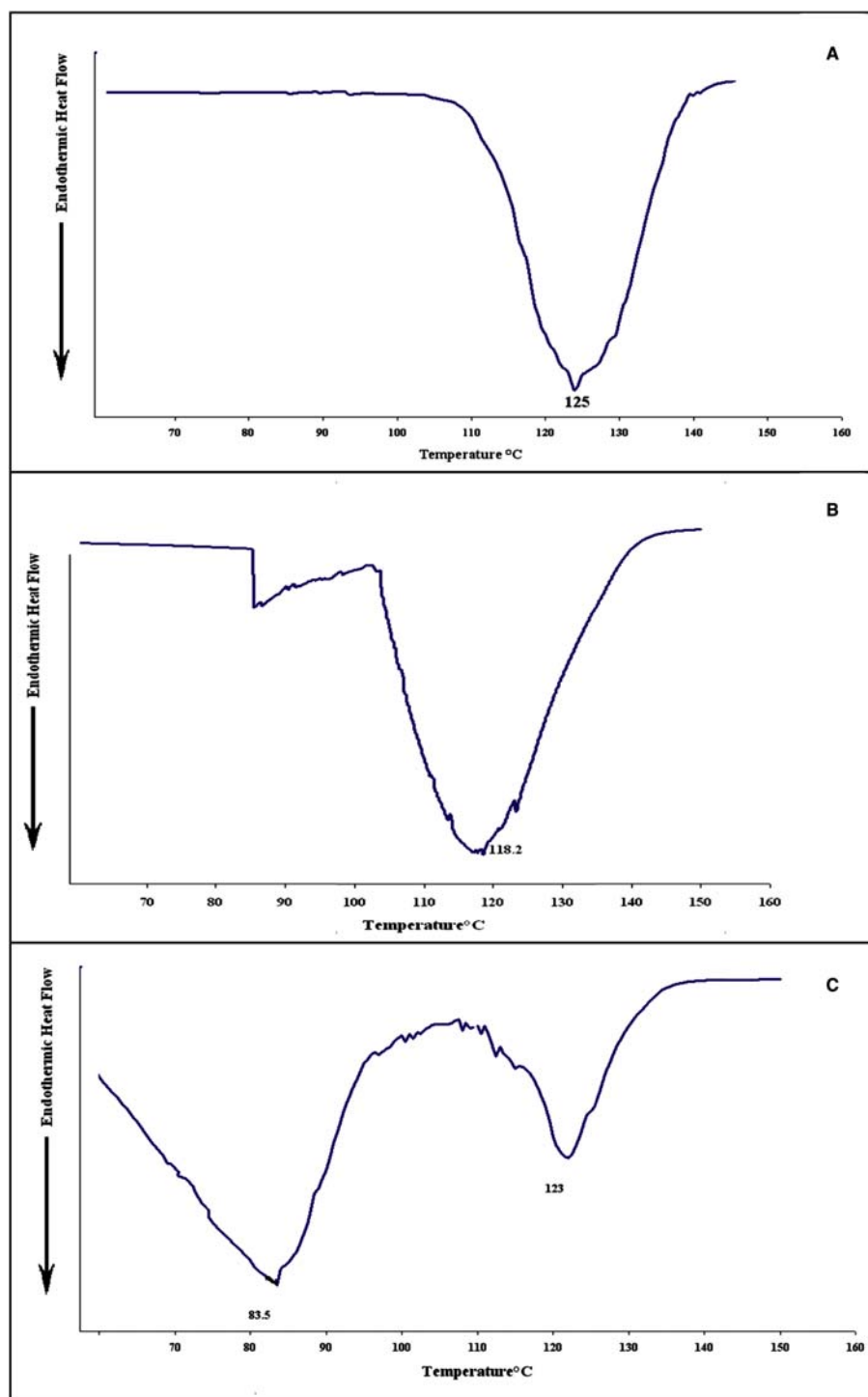
**Figure 1** Effect of the removal of free and bound phenolics from isolated proteins from defatted soybean on thermal stability assessed by differential scanning calorimetry (A) before removal free phenolics, (B) after removal of free phenolics, (C) after removal of free and bound phenolics (Alu'datt, 2006; Alu'datt et al., 2014).



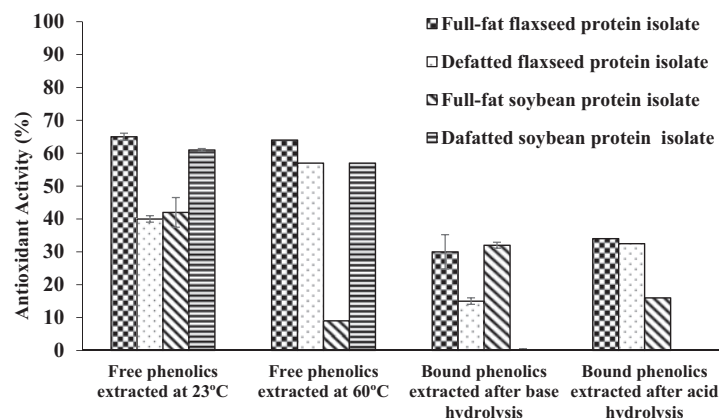
**Figure 2** Effect of the removal of free and bound phenolics from isolated proteins from full-fat soybean on thermal stability assessed by differential scanning calorimetry (A) before removal free phenolics, (B) after removal of free phenolics, (C) after removal of free and bound phenolics (Alu'datt, 2006; Alu'datt et al., 2014).



**Figure 3** Effect of the removal of free and bound phenolics from isolated proteins from defatted flaxseed on thermal stability assessed by differential scanning calorimetry (A) before removal free phenolics, (B) after removal of free phenolics, (C) after removal of free and bound phenolics (Alu'datt, 2006; Alu'datt et al., 2014).



**Figure 4** Effect of the removal of free and bound phenolics from isolated proteins from full-fat flaxseed on thermal stability assessed by differential scanning calorimetry (A) before removal free phenolics, (B) after removal of free phenolics, (C) after removal of free and bound phenolics (Alu'datt, 2006; Alu'datt et al., 2014).



**Figure 5** Antioxidant activity of extracted free and bound phenolics from isolated proteins from full-fat and defatted flaxseed and soybean meals (Alu'datt, 2006; Alu'datt et al., 2013).

isolation from flaxseed and soybean only showed a weak positive correlation with antioxidant activities (Alu'datt, 2006; Alu'datt et al., 2016b). Alu'datt et al. (2017e) found that the contents of free, bound and total phenolics in sonicated protein isolates from soybean were lower than in non-sonicated protein isolates. They indicated that sonication increased the efficiency of the extraction of bound phenolics from soybean protein isolates.

## Conclusion

Protein-rich plants like soybean and flaxseed and their dietary products are of high nutritional importance worldwide. Their regular consumption is increasingly associated with appreciable health-promoting effects beyond their nutritional roles. In this respect, the protein-phenolic interactions that involve plant proteins have attracted a great deal of interest in the food and healthcare industries. Interactions with lipids may contribute additional beneficial effects. The interactions of phenolics with proteins and lipids in edible seeds (including fresh beans and pulses), cereals, fruits, vegetables, herbs and their derived food products warrant further research to better understand how such complexes form and exert their beneficial physiological effects *in vivo* and contribute to health promotion and disease prevention. Further research will also help elucidate how these complexes contribute to important functional properties in food matrices. Advancements in this field will support the development of novel food and non-food products with enhanced properties.

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# Interactions Between Dietary Antioxidants and Plant Cell Walls

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## Glossary

**Pectin** one of the major polysaccharide in primary plant cell walls and food additive E440, pectin is characterized by its high content of galacturonic acid, forming either homogalacturonan chains or rhamnogalacturonans. The carboxylic acids of galacturonic acids may be methylesterified, and the degree of methylation determines the conditions for gelification.

**Proanthocyanidins** oligo or polymeric flavan-3-ols; they are divided in various subclasses, the most common being procyanidins, which are composed exclusively of catechin or epicatechin subunits, and prodelphinidins, which include gallocatechin or epigallocatechin subunits. Proanthocyanidins are also known as condensed tannins.

**Carotenoids** C40 tetraterpenoid pigments presenting highly unsaturated carbon chains. Carotenes (like lycopene,  $\beta$ -carotene or  $\alpha$ -carotene) are strictly hydrocarbons; xanthophylls (like lutein, zeaxanthin, b-cryptoxanthin) are oxygenated.

**chromoplasts** plastids that accumulate carotenoid pigments, resulting in the yellow-red colours of ripe fruit, some flowers and roots, or senescent leaves.

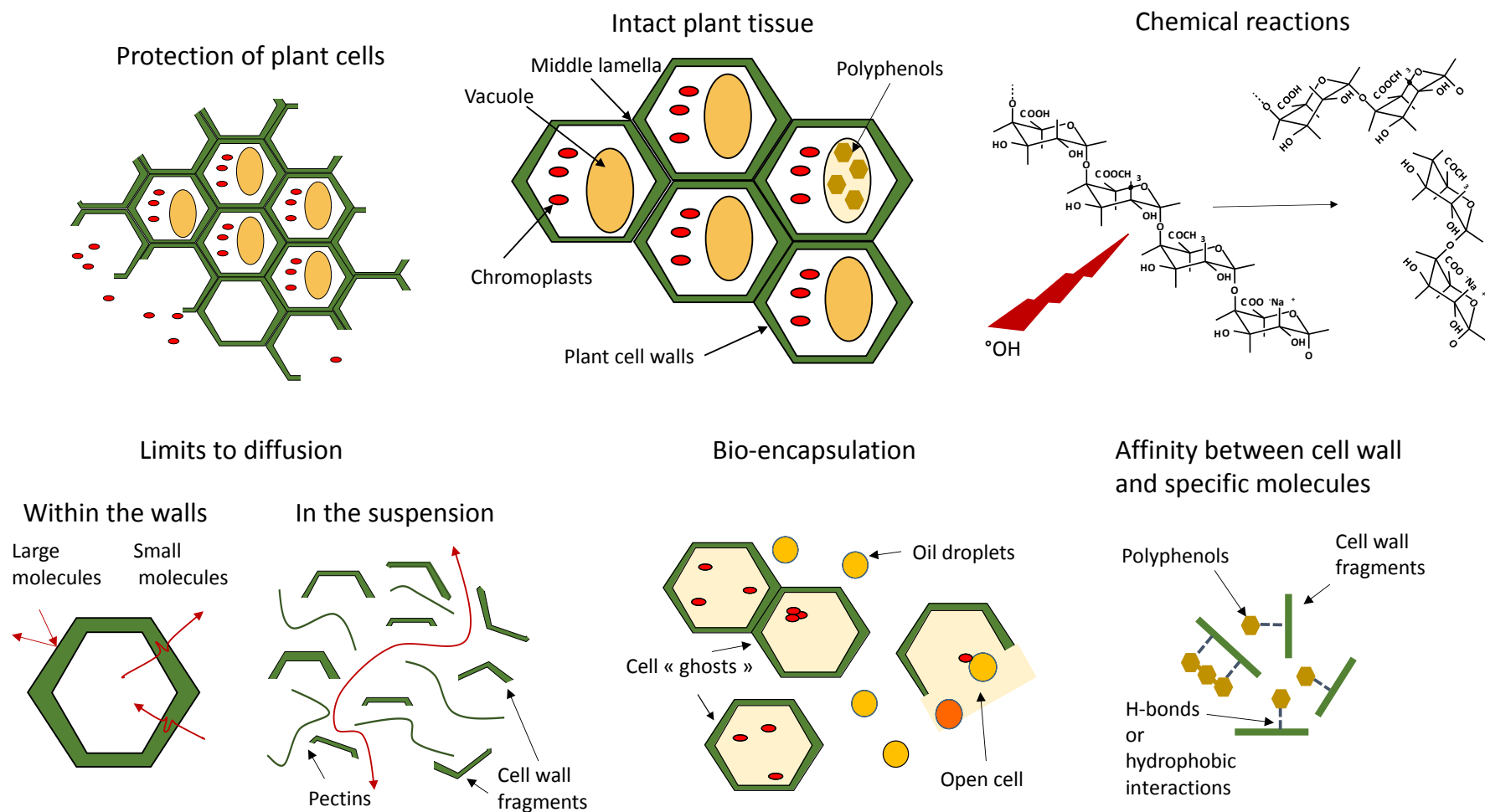
## Introduction

“Antioxidant” is a generic terms that covers very diverse chemical structures with a common property, namely molecules that may prevent the oxidation of other molecules. This can involve different mechanisms: acting as electron donors, reducing reactive oxygen species, complexing transitions metal ions or regenerating other antioxidants such as tocopherols (vitamin E) (Dangles, 2012). Antioxidants, whether synthetic or of natural origin, are used in the food industry to prevent degradation of products leading to destabilization of emulsions (by protein oxidation for example), off-tastes (and toxic by-products, for example by lipid oxidation), or off-colours (oxidation of polyphenols). However “antioxidant” has become a household term for a diversity of natural plant molecules (dietary antioxidants) which were believed to participate to prevention of some degenerative diseases by contributing to the oxidative balance in vivo. This includes notably some vitamins (vitamin C, E), provitamin A and other carotenoids, and polyphenols. Many more secondary plant metabolites may have some antioxidant activity as assessed by the common colorimetric assays. This was a seductive simple hypothesis for the health effects of fruit and vegetables, which has been largely disproved. The scientific consensus is now that some of these molecules (or their metabolites) can interact at minute concentrations with specific sites in the body, which may lead to positive health effects for some, but detrimental for others if taken in excess (Bjelakovic et al., 2012), and which are in any case not related to their antioxidant property. It is only in the gastric compartment that dietary antioxidant properties may be relevant, as dietary antioxidants may be present in millimolar concentrations and exert protective actions on other food components (Dangles, 2012).

Three classes of dietary antioxidants have been specifically studied for their interactions with the plant cell walls: polyphenols, carotenoids, and ascorbic acid, with very different mechanisms and consequences. Polyphenols may bind to the cell wall components; this is a major factor in extraction of polyphenols to some beverages, notably wine, with consequences on their colour, bitterness and astringency (Le Bourvellec and Renard, 2012; Renard et al., 2011, 2017; Bindon et al., 2010a, 2012). This binding also decreases their bioaccessibility but ensures that a high load of polyphenols reaches the colon where they may be metabolised in bioavailable and active metabolites (Del Rio et al., 2010; Jaganath et al., 2006; Aura et al., 2005, 2013; Appeldoorn et al., 2009). Bio-encapsulation of the lipophilic intracellular carotenoids by the hydrophilic cell walls decreases their bioaccessibility and notably explains the very low efficiency observed for provitamin A carotenoids from raw fruit and vegetables (Van Buggenhout et al., 2010; Lemmens et al., 2009, 2010, 2011; Tiback et al., 2009; Colle et al., 2013; Mutsokoti et al., 2016, 2017; Palmero et al., 2014; Edwards et al., 2002). Ascorbic acid, in spite of its antioxidant reputation, actually appears to act as prooxidant and degrade cell wall polysaccharides (Airianah et al., 2016; Fry, 1998; Fry et al., 2001).

## General Mechanisms

Five mechanisms can be involved in interactions between dietary antioxidants and the plant cell walls: protection of plant cells, limit to diffusion (of soluble molecules or enzymes), bio-encapsulation, affinity for specific molecules, and chemical reactions (Fig. 1).



**Figure 1** The five mechanisms involved in interactions between dietary antioxidants and plant cell walls.

## The Plant Cell Wall

The plant cell wall is an organite that surrounds plant cells; its main roles are support for the plant organs and exchange with the external medium. It defines the shape and size of cells and is responsible for intercellular adhesion within a plant tissue. In foods commonly consumed by Humans, type I cell walls are dominant: They are thin, hydrophilic and highly hydrated. They are composed primarily of polysaccharides, with load-bearing cellulose microfibrils, embedded in an amorphous matrix composed mainly of pectins and hemicelluloses (xyloglucans, mannans, arabinoxylans) (Carpita and Gibeau, 1993). Pore size in the plant cell walls have been estimated to be about 3–4 nm (Carpita et al., 1979), i.e. while small water-soluble molecules may diffuse through plant cell walls, proteins above 10–20 kDa are restricted. Pectin is responsible for tensile strength of the cell wall, and is also extensively used as a gelling agent or stabilizer in foods. It is also the plant cell wall component which changes the most with ripening (Brummell, 2006; Goulao and Oliveira, 2008; Redgwell et al., 1997) or processing (Ng and Waldron, 1997), and its degradation may increase pore size, while its reticulation (typically by calcium after partial demethylation) increases resistance (Cybulska et al., 2011; Muller and Kunzek, 1998). Plant cell walls are also the main origin of dietary fibers in Human foods. They determine the texture of fruit and vegetable-based foods (Waldron et al., 1997): plant cell walls are responsible for the cohesion and structure of intact fruit or vegetable pieces, cell wall “ghosts”, more or less aggregated, produce the texture of pureed fruit and vegetables (Espinosa-Munoz et al., 2013; Leverrier et al., 2016), and solubilized polysaccharides, mainly pectins, or microfibrillar complexes have gelling and thickening properties (Chan et al., 2017).

## Protection of Plant Cells

This is a mechanical effect linked to the *in vivo* role of cell walls. It has not been studied specifically for dietary antioxidants, but one may assume that they will be affected similarly to macrocomponents. Soft plant foods (e.g. tomato, ripe fruits ...) are swallowed as millimetric or centimetric pieces. Median particle sizes of 2 mm are reported for raw carrots or olives, and 1 cm for tomato or ripe papaya (Jalabert-Malbos et al., 2007; Peyron et al., 2004). Within these pieces plant cells are intact and intracellular components are still compartmented. This delays their liberation in the gut as action of gastrointestinal enzymes and peristalsis actually have limited efficiency in plant tissue breakdown or plant cell lysis: recognizable shredded carrots and intact plant cells have been reported after digestion in ileostomic patients (Faulks et al., 2004; Livny et al., 2003; Tydeman et al., 2010). In simulated gastric digestion conditions, raw carrots lose less than 10% of their dry weight (mainly sugars) in weakly acidic conditions (pH 5.3, i.e. start of digestion) but up to 60% in acidic conditions (pH 1.8) (Kong and Singh, 2011); this is accompanied by swelling of plant cell walls, probably due to degradation of pectins. Size of particles, either after processing (grinding) or mastication, and the proportion of open cells is thus an important factor in bioaccessibility.

## Limit to Diffusion

Once plant cells are lysed (degradation of the plasmalemma and intracellular membranes), the plant cell walls constitute a barrier with a limited porosity; as such they slow the diffusion of water-soluble molecules and the ingress of enzymes to the plant tissue (Kong and Singh, 2004; Mandalari et al., 2008). There is little scientific information on this phenomenon during food processing, and almost none during digestion. Diffusion and leaching has been modelled for glucosinolates in cabbages, as they may either diffuse and leach as such (e.g. during processing in a large amount of water) or be degraded by myrosinase to isothiocyanates (Volden et al., 2008; Nugrahi et al., 2015). As myrosinase is present in the same tissues but different cells than glucosinolates, enzymic degradation depends on cell lysis followed by diffusion and leaching (Verkerk and Dekker, 2004), and is affected differentially by processing and cooking methods.

Much more attention has been devoted to the effect of dietary fibers as a limit to bioaccessibility in the gut: dietary fibers increase the viscosity of the gastrointestinal liquid, and this acts *per se* to slow diffusion of all molecules, including digestive proteases, lipases or amylases (Capuano, 2017; Kumar et al., 2012; Gidley, 2013). This has been highlighted for the glycaemic index: increased viscosity of the chyme slows down gastric emptying, it slows starch digestion by limiting amylase binding to their substrate, and it slows diffusion of glucose to the gastrointestinal mucosa. Though rarely specifically studied, these mechanisms are also involved in bioaccessibility of dietary antioxidants, and their relevance has been shown for carotenoids (Carrillo et al., 2017; Mutsokoti et al., 2015; Palmero et al., 2016).

## Bio-Encapsulation

Bio-encapsulation describes the mechanisms by which the plant cell walls (among others) as such act as a barrier between fat-soluble dietary antioxidants and the outer medium. For carotenoids and vitamin E, a lipid continuum is needed between the cell and the fat phase of the food to allow integration during digestion in mixed micelles (Castenmiller and West, 1998; Castenmiller et al., 1999; Kopeck and Failla, 2017; Desmarchelier and Borel, 2017; Fernandez-Garcia et al., 2012; Rodriguez-Amaya, 2010; Schweiggert and Carle, 2017). The plant cell walls prevents establishment of this continuum, and its degradation, either chemical or mechanical, allows rupture of this barrier. This will be detailed below for carotenoids, for which this mechanism and the effects of food processing on bioaccessibility have been extensively studied.

### Affinity for Specific Molecules

Polyphenols are highly reactive molecules that bind to macromolecules (Le Bourvellec and Renard, 2012). Though this is mostly known for proteins, with perception of astringency (Schwarz and Hofmann, 2008; Zanchi et al., 2008; de Freitas and Mateus, 2012; McRae and Kennedy, 2011) or inhibition of (digestive) enzymes (Rohn et al., 2001, 2002), they also have affinity for polysaccharides such as constitute the plant cell walls (Renard et al., 2017; Bindon et al., 2010a,b, 2012; Braham et al., 2017; Watrelot et al., 2013, 2014; Bindon and Kennedy, 2011; Bindon and Smith, 2013; Cerpa-Calderon and Kennedy, 2008; Carn et al., 2012). Affinity constants are much lower than for proteins, but the plant cell walls constitute a quantitatively major component of the plant tissues in which polyphenols are present. Impact of binding of polyphenols to plant cell walls has mostly been studied for tannins, and will be presented below.

### Chemical Reactions

Vitamin C is a small, water-soluble molecule and as such may diffuse almost freely from plant tissues, with only limited hindrance by plant cell walls. However it has been demonstrated to exert a pro-oxidant effect and cleave the cell wall polysaccharides, with consequences on texture (Airianah et al., 2016; Fry, 1998; Fry et al., 2001).

## The Interactions Between Polyphenols and Plant Cell Walls

Mechanisms of these interactions are described in Chapter “Interactions between polyphenols and macromolecules”. This paragraph will mostly focus on consequences of the interactions in terms of food processing and digestion.

### Polyphenols

Polyphenols are a very diverse class of molecules (Fig. 2), and their antioxidant properties are linked to the presence of ortho-diphenol groups (Dangles, 2012). Polyphenol intake in a typical Western diet is mostly from beverages (Manach et al., 2004): tea (with flavan-3-ols such as epigallocatechin gallate), chocolate (with procyanidins i.e. oligomers of catechin and epicatechin), coffee (with caffeic acid and its quinic esters chlorogenic acids), wine (with complex structure derived from proanthocyanidins and condensation of anthocyanins, or stilbenes such as resveratrol). Fruit and vegetables have a lower contribution, in which proanthocyanidins, flavonols, anthocyanins (characterized by their red colour), or hydroxycinnamic acids are the most common classes. All these polyphenols are located in the vacuoles of the plant cells.

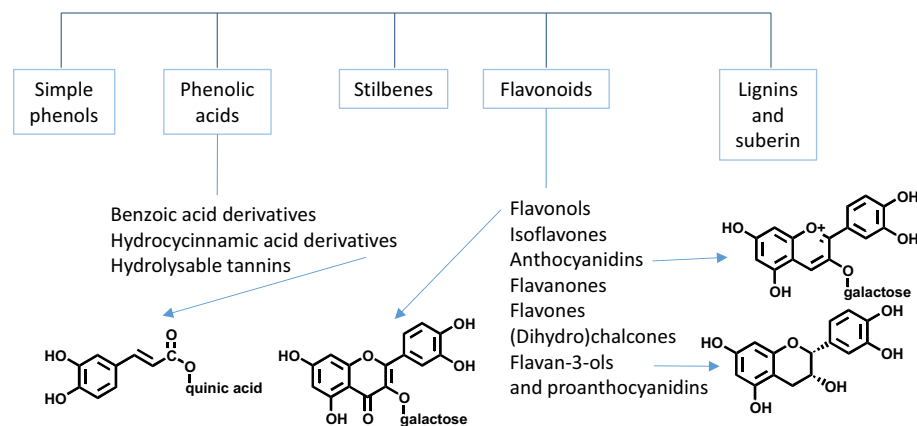
### Investigated Impacts

Polyphenols, initially present in the vacuoles, bind spontaneously and non-covalently to macromolecules, including the plant cell wall polysaccharides (Le Bourvellec and Renard, 2012). This binding gives rise to polyphenol – plant cell wall complexes whenever the plant cells are lysed or ruptured, either physically (e.g. grinding, pressing) (Le Bourvellec et al., 2007), by physical phenomena that lead to loss of membrane integrity (e.g. heating (Le Bourvellec et al., 2011; Le Bourvellec et al., 2013; Le Bourvellec et al., 2012), pulsed electric fields, power ultrasound and others) or by senescence. These interactions have mostly been studied in relation with the extractability of polyphenols to beverages, and in particular to wine by the groups of Smith, Kennedy & Bindon (Bindon et al., 2010a,b, 2012; Bindon and Kennedy, 2011; Bindon and Smith, 2013), Gidley at University of Queensland (Phan et al., 2015, 2017; Liu et al., 2017; Padayachee et al., 2012), and Gomez-Plaza (Bautista-Ortin et al., 2015; Bautista-Ortin et al., 2015; Bautista-Ortin et al., 2014; Bautista-Ortin et al., 2016) or to apple juice by Le Bourvellec & Renard (Renard et al., 2011, 2017; Le Bourvellec et al., 2007). The main focus was on proanthocyanidins (or condensed tannins), because of their role in wine or cider body and astringency, and on anthocyanins, because of their role in wine colour.

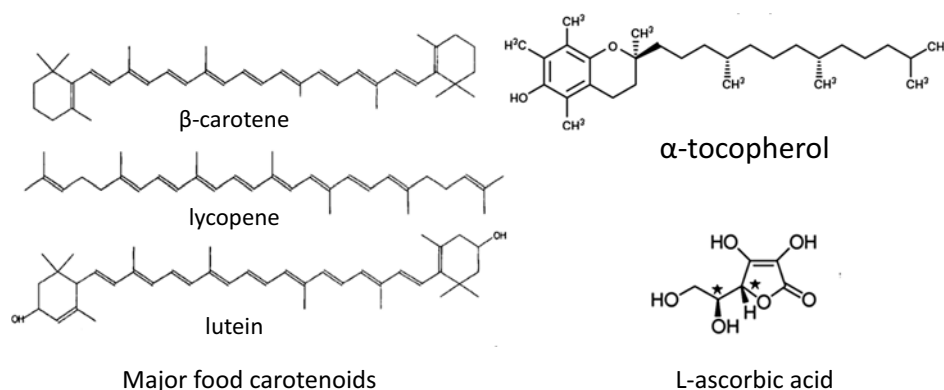
These polyphenol – cell wall complexes are also the major source of “non-extractable polyphenols” such as studied by Perez-Jimenez & Saura-Calixto (Perez-Jimenez et al., 2013; Arranz et al., 2010), and are indeed a very common artefact explaining detection of polyphenols in plant cell walls or “antioxidant polysaccharides” by numerous authors.

A special notice should be taken for the existence in the cell walls of polysaccharides which are biosynthetically esterified by hydroxycinnamic acids, primarily ferulic acid. Feruloylated arabinoxylans are present in cereals (with higher concentrations in the bran) (Saulnier and Thibault, 1999) and feruloylated pectins in some Chenopodiaceae such as sugar-beet (Saulnier and Thibault, 1999; Fry, 1983) or quinoa (Renard et al., 1999). They can contribute to antioxidant properties of derived foods, with marked effect of particle size. However it should be kept in mind that presence of feruloylated polysaccharides is the exception and not the rule for primary plant cell walls. Lignin would also be an unextractable polyphenol, but it is not normally encountered in significant amounts in Human foods as lignin deposition is a sign of plant cell wall secundarization leading to hard textures, such as in wood, straw or bran.





Classification and typical structures of major food polyphenols



**Figure 2** Chemical formulae of some major dietary antioxidants in plant foods.

### Transfer of Polyphenols From Plant Tissue to Beverages

The main technological impact of the interactions between polyphenols and cell walls (or other polysaccharides) concerns proanthocyanidins and the extraction of juices. There is a marked discrepancy between the amounts of proanthocyanidins present in the fruits and the low concentrations often found in the juices or musts (Renard et al., 2011; Brahem et al., 2017; Le Bourvellec et al., 2007; Bautista-Ortin et al., 2015; Guyot et al., 2003). Not only the concentrations but also the structures are different: the extracted proanthocyanidins generally have much lower degrees of polymerization. There has been attempts to link the evolution of cell walls (notably in grape skin) with the extraction of tannins (Bindon et al., 2010a, 2012; Bautista-Ortin et al., 2015a,b, 2016). In pear or apple, which are simply pressed after grinding, the extraction decreases with increased maturity levels (Brahem et al., 2017). In wine phenomena are more complex due to the importance of the diffusion of polyphenols from skin or seed during maceration and fermentation (Bindon et al., 2010, 2012; Bindon and Kennedy, 2011).

### Bioaccessibility and Colonic Fermentation

Bioaccessibility of polyphenols in relation to their health effects is also impacted by binding to plant cell walls (Saura-Calixto et al., 2007). Many polyphenols have low to very low bioavailabilities even in pure form (Landete, 2012), and binding to plant cell walls or to plant cell wall components such as pectin further decreases their bioaccessibility. However there is strong evidence that the colonic fermentation metabolites (Dall'Asta et al., 2012) play a major role in the health effects of many polyphenols (Jaganath et al., 2006). For flavonoids, colonic fermentation metabolites, notably ring fission metabolites such as hydroxyphenyl- $\gamma$ -valerolactones, are efficiently absorbed. Presence of plant cell walls facilitates colonic fermentation of polyphenols, probably by providing substrates for bacterial growth (Aura et al., 2005; Perez-Jimenez et al., 2013; Bazzocco et al., 2008; Saura-Calixto, 2010). Therefore it is likely that the interactions between polyphenols and plant cell walls are favorable rather than detrimental when taking into account the whole picture and the whole gut.

### Structure–Affinity Relationships

The affinity of polyphenols for plant cell walls increases with molecular weight (specially for oligo and polymeric proanthocyanidins) and number of hydroxyl groups of the polyphenols (Le Bourvellec et al., 2004; Le Bourvellec and Renard,

2005; Renard et al., 2001). Galloylation increases the affinity, but less than molecular weight increase, as observed by comparing prodelphinidins and procyanidins (Le Bourvellec et al., 2004). The nature of the interactions is not totally elucidated yet: both hydrophobic and hydrogen bonds are involved for proanthocyanidins (Le Bourvellec and Renard, 2012; Watrelot et al., 2013, 2014; Le Bourvellec et al., 2004). In addition for anthocyanins ionic bonds might form between the positively charged anthocyanins and the negatively charged pectins (Padayachee et al., 2012; Buchweitz et al., 2012a,b). Concerning plant cell wall polysaccharides, highest affinities are found for pectins, and specially highly methylated pectins (Watrelot et al., 2013), but cellulose is also involved (Phan et al., 2015, 2016, 2017).

## Bio-Encapsulation of Carotenoids by Plant Cell Walls

### Carotenoids

Common dietary carotenoids are highly hydrophobic C40 tetraterpenoid pigments (from yellow to red) (Fig. 2) (Saini et al., 2015). They are subdivided in 2 classes: carotenes, which are exclusively hydrocarbons ( $\beta$ -carotene and lycopene are the main molecules in foods) and xanthophylls, which are oxygenated (such as lutein). They are located in the plant cells inside plastids: they are found in chloroplasts of leaves and accumulate in chromoplasts of fruit and vegetables where they are embedded within a complex organisation of lipids and proteins with very diverse substructures (crystalline, globular, fibrillar, membranous, tubular) (Schweiggert and Carle, 2017; Schweiggert et al., 2012). Their antioxidant properties are linked to the polyene chain and the presence of functional groups (hydroxyl or carbonyl) in the terminal ring (Kopeck and Failla, 2017; Rodriguez-Amaya, 2010).

### Bioaccessibility of Carotenoids

Carotenoids are better absorbed from processed fruit and vegetables, and many investigations have been carried out in the last ten years to understand the mechanisms (Castenmiller and West, 1998; Desmarchelier and Borel, 2017; Nagao, 2014). There are many barriers to dissolution of carotenoids from within a plant matrix, which explain why very low bioaccessibilities are reported. To become bioaccessible, they must be liberated from the food matrix to dissolve in the fat phase of the gastro-intestinal lumen. This is a prerequisite for their incorporation in bile salt mixed micelles produced by digestion of triglycerides and phospholipids, and which may reach the intestinal mucosa for absorption. An important point is that the effects of bio-encapsulation also vary with the carotenoid, and in particular with its hydrophobicity. In the same food matrix, xanthophylls are generally much more bioaccessible than carotenes.

Carotenoids bioavailability is further influenced by many factors which will not be treated here, such as the carotenoid itself and its conformation, the presence of other effectors such as naringenin, and the host's nutritional status and genotype (Humans have difference expression of receptors for carotenoids) (Desmarchelier and Borel, 2017; Borel, 2003; Borel et al., 2005; Reboul et al., 2006).

Carotenoids are found in diverse physical forms inside plastids, where they may be dissolved in lipids but also form more of less complex aggregates or associate with proteins. Next, the hydrophilic cell walls of the individual cell but even more of the neighbouring cells forming a plant tissue constitute natural barriers with a porosity which is not compatible with the size of the lipid droplets. Carotenoids liberation from plant tissues is thus limited by their structural features and by at least two levels of bio-encapsulation, by substructures of the plastids and by cell walls. In the next paragraph only encapsulation by the cell walls will be detailed, as it has been demonstrated that cell breakage is the most efficient step in increasing bioaccessibility. This has been studied in detail notably by Hendrickx and Van Loey's group in relation with diverse food processing techniques.

### Food Processing and Bioaccessibility of Carotenoids

Thermomechanical processes appear to be the most efficient to increase carotenoid bioaccessibility (Lemmens et al., 2011; Livny et al., 2003; Knockaert et al., 2011). Bioaccessibility decreases as the tissue structure increases: enriched oils > chromoplast fractions > small cell clusters > large cell clusters (Palmero et al., 2013). Decreasing the size of cell clusters facilitates carotenoids bioaccessibility, which has been demonstrated with raw carrot tissues to be proportional to the available surface of the particles (Lemmens et al., 2010; Tydeman et al., 2010). Heat treatments also generally lead to increased bioaccessibility of carotenoids, though some conflicting observations are reported for example for lutein from spinach (Faulks et al., 2004) or when intact cell ghosts are generated (Tydeman et al., 2010a, b). The sequencing of homogenization and cooking (homogenization prior to or after cooking) has contrasting impacts: for example while homogenization prior to cooking leads to higher bioaccessibility from sweet potato (Bengtsson et al., 2009, 2010), cooking prior to homogenization increases bioaccessibility for tomato (Page et al., 2012). Heat treatments rupture the natural barriers to carotenoid bioaccessibility and enhance cell cluster dissociation. This has been explained by pectin degradation, which may lead to cell breakage, facilitates cell separation and increases cell wall porosity. Ripening of fruits, which is also characterized by softening caused by pectin degradation, also increases carotenoid bioaccessibility (Ornelas-Paz et al., 2008; Victoria-Campos et al., 2013). By contrast, high pressure processing decreases carotenoid bioaccessibility (Gupta et al., 2011; Barba et al., 2017). High pressure processing better preserves tissue structure, by favouring pectin reticulation through action of endogenous pectinmethylesterases that liberate free galacturonic acids which form a calcium network, thus strengthening cell walls and intercellular adhesion (Van Buggenhout et al., 2009; Duvetter et al., 2009).

Recent experiments with non-thermal food processing technologies mitigate the importance of bio-encapsulation by more or less intact plant cell walls for carotenoid bioaccessibility. High pressure homogenization consists in forcing a fluid through a narrow valve. This generates strong shear stresses, cavitation and turbulence that rupture cell walls and decrease the size of particles in plant tissue suspension below that of intact cells. However, high pressure homogenization only has slight effects on carotenoid bioaccessibility, and even decreases the bioaccessibility of carotenoids added in oil (Mutsokoti et al., 2015; Palmero et al., 2016; Knockaert et al., 2012; Panozzo et al., 2013; Svelander et al., 2011). This was also observed when high pressure homogenization was applied to oil droplets in pectin-containing suspensions, specially with pectins of low degree of methylation (Aschoff et al., 2015; Sriwichai et al., 2016; Verrijssen et al., 2016). A similar effect i.e. decrease of bioaccessibility in spite of cell rupture is observed with ultrasound treatments (Anese et al., 2015). This phenomenon was ascribed to formation of a fibrillar network or gel during food processing. Thus, plant cell walls and their solubilised pectins may decrease bioaccessibility of carotenoids which were not initially encapsulated in plant cells, by formation of a new network or more generally by increased viscosity slowing the complex mechanisms by which carotenoids are transferred to mixed micelles.

## Conclusion

Because most dietary antioxidants are of plant origin, they can come in contact with plant cell walls at various stages: during processing, where plant cell walls act to limit their extractability, during digestion, where plant cell walls or their derived dietary fibers limit bioaccessibility, or during colonic fermentation where both are modified by the gut microbiota. Sequestration and protection can both be found.

Although the first decade of the XXI<sup>st</sup> century has seen increased awareness of these interactions, and more generally of the role of food structure on nutrition, there are still many unanswered questions. Major complexity is linked to the nature and diversity of the plant cell walls. Although the main cell wall constituents are by now well identified, their precise structures and interactions are still only basically understood. For example, knowledge on plant cell wall porosity and its evolution relies on very few data. Different plant cell walls are synthesized in different plant species, varieties and tissues, and they evolve differently during growth, maturation and senescence, to say nothing of food processing. All of this will affect their physicochemical properties (porosity, hydrophobicity, swelling) and capacity to interact with antioxidants.

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## Interactions of Some Common Flavonoid Antioxidants

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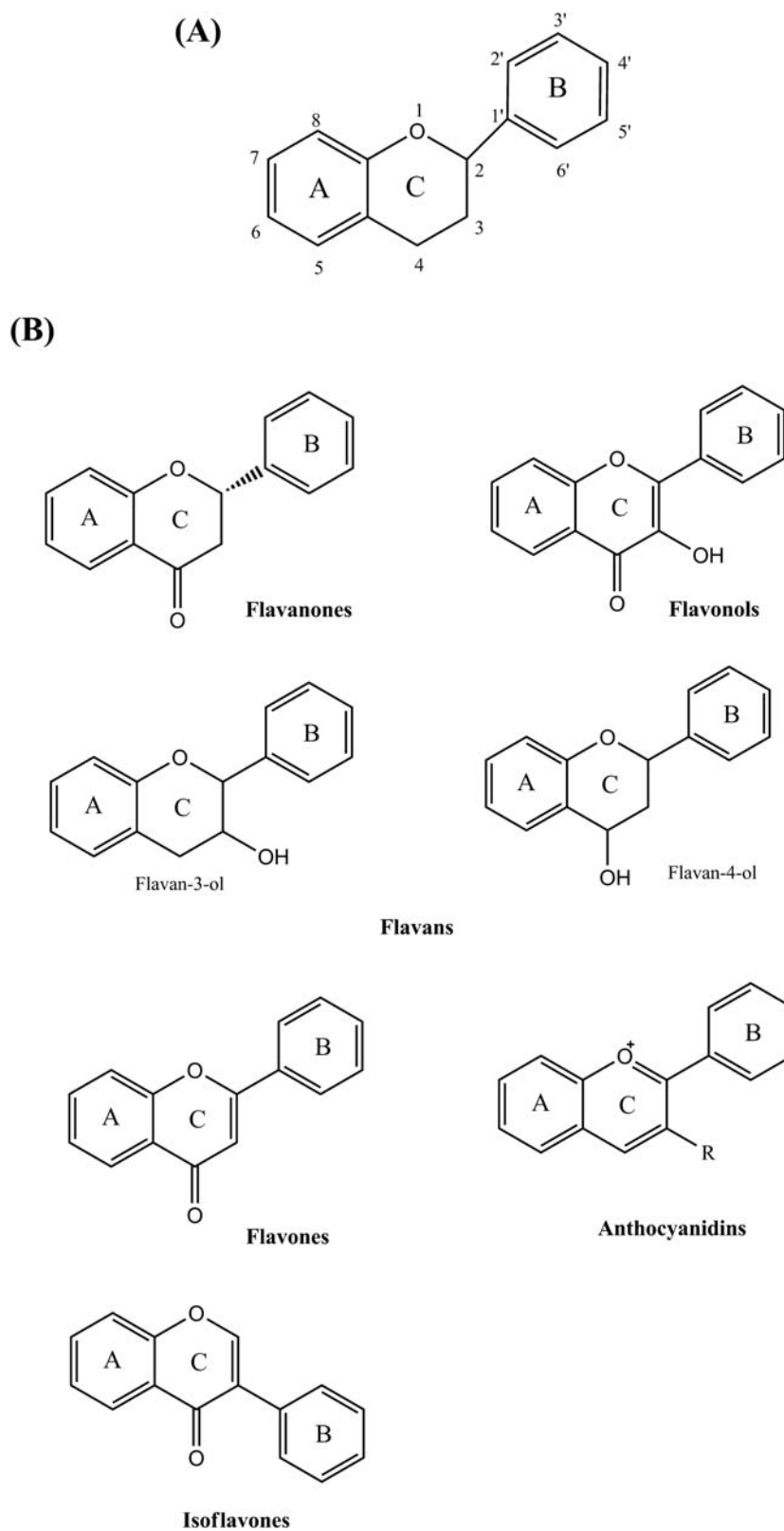
### Introduction

Since the free radical theory of ageing was proposed by Harman (1956), a broad range of investigations have been conducted on the relationship between oxidative damage and various degenerative diseases and have led to various publications in the scientific literature. Reactive oxygen species (ROS), including superoxide anion radical ( $O_2^{\cdot-}$ ), singlet oxygen ( $^1O_2$ ), hydrogen peroxide ( $H_2O_2$ ) and the highly reactive hydroxyl radical ( $\cdot OH$ ), are formed as natural by-products of the normal metabolism of molecular oxygen. ROS behave like a double-edged sword in the prevention and promotion of some diseases. Normally ROS exist in all aerobic cells in a physiological balance with biochemical antioxidants, and are essential signalling molecules of certain normal biological and physiological processes such as cellular responses to noxia, immune responses, and defences against infectious agents. Excessive ROS production in the absence of adequate antioxidant defences would lead to an imbalance between prooxidant load and body's antioxidant defence causing nutritional and physiological oxidative stress. Over accumulation of ROS cause oxidative damages to cellular membranes, structure and biomolecules such as proteins, lipids, carbohydrates and DNA, resulting in many degenerative diseases or disorders such as inflammation, cardiovascular diseases, cancers ischaemia-reperfusion injury and ageing. Therefore, in the past decades, a great deal of interest has been directed towards searching potent antioxidants, particularly those naturally occurring species such as polyphenols like flavonoids, that have the ability to minimize or prevent oxidative damages by preventing or inhibiting ROS formation and by quenching the generated ROS. Efforts have also been made to promote the health-promoting properties and counteract the disadvantages of polyphenols in practical applications, through exploring effective preservation of polyphenols or examining desired synergies between antioxidants under simulating or real handling, manufacturing and digestive conditions.

### Chemical Structure and Antioxidant Activity of Flavonoids

Flavonoids consist of a large group of secondary plant metabolites that are mainly synthesized from the aromatic amino acid phenylalanine (via the shikimic acid pathway) and acetic acid or their derivatives. So far, more than five thousand of flavonoid compounds have been identified. Flavonoids have received increasing attention mainly owing to their intrinsic structures and properties. Flavonoids contain three phenolic rings (two benzene rings, A and B rings; one heterocyclic pyrane ring, C) and present a unique benzo- $\gamma$ -pyrone structure (Fig. 1). The differences in substitution on three rings (e.g. the type, number, distribution and orientations in space) lead to various flavonoid derivatives with distinct structures and properties such as those commonly included in human diets like flavonols, flavones, anthocyanidins, flavanols (catechins), flavanones and isoflavones. Flavonoids occur as glycoside and methylated derivatives besides aglycones with the majority as glycosides in plants (e.g. arabinosides, galactosides, glucosides, rhamnosides and xylosides), except for catechins (i.e. mainly in the form of aglycones) (Smith et al., 2000). The chemical structures of flavonoids determine their modes of molecular action such as delocalization of electrons, rearrangements of molecules, ROS scavenging, intra-/inter-molecular hydrogen bonding, metal ion chelation, steric effects and electronic properties. The geometry and electronic properties are greatly affected by the position of OH groups in flavonoids. O-Methylation of the hydroxyl groups of flavonoids generally leads to reduced radical scavenging capacity. Procyanidins with a higher degree of polymerization tend to exhibit greater effectiveness against various radical species.

Epidemiological, animal and clinical studies have linked the consumption of flavonoid-rich diet with a reduced risk of cardiovascular diseases, and indicated the underlying mechanisms involving the potent antioxidant capacity, improved endothelial function, anti-thrombotic effect and lipid-lowering property of various flavonoids. Dietary flavonoids act as important endogenous antioxidants to mediate cardioprotective effects (Stangl et al., 2007), as shown in chemical-based and cellular assays as well as *in vivo* experiments. Certain flavonoids exhibit higher antioxidant activity than some commonly known antioxidants such as vitamins C and E (Prior and Cao, 2000). The biological behaviours and activities of flavonoids depend on their chemical structures (including basic structure, configuration, number and distribution of hydroxyl groups and type and degree of substitution of functional groups) as well as physicochemical characteristics such as molecular size, solubility,  $pK_a$  and lipophilicity. Poor solubility of flavonoid aglycones in water, along with their short residence in the intestine and low absorption, presents minimal risks to humans in terms of irritancy, allergenicity and toxicity. Flavonoid glycosides generally have greater absorption than corresponding aglycones e.g. the absorption of quercetin glucosides could be twice as much as quercetin in humans (Hollman et al., 1995). The dietary flavonoids liberated from foods, depending on chemical structures, may be absorbed from the small intestine or transported to the colon to undergo molecular changes before absorption. Oligomeric flavonoids may be broken down to monomers or dimers under the acidic environment in the stomach. Hydrophilic flavonoid glycosides such as quercetin glucoside may be transported across the small intestine by the intestinal  $Na^+$ -dependent glucose cotransporter (SGLT1), hydrolysed by  $\beta$ -glucosidases (e.g. lactase phloridzin hydrolase) outside the brush border membrane of the small intestine, or hydrolysed by microflora in the colon (Day et al., 2000;



**Figure 1** Basic structure of flavonoids (A) and the chemical structures of six primary groups of polyphenols (B).

Hollman et al., 1999; Scheline, 1973). Isoflavones probably exhibit the highest bioavailability among the flavonoid family (Spencer et al., 2000).

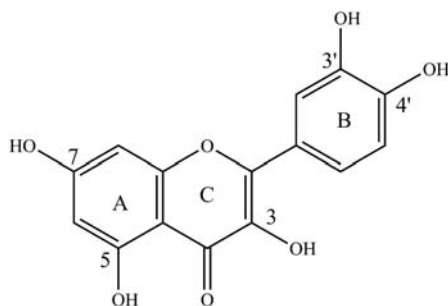
The bioactivities of flavonoids and associated mechanisms of action depend on not only their native form (aglycones or glycosides) but also their metabolites resulting from phase 1 and/or phase 2 metabolisms. Aglycones of flavonoids usually exhibit greater antioxidant capacity but lower bioavailability compared to their corresponding glycosides. The flavonoid products derived from phase 2 metabolism often exhibit lower antioxidant activity compared with the initially ingested flavonoids. The pathological mechanisms are involved in the antioxidant actions of consumed flavonoids through 1) upregulating or protecting the endogenous antioxidant defences e.g. inducing antioxidant enzymes; 2) inhibiting pro-oxidant enzymes such as oxidases; 3) inhibiting redox sensitive transcription factors; 4) mitigating oxidative stress caused by nitric oxide; 5) suppressing lipid peroxidation in the local biological environment; 6) complementing and/or enhancing antioxidant properties of small molecule antioxidants (Babu et al., 2006; Cabrera et al., 2006; Chan et al., 1997; Khan et al., 2006; Procházková et al., 2011; Suzuki et al., 2006; Vinson and Zhang, 2005). Further, the necessity of balance between the antioxidant and prooxidant characteristics of flavonoids enables them as multitarget agents to exert effective biological activities. Only a small proportion of flavonoids' therapeutic properties probably result from their direct antioxidant properties, with the majority of efficacy likely due to their pro-oxidant action and direct interaction with target biomolecules (Chen et al., 2000; Clifford, 2004; Crozier et al., 2009). Flavonoids can bind cellular targets with high sequence selectivity such as those planar molecules with an extended aromatic core (like quercetin) can bind to and stabilize human telomeric G-quadruplex DNA Tel7 or Tel22 sequence via end-stacking ( $\pi$ - $\pi$  stacking) and external binding (Tawani and Kumar, 2015). Flavonoids containing a 1,2-diphenol (catechol moiety), 1,4-diphenol (hydroquinone moiety) or 1,2,3-triphenol (galloyl moiety) would undergo oxidation upon ingestion and for flavonoid quinones. These flavonoid quinones possess electrophilic properties and can readily react with the thiols in protein Cys residues to form cysteinyl flavonoid adducts and induce cytoprotective proteins, which represents a major mechanism for their anti-inflammatory and anticancer effects (Awad et al., 2003; Crozier et al., 2009; van Zanden et al., 2003). Some flavonoids, although do not contain a catechol, hydroquinone or galloyl moiety, can still induce cytoprotective proteins and exhibit anti-inflammatory and anti-cancer properties, through transforming to intermediates like 1,2-diphenols by phase 1 enzymes and then further oxidized into quinones (Sahin et al., 2012; Yu et al., 2012). Further, flavonoids are well known natural pigments that can undergo excited-state intramolecular proton transfer (ESIPT) (via an intramolecular hydrogen bond between the C=O and 3-OH groups) and exhibit dual fluorescence behaviours and chromophore frame-based selective binding to proteins (Xiao et al., 2011). The ESIPT in flavonoids is a microenvironment-dependent process e.g. considerably sensitive to the external hydrogen-bonding interference from the surrounding environment.

### Insight Into the Antioxidant Interactions Involving Some Common Flavonoids

Various flavonoids co-exist in Nature and in human diets. The bioactivity of a natural mixture or a formulated combination of flavonoids is determined mainly by individual chemical structure and their mass ratio (rather than individual activity) as well as the interactions among these phenolic compounds. Flavonoids may interact with one another, or with other water-soluble and lipid-soluble substances because of their amphiphilicity, prior to consumption and after ingestion, digestion and absorption. Such interactions are rather complicated considering the diversity and complexity of the flavonoids in human daily diets and the variations in individual's metabolic conditions along with the whole digestion and absorption process. Therefore, the detected health-promoting properties of flavonoid-rich diets in *in vitro* or *in vivo* studies resulted from different interactive effects, additivity, synergism or antagonism, which reflect the combined effect owing to the interaction of two or more flavonoids is equal, greater and lesser than the theoretical sum of the separate effect of each flavonoid, respectively. Some examples of such interactions between flavonoids are provided as follows.

#### Quercetin and Other Flavonoids

Quercetin (3,5,7,3',4'-pentahydroxyflavone) is a member of the flavonol family and has two aromatic rings (A and B) linked by an oxygen heterocycle (ring C) (Fig. 2). It accounts for approximately 60%–75% of total flavonoid intake (Sampson et al., 2002).



**Figure 2** Chemical structure of quercetin.

Accumulating evidence has shown that quercetin is a powerful antioxidant with a potent antiradical property towards hydroxyl/peroxyl radicals and superoxide anions, and possesses a range of biological effects including anti-inflammatory, antineoplastic and cardio-protective activities. The antioxidant properties of quercetin are greatly attributed to its ability to decrease the amount of ROS by inhibiting ROS formation and promoting ROS removal, through mechanisms such as inhibition of various ROS-generating enzymes, chelation of metals involved in ROS generation, down-regulation of expression of relevant enzymes, up-regulation of expression of endogenous synthesizing enzymes, inducing of the synthesis of ROS-removing enzymes, and/or direct ROS scavenging action via single-electron transfer and hydrogen atom-donation. Both synergic and antagonistic effects were found for the combinations of quercetin and other flavonoids. Quercetin, epicatechin, rutin and resveratrol in red grape exhibited a dose-dependent synergy in antiradical effect towards ONOO<sup>-</sup> (Iacopini et al., 2008). The combination of 25 µM catechin and 5 µM quercetin, although using either alone had no effect on platelet function, could act synergistically to inhibit platelet adhesion to collagen and collagen-induced platelet aggregation by virtue of their antioxidant effect (Pignatelli et al., 2000). In the ferric reducing antioxidant power (FRAP) assay, the combined use of epicatechin and quercetin-3-β-glucoside exhibited the greatest synergistic effect whilst myricetin along with quercetin resulted in an antagonistic effect. Quercetin and epicatechin may provide a synergistic neuro-protection against ischaemic injury via activation of Akt- and Ca<sup>2+</sup>-mediated signalling pathways (Nichols et al., 2015). A negative synergistic effect on the antiradical activity was reported for an ethanol solution containing catechin, resveratrol and quercetin (Pinelo et al., 2004). No synergistic interaction but additive effects on vascular function were found between quercetin and catechin in systemic and pulmonary vascular smooth muscle (Menendez et al., 2010). Strong antagonistic effects were found on *in vitro* radical scavenging capacity when quercetin-3-glucoside was paired with the five anthocyanins (Hidalgo et al., 2010).

### Flavonoids and Tocopherols

Tocopherols (also called methylated phenols) belong to the vitamin E family and occur in α, β, γ and δ forms (which differ in the number and position of methyl groups on the chromanol ring). They are lipid-soluble antioxidants and can effectively prevent oxidative damage to lipids. Once being oxidized to tocopheroxyl radicals, tocopherols can be regenerated by ascorbate alone or in co-operation with certain flavonoids like quercetin. Green tea catechins and procyanidins were found to regenerate α-tocopherol via one-electron reduction of α-tocopheroxyl radicals (Pazos et al., 2007). Catechins might reduce the consumption of α-tocopherol to retain α-tocopherol as a scavenger within cell membranes while scavenging the aqueous peroxy radicals near the membrane surface (Zhu et al., 1999). In terms of the structure-activity relationship of the tocopherol-regeneration reaction by catechins (EC, ECG, EGC, and EGCG), Mukai et al. (2005) found that anionic character of catechins was positively correlated to the reaction rates of catechins, with the monoanions at catechol B- and resorcinol A-rings as well as dianions at pyrogallol B- and gallate G-rings exhibiting the highest free radical scavenging activity.

The bioactivity outcome of the interactions between flavonoids and tocopherols varies with the flavonoid species possessing different chemical structures and degree of lipophilicity. Significant antioxidant synergisms were found between α-tocopherol and catechin monomers or oligomers (procyanidins) in antioxidant assays (Arnous et al., 2001; Vinson et al., 2001). Quercetin in combination with vitamins (C and E) significantly ameliorated cadmium-induced oxidative stress and anomalies in renal biochemical and histological indices (Milton et al., 2010). The presence of flavonoids in the macrophages could conserve the content of α-tocopherol while delaying the onset of lipid peroxidation (Middleton et al., 2000). Tea catechins can take part in vitamin E recycling to complement some functions of glutathione antioxidant that is essential for cellular protection (Zhu et al., 1999).

### Interactions of Some Flavonoids Presented in the Same Fruit

Epidemiological studies have shown a strong association between the consumption of whole fruits with a range of potential health benefits. Thus, it is of interest to understand what natural polyphenols in fruits may exert such biological effects, and whether or not these purified antioxidants act alone or in a combination and behave in the same way. Recently, antioxidant extracts have been obtained from certain flavonoid-rich fruits, or dietary flavonoids that naturally occur in these fruits have been isolated, for the examinations on the possible interactions of flavonoids. For example, tart cherry (*Prunus cerasus*) fruit is rich in a variety of flavonoids antioxidants (such as cyanidin derivatives, kaempferol, quercetin and isorhamnetin and their derivatives), and the health benefits of tart cherries are likely associated with interactions of these compounds (Seymour et al., 2008). The pairs of antioxidant compounds exhibiting the highest antioxidant capacity and strong synergism included kaempferol and melatonin at a dose ratio of 2:1, and cyanidin 3-rutinoside and isorhamnetin 3-rutinoside at a dose ratio of 1:4, whereas, other pairs of antioxidants had lower antioxidant capacities and exhibited either additive or negative effect (Kirakosyan et al., 2010). Grapes are berries rich in natural antioxidants, among which the polyphenol compounds exhibit a range of health benefits. Extracts were produced from grape seed (GSD, which contained polygalloyl polyflavan-3-ol accounting for 47.16% of GSD's total phenolic content) and from grape skin (GSK, which contained anthocyanins, flavonols, polygalloyl polyflavan-3-ol and hydroxycinnamic acids accounting for 28.18% of GSK's total phenolic content). *In vitro* incubation experiments with human platelets and *ex vivo* feeding studies in dogs found that maximal antiplatelet benefits were achieved only when GSD and GSK were combined, whilst the concentration of GSD or GSK exerted little or no effect on platelet activity when either extract was used alone (Shanmuganayagam et al.,

2002). Accordingly, the combined use of these two extracts can provide the health benefits of grape polyphenols at a relatively low daily dose compared to use of one extract alone.

## Conclusion

Flavonoids are one of the most studied phytochemicals due to their diverse health benefits. Flavonoids have varying chemical structures. Such structural differences determine their biological behaviours and the interactions among flavonoids or between flavonoids and other non-flavonoid antioxidants from the same whole fresh foods like fruits. The bioactivities of flavonoids depend on not only their native state (aglycones or glycosides) but also their metabolites. The findings associated with the structure-bioactivity relationships and interactive effects of flavonoids obtained previously were mostly based on purified or isolated flavonoids. These results could only provide some evidence of a more basic character, but probably not transferable for real food matrices. Therefore, more investigations are required on the interactions of bioactive substances including flavonoid–flavonoid or flavonoid–non-flavonoid interactions in a near native state to obtain a better understanding of the health benefits associated with the consumption of flavonoid-rich diets. Future design of new dietary supplements or fortified foods should select the combination of flavonoids with the most desirable properties.

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# Polyphenol Interactions and Food Organoleptic Properties

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## Introduction

Polyphenols are present in all vegetables, fruits and derived foodstuffs, e.g. coffee, beer, tea and wine. These compounds are referred as the phytonutraceuticals of the century due to important biological properties linked to their intake, e.g. cardiovascular protection and antioxidant activity (Valavanidis and Vlachogianni, 2013).

Besides health-promoting properties, polyphenols are also associated with some sensory properties of foodstuffs, namely colour and taste. Regarding colour, anthocyanins are one of the most important natural pigments but they present a problem due to their high instability. Regarding taste, polyphenols are responsible for some unpleasant organoleptic properties, namely astringency and bitterness. Astringency is a tactile sensation defined as dryness, tightening and puckering sensations perceived in the oral cavity during the ingestion of astringent molecules, mainly tannins, alums and some metal ions. It is desired if these molecules are in balanced levels for some food products such as red wine, being a parameter of wine quality, but it is unpleasant when perceived in excess. Currently, oral astringency onset is mainly explained by the precipitation of salivary proteins by tannins. Bitterness is a taste and so it is perceived via activation of specific bitter taste receptors (TAS2Rs) by some polyphenols. Usually, excessive bitterness is also undesirable.

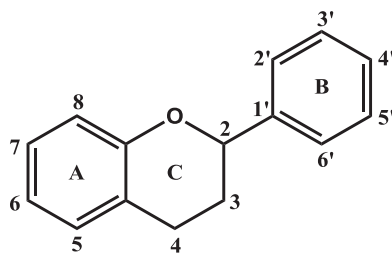
Polyphenols are widely known to interact with many different compounds, both with food compounds, such as carbohydrates, lipids and proteins, as well as with biological compounds, namely proteins. Interactions between polyphenols and molecules from food are mostly based on non-covalent hydrophobic interactions but in the cases of interactions between polyphenols and proteins or/and carbohydrates, hydrogen bonds also contribute significantly. Furthermore, some covalent bonds may also occur under certain food processing conditions. All these interactions can influence the overall sensory perception of foodstuffs and consumer acceptance. This article revisits the current knowledge about these interactions at a molecular level, how these interactions could affect the sensory properties, e.g. taste, colour, texture, with the purpose to modulate them toward increasing consumer acceptance and intake of healthy foodstuffs rich in polyphenols.

## Polyphenols

Polyphenols are usually divided into two major groups: flavonoids and non-flavonoids. Flavonoids share a common flavanic core (Fig. 1) and are further divided into several classes such as flavanols [(epi)catechin], flavonols (e.g. myricetin and quercetin), anthocyanins (e.g. malvidin-3-glucoside), flavanones, flavones and isoflavones; non-flavonoid compounds include phenolic acids, phenols and stilbenes. These classes differentiate according to the degree of unsaturation and degree of oxidation of the heterocyclic pyranic ring C (Fig. 1). Flavonoids are the most relevant and widely present polyphenols in the human diet ( $\frac{2}{3}$  of ingested polyphenols are flavonoids) (Scalbert and Williamson, 2000). Among flavonoids, the most consumed groups are flavanols and anthocyanins (Rasmussen et al., 2005).

Flavanols differ in the stereochemistry of C3 of ring C and pattern of hydroxylation of ring B (Fig. 1). They share a structural unit of (epi)catechin and can range from monomers to polymers (proanthocyanidins). Conversely to other flavonoids classes, flavanols usually occur as aglycone molecules without sugar molecules.

Anthocyanins are the main pigments in many flowers and fruits (e.g. grapes) responsible for the great diversity of colours (i.e. red, violet and blue) found in nature and in some foodstuffs. In aqueous solution, the colour of anthocyanins is dependent on their structure and also strongly affected by the pH (Brouillard and Lang, 1990). At acidic pHs (pH~1), anthocyanins are present in their flavylium cation form which has a red colour. As the pH increases, anthocyanins can be present in other different forms yielding from the violet quinonoidal base to the non-coloured hemiketal form or to the *trans*-chalcone forms which present a yellow colour (Pina, 1998).



**Figure 1** Structure of the flavanic core common to flavonoids.

Another common designation to some polyphenols is tannins. Tannins comprise a group of usually structural complex polyphenols that share the ability to interact with proteins. Tannins are usually divided in two major subgroups: condensed and hydrolysable tannins. Condensed tannins (proanthocyanidins) are polymers of flavanols, usually gallo- or (epi)catechin units, which can form very complex and high-molecular weight structures. Hydrolysable tannins are further divided into gallotannins and ellagitannins. They are esters of gallic acid or hexahydroxydiphenic acid, respectively, with a polyol, usually glucose.

In summary, polyphenols present a high structural diversity and possess a significant number of hydroxyl groups and planar rings, which makes them susceptible to a wide range of interactions. Therefore, polyphenols can interact with one another and with other types of molecules that surround them.

## Interactions Between Polyphenols and Other Food Compounds

Lipids, proteins and carbohydrates are major food compounds often found together with polyphenols in the same food matrices. An excellent review on these interactions and their biological effects was published by Jakobek (2015). Herein, these interactions will be focused on a sensory perspective.

### Interaction With Food Lipids

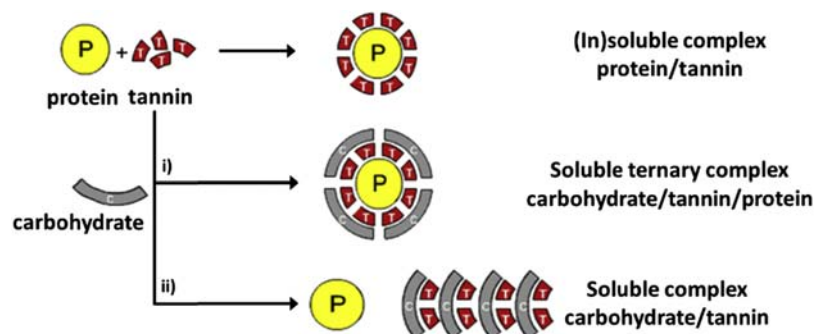
Lipids affect flavour perception by their effects on aroma, taste and mouthfeel. Surprisingly, only a few studies have investigated the effect of dietary lipid–polyphenol interactions on taste. Dietary fats go through a digestion process that starts with mastication in which lipids are often transformed into emulsions. The particle size of fat droplets present in these emulsions is important since it influences the rheological properties, texture, shelf life and appearance (Dapčević Hadnadev et al., 2013). The association of polyphenols with milk fat globules has been widely studied. Several studies have been focused on the interaction associated with tea catechins and this interaction as shown to affect the size and the surface properties of milk fat globules (Rashidinejad et al., 2016). In general, the globules seem rougher and larger in size in the presence of catechins, and sharp and rough particles are known to cause more friction than rounder particles (De Wijk and Prinz, 2006).

Considering dietary lipids, it is inevitable to refer oxidation, which is an expected process in fat rich products, such as olive oil, leading to deterioration that can become more pronounced during oil storage. Initially, lipids are radically oxidized to hydroperoxides, which are odourless and tasteless. However, cleavage of these products gives rise to various volatile compounds, known as secondary oxidation products, which are responsible for typical unpleasant sensory characteristics of fat-rich foodstuffs. Oxygen, light, temperature, metals, pigments, unsaturated fatty acid composition, as well as the quantity and kind of natural antioxidants, are all factors that can influence the free radical mechanism of the autoxidation process but in a different manner. The addition of phenolic extracts to olive oil to improve oxidative stability is not straightforward as expected, especially in the presence of hydrocolloids, where their interactions with hydrocolloids can exert a significant influence and lead to different creaming rates, turbidity and rheological behaviours, but can also have pro-oxidant effects under certain conditions (Caporaso et al., 2016). Shishikura et al. (2006) studied the emulsification process of a model emulsion from olive oil and phosphatidylcholine, *in vitro*, and the influence of polyphenols on this process. It was found that green and black tea polyphenols affected the emulsions by increasing droplet size and decreasing specific surface area. Phosphatidylcholine possesses hydrophilic heads which face the exterior of the emulsion droplet. Polyphenols have many hydroxyl groups and may interact with phosphatidylcholine creating complexes. Furthermore, polyphenols may have acted as a linker between complexes to form complex aggregates, thus increasing droplet size.

### Interaction With Food Carbohydrates

Food is also usually rich in carbohydrates and the interaction of some of these compounds with polyphenols is well reported. The first ideas that some polyphenols interact with carbohydrates arise to explain fruit ripening process and subsequent loss of astringency. This interaction has also been the justification to explain the decrease of anthocyanins extracted during red wine making process.

Fruit ripening process includes several physicochemical and sensory changes, namely texture changes where fruits become softer and smoother, with reduction of astringency and bitterness (Prasanna et al., 2007). A typical example of these changes is persimmon. One hundred years ago, Lloyd and co-workers (Lloyd, 1911) suggested that the decrease in persimmon astringency was related to the loss of tannins' solubility due to their interaction with carbohydrates presented in tannin-rich cells. Recent evidence revealed that during grape ripening, tannins bind to cell wall carbohydrates (Braidot et al., 2008). Texture changes during fruit ripening result from the enzymatic degradation of structural carbohydrates of cells wall, namely pectins, hemicellulose and cellulose, and also storage carbohydrates (Prasanna et al., 2007), causing the release of small soluble fragments of carbohydrates. So, another hypothesis for reducing astringent sensation is that these small, soluble fragments would be able to inhibit the interaction of tannins with salivary proteins (Luck et al., 1994) via two mechanisms (Luck et al., 1994; Ozawa et al., 1987; Mateus et al., 2004) (Fig. 2): (i) carbohydrates form ternary complex protein–polyphenol–carbohydrates, which increase solubility in an aqueous medium; (ii) molecular association occurs in solution between carbohydrates and polyphenols hence competing for salivary protein aggregation.



**Figure 2** Possible mechanisms to explain the effect of carbohydrates on restraining the formation of tannin/protein aggregates (Mateus et al., 2004).

Polydextrose (Ares et al., 2009) and carboxymethylcellulose (Troszynska et al., 2010; Smith et al., 1996) can be used to reduce the astringent properties of aqueous tannin solutions and polyphenol-rich plant extracts. Arabic, guar and xanthan gums also demonstrated a significant capability to reduce astringent sensation (Troszynska et al., 2010). In general, pectins are the most effective carbohydrates in restraining the interaction between tannins and proteins (Le Bourvellec et al., 2005; de Freitas et al., 2003; Soares et al., 2012), and their high affinities to tannins resulting from their ability to develop a gel-like network and form hydrophobic pockets to encapsulate procyanidins (Le Bourvellec et al., 2005). Whereas, filamentous and globular polysaccharides, like cellulose and xyloglucan, would bind procyanidins weakly. Another common trait is that acidic carbohydrates seem to have a more significant effect on decreasing astringency than neutral carbohydrates from the same origin (Vidal et al., 2004). Besides influencing astringency perception, the tannin-carbohydrate interaction also affects the physical properties of carbohydrates e.g. the interaction between amylopectin and tea polyphenols favours gelatinization by decreasing the gelatinization temperature and enthalpy of starch (which is possibly related to easier hydration of starch granules).

Besides tannins, anthocyanins were found to interact with carbohydrates. During wine making, the extent and yield of anthocyanin extraction depend not only on their concentration but also the carbohydrate composition of grape cell wall (Busse-Valverde et al., 2011). In fact, Romero-Cascales and co-workers found that the anthocyanin concentration in the grapes is not directly correlated with the final concentration in the resulting red wine and the barrier effect of carbohydrates polymers is likely the reason (Romero-Cascales et al., 2005; Ortega-Regules et al., 2006). Several studies with model carbohydrates (e.g. cellulose and cellulose-pectin mixtures) or those isolated from grape cells walls have shown their high affinity to anthocyanins and their effect on anthocyanins extraction (Padayachee et al., 2012). In general, acylated anthocyanins have greater affinity to carbohydrates than non-acylated forms. The physicochemical characteristics of carbohydrates, namely porosity, monosaccharide composition and hydrophobicity affect their interaction with anthocyanins. Differences in the galactose and arabinose contents along with variations in the cellulose and pectin esterification degree (the lower esterification, the greater interaction) affect the interaction with anthocyanins (Ortega-Regules et al., 2006; Buchweitz et al., 2013; Kopjar et al., 2009). Further, anthocyanins occur in different forms with different hue and intensity in colour (otherwise, colourless) at different pHs. In nature, copigmentation interactions can occur which promotes the stability of the flavylium form of anthocyanins and associated colour of anthocyanin-rich foodstuffs. Interaction between anthocyanins and pure carbohydrates (e.g. pectins, arabic gum or  $\beta$ -cyclodextrin) have been examined mostly on the pectin copigmentation effect i.e. a slight stability enhancement of anthocyanins colour (Chung et al., 2016; Howard et al., 2013). This effect was mostly ascribed to the intermolecular association between the carbohydrate and anthocyanins mainly through hydrogen bonds and hydrophobic interactions (Fig. 3) (Fernandes et al., 2014).

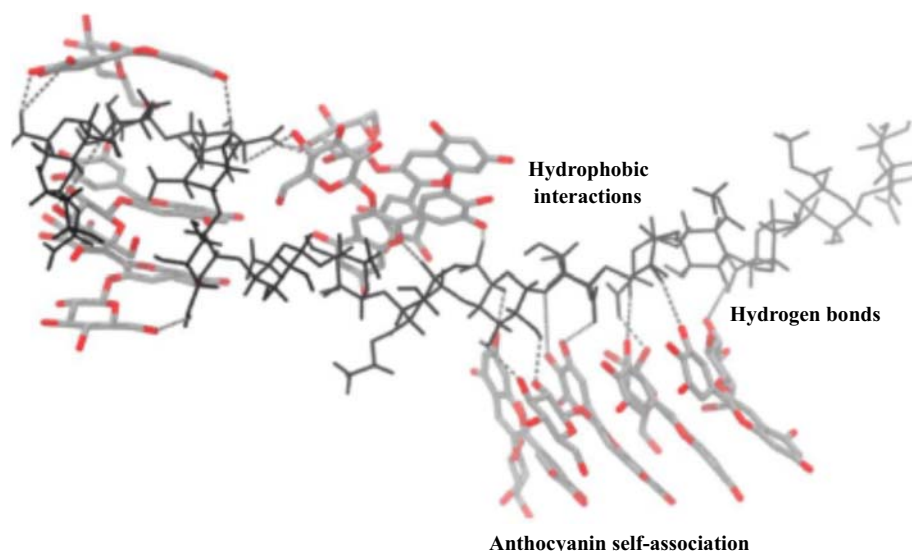
In summary, the studies discussed here showed that different polyphenols could bind to different food carbohydrates. Depending on the compounds these interactions could have positive and negative effects. The interaction of tannins with food carbohydrates can lead to an astringency and bitterness modulation while anthocyanins interaction with carbohydrates could lead for, on one hand, to a lower extraction yield, and therefore lower colour intensity on the final product, and on the other hand to stabilization and enhancement of anthocyanins colour.

## Interaction With Proteins

### Food Proteins

Plant phenolic compounds are known to interact with proteins producing changes in the food (e.g. biological value (BV), colour, taste).

Polyphenols are known to interact with proteins causing changes in food, e.g. biological value, colour and taste. In some foods, proteins and polyphenols can form soluble complexes with sizes similar to colloidal particles, causing turbidity of beverages and limited shelf life. In beer, wine and juice production, polyphenols may induce the development of protein precipitates causing undesired sensory properties in consumers' perception (Siebert, 1999; Siebert et al., 1996). Most of the studies on the interaction of polyphenols with food proteins examined milk proteins, soy proteins, myoglobin (meat protein), lysozyme (egg-white protein)



**Figure 3** Schematic representation of some interactions established between anthocyanins and a low methoxylated pectin model involving hydrophobic interaction and H bonding. Pectin molecule is depicted with sticks coloured in dark grey (Fernandes et al., 2014).

and gelatin. Beside noncovalent interactions like  $\pi$ - $\pi$  bonding (aromatic interaction), hydrogen bonding, hydrophobic or ionic interactions (Siebert, 1999; Siebert et al., 1996), it has been assumed that covalent bonding is likely to play an important role in protein-polyphenol interactions (Rawel et al., 2001; Beart et al., 1985).

Beside noncovalent interactions like p-p-bonding (aromatic interaction), hydrogen bonding, hydro-phobic or ionic interactions (Valavanidis and Vlachogianni, 2013; Scalbert and Williamson, 2000), it has been assumed that covalent bonding is likely to play an important role in protein-phenol interactions.

Polyphenols, in general, interact with globular proteins and can cause structural and conformational changes in proteins. Interaction of milk proteins with tea polyphenols induces structural changes in both whey proteins and ( $\alpha$ - and  $\beta$ -) caseins (Hasni et al., 2011; Kanakis et al., 2011). This interaction leads to reduced protein digestibility and altered antioxidant activity although no change in polyphenol structure (Stojadinovic et al., 2013; Xiao et al., 2011), as well as reduced tea astringency. An extensive study on the interactions of fifty-five dietary polyphenols with milk proteins revealed that the binding was strongly influenced by the structural features of polyphenols (Xiao et al., 2011). Methylation and methoxylation as well as glycosylation of flavonoids decreased the affinities to milk proteins. Hydroxylation on the rings A and B of flavones and flavonols enhanced slightly the interaction, whilst hydroxylation on the ring A of flavanones significantly improved the affinities. Galloylation of catechins significantly increased the affinity by 100–1000-fold. Glycosylation of resveratrol decreased its affinity for milk protein. Esterification of gallic acid increased its binding affinity. Soy proteins, especially soy glycinin and soy trypsin inhibitor, were shown to react with several phenolic acids (gallic, chlorogenic and caffeic acids), flavones (flavone and apigenin) and flavonols (kaempferol, quercetin and myricetin) giving protein derivatives. The secondary and tertiary structure of the proteins is changed as a result of these interactions, influencing their surface properties and making them hydrophilic in nature (Rawel et al., 2002). In the same way, myoglobin also suffers structural changes upon interaction with *o*-, and *p*-hydroxyphenols, *p*-quinone and gallic acids (Kroll and Rawel, 2001), causing lower digestion by tryptic,  $\alpha$ -chymotryptic, peptic and pancreatic proteases. Lysozyme is also modified upon its covalent attachment to certain phenolic compounds (*m*-, *o*-, *p*-dihydroxybenzenes, ferulic and gallic acid) (Rawel et al., 2001). The derivatization was accompanied by the reduction of solubility over a broad pH range and increase of hydrophobicity, with the isoelectric points shifted to lower pH values and high molecular weight fractions formed. Furthermore, the peptic digestion of the derivatized lysozyme was adversely affected, whereas the tryptic, chymotryptic and pancreatic hydrolysis seemed in favour.

Besides modifications on the biological value of these proteins due to reduced digestibility, changes in protein hydrophilic/hydrophobic properties are induced by the interaction with polyphenols causing altered protein solubility and different functional and sensory properties of food (e.g. emulsion- and foaming properties, gel building, etc.) (Rohn et al., 2006). Strong interactions occur between polyphenols and proteins with flexible and open structures (usually with a high proline content, e.g. gelatin) (Asano et al., 1982; Oh et al., 1980; Hagerman and Butler, 1981; Frazier et al., 2010). Gelatin is a mixture of polypeptides derived from collagen and often used as wine fining agent. In fact, fresh wine is a turbid, complex and physicochemically unstable hydroalcoholic solution. Its limpidity, which is highly appreciated and required by consumers, is achieved through several processes like natural sedimentation, filtration and centrifugation. Still, a limpid wine has to remain that way during the period of bottle ageing and storage. In white wine production, chemical oxidation of catechins and proanthocyanidins is the main source of browning, affecting the colour, aroma and taste of white wine (Spagna et al., 2000). Accordingly, clarification and stabilization of wines are mainly achieved through addition of proteins, a process known as “fining.” Protein components in gelatin bind to wine polyphenols to form complexes which would precipitate out of solution and thereby potential browning is minimized (Spagna et al., 2000; Sarni-Manchado et al., 1999). Maury et al. (2001) characterized the fining abilities of a commercial gelatin (average molecular



weight 25,000 Da) and gelatin fractions (16,000–190,000 Da) for tannin removal. Selective precipitation occurred between gelatin and highly polymerized or galloylated tannins, and more polymerized tannins was precipitated by the 16,000 Da gelatin than by the 190,000 Da gelatin. In addition, the gelatin-treated wines were significantly less astringent than the unfined wine. Another study also reported the significant differences caused by various hydrolysed gelatins in wine chemical composition and polyphenol profile, and a preference for the treated wines over untreated ones (Karamanidou et al., 2011).

It has been shown that the protein-polyphenol binding increases with the molecular size of the polyphenol molecule (de Freitas and Mateus, 2001) and is affected by polyphenol conformational flexibility (Haslam et al., 1992). The above-mentioned studies indicate that some structural features of polyphenol molecules are important for this kind of interaction: (i) molecular weight, (ii) structural flexibility and (iii) number of OH groups.

### Salivary Proteins

The most recognized mechanism for astringency perception involves the interaction between tannins and salivary proteins. In 1954 Bate-Smith (Bate-Smith, 1954) proposed that astringency results from the interaction of tannins with salivary proteins in the oral cavity, mainly proline-rich proteins (PRPs), and since this idea has been generally accepted and supported by numerous studies in the literature (Sun et al., 2013; Rinaldi et al., 2012; Kallithraka et al., 1998; Bennick, 2002; Mehansho et al., 1987; Soares et al., 2011). Moreover, some studies reported the differences in reactivity of several families of salivary proteins. HPLC and SDS-PAGE analyses demonstrated that when all the major families of salivary proteins were presented in a competitive assay, which simulated the oral cavity, condensed tannins interacted firstly with acidic PRPs and statherin, then with histatins, glycosylated PRPs and finally basic PRPs (Soares et al., 2011). It seems that *in vivo* astringency intensity and development with time may be related to the modifications of different salivary proteins. In the case of a low concentration of tannins for the first exposure to PRPs, the precipitation of acidic PRPs and statherin correlated with the intensity of astringency. However, for higher concentrations of tannins upon subsequent exposures, the relative astringency likely correlated to gPRPs precipitation. These results were reported for the first time that the several families of salivary proteins could be involved in different stages of astringency development (Soares et al., 2011; Brandão et al., 2014).

In summary, polyphenol–protein interactions might potentially influence the availability of certain amino acids and protein structure, which could affect the functionality and digestibility of proteins. However, the extent of this influence depends on the origin and nutritional quality of proteins, and food processing conditions and other co-existing food nutrients. Furthermore, polyphenols bind to salivary proteins inducing the perception of astringency in the oral cavity, whilst the binding of polyphenols to some other (food) proteins can modulate this perception. So, at the end, interaction of polyphenols with proteins, as one of the most important interactions, is very complex with many positive and negative effects thus its characterization requires many different areas of knowledge.

### Concluding Remarks

Studies discussed here show that polyphenols are able to interact with other food compounds. The referred interactions have many side effects on sensory properties. Most of the current discussion focused on colour and taste. Also, the effects of these interactions, positive or negative, depend on the end use. Although polyphenol–lipid interactions have scarce data, it seems that this interaction can affect lipid emulsion properties; Polyphenol-carbohydrate interactions can lead to an astringency and bitterness modulation as well as affect anthocyanin colour; Polyphenol-protein interactions can affect astringency and also affect some functionalities of proteins in food. Literature review revealed that most studies on these interactions are only focused on the bioavailability of the involved compounds. More studies are needed to investigate other sensory properties that could be influenced by these interactions such as food texture and pleasantness, especially in relation to the interaction with food lipids.

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# Effects of Interactions Between Antioxidant Phytochemicals and Coexisting Food Components on Their Digestibility

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## Glossary

**Antagonistic effect** The effect of two or more substances that the combined effect is less than the algebraic sum of the individual effect of each substance.

**Digestibility** The proportion of a foodstuff absorbed from the digestive tract into the bloodstream. This is measured as the difference between food intake and output, including excretion of feces.

**Digestive tract** The system composed of the gastrointestinal tract along with the accessory organs that break down food into smaller components and then digestible molecules for the human body to absorb and assimilate. The digestive tract includes mouth, salivary glands, esophagus, stomach, pancreas, liver, gallbladder, small intestine, large intestine, and rectum.

**Phytochemicals** Nonnutritive plant chemicals that play important roles in human health through providing protective properties such as antioxidant and antiinflammatory effects to reduce the incidence of certain chronic diseases.

**Synergistic effect** The joint action of two or more substances to create a combined effect greater than the algebraic sum of the individual effect of each substance. A synergistic effect can be beneficial or harmful.

## Introduction

Antioxidant phytochemicals are secondary plant metabolites found in plants such as cereal grains, fruits, vegetables, and spices, among which polyphenols and carotenoids are two major groups that have demonstrated various beneficial effects on human health, including preventive properties on certain chronic diseases such as diabetes and obesity (Zhang et al., 2015). Polyphenols are compounds that contain one or more aromatic rings connected to at least one hydroxyl group. More than 10,000 polyphenols have been identified and they can be classified differently; for example, according to the number of phenol rings. Flavonoids, stilbenes, and phenolic acids are among the major groups of polyphenols in nature. Polyphenols may be soluble in polar solvents such as water, ethanol, and methanol. Their solubility depends on hydroxyl group, molecular size, and length of hydrocarbon (Franco et al., 2008). Carotenoids are compounds found in orange and red fruits and vegetables that play important roles in human health. More than 600 types of carotenoids have been identified from natural sources with about 40 types found in the human diet containing basic structures of 40-carbon terpenoids linked to isoprene (Kiokias et al., 2016). Most carotenoids are slightly soluble in oil at room temperature and readily soluble in nonpolar organic solvents such as acetone and chloroform, with exceptions like crocin that can be dissolved in water (Alavizadeh and Hosseinzadeh, 2014; Kiokias et al., 2016).

The consumption of foods containing antioxidant phytochemicals leads to many health benefits to humans (Li et al., 2014). However, several factors would affect the digestibility and bioavailability of phytochemicals in foods, of which chemical interactions are important factors that influence food metabolites in the human body (Chitindingu et al., 2015).

## Sources and Structures of Antioxidant Phytochemicals

Most natural antioxidant phytochemicals have been found in cereal grains, herbs, microalgae, beans, fruits, vegetables, and tea, including edible and wild flowers. Vegetables and cereal grains, especially pigmented rice, soybean, broccoli, and cowpea, are important sources of polyphenols (Li et al., 2014). Most polyphenols in cereal grains exist in a bound form in the aleurone layer, pericarp, and embryo cell walls (Abdel-Aal et al., 2006; Liu, 2007) and in flesh and peels of various fruits and vegetables (Boyer and Liu, 2004; Xu, 2012). Citrus fruits, tomato, papaya, and carrot contain important dietary sources of carotenoids, in particular, lycopene and  $\beta$ -carotene (Kiokias et al., 2016; Xu, 2012; Zhang et al., 2015). Thus, cereal grains, fruits, and vegetables are large food groups that are rich in polyphenols and carotenoids.

Flavonoids occur in the forms of aglycone, glycosides, and methylated derivatives and can be divided into six subclasses: anthoxanthins (including flavones and flavonols), flavanones, flavanonols, flavans, anthocyanidins (including derived anthocyanins), and isoflavonoids (including isoflavones). Phenolic acids normally occur in a bound form, which can be divided into two major groups: hydroxybenzoic acids (e.g., *p*-hydroxybenzoic, vanillic, syringic, gallic, and protocatechuic acids) and hydroxycinnamic acids (e.g., caffeic, *p*-coumaric, sinapic, and ferulic acids). Hydrocarbons (carotenes; e.g.,  $\alpha$ -carotene,  $\beta$ -carotene, and lycopene) and their oxygenated derivatives (xanthophylls; e.g., lutein, bixin, and capsanthin) are the major subclasses of carotenoids in fruits and vegetables.

## Health-Promoting Effects of Foods Rich in Antioxidant Phytochemicals

The overproduction of free radicals in humans can generate oxidative stress and cause severe damage to biomolecules of cells and tissues, resulting in various chronic diseases such as cardiovascular disease, obesity, cancers, and diabetes (Zhang et al., 2015). Many studies have shown that the intake of food rich in antioxidant phytochemicals would lead to reduced risk of chronic diseases (Kim et al., 2013; Munirah et al., 2012). The consumption of whole grain along with three or more servings of fruits and vegetables exhibited positive effects on glycemic control, blood lipid profile, and body weight of diabetic patients (Munirah et al., 2012). The study involving 38 healthy volunteers demonstrated that a daily consumption of red and black cabbage in a portion of 300 g could increase the plasma carotenoid concentration resulting in decreased lipid hydroperoxide levels and oxidized low-density lipoprotein (Bacchetti et al., 2014). The consumption of black rice containing high concentrations of anthocyanins for 8 weeks has caused the reduction of weight gain and cellular lipids in an animal study (Kim et al., 2013). Quercetin could reduce body weight gain along with improved sensitivity and glucose intolerance in mice (Dong et al., 2014). Moreover, antioxidant phytochemicals may exert direct impacts on the enzymes that intrinsically occur inside the human body and contribute to food component digestion and absorption. Citrus fruits containing significant amounts of antioxidant phytochemicals exhibited  $\alpha$ -glucosidase inhibitory activity in *in vitro* study (Girones-Vilaplana et al., 2014). In addition, anthocyanins in purified form showed suppressive effects against porcine pancreatic  $\alpha$ -amylase, and cyanidin-3-glucoside seemed to be the most effective compound (Sui et al., 2016). A previous study (Akkrachiyasit et al., 2010) reported that cyanidin or cyanidin-3-glucoside (1.0  $\mu$ M) combined with acarbose (3.12  $\mu$ M) exhibited a synergistic inhibition of pancreatic  $\alpha$ -amylase. A study of 14 chili pepper varieties revealed the synergism among their phytochemical components such as carotenoids, phenolics, and capsaicinoids to reduce the sugar level via inhibition of  $\alpha$ -amylase activity (Sricharoen et al., 2017). The study of tomato leaf extracts also revealed the synergism of flavonoids, phenolic acids, and chlorogenic acid to inhibit both  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (Figueiredo-González et al., 2016). Hydrogen bonding between the hydroxyl groups of polyphenols and the catalytic residues of the active site of the digestive enzyme could lead to inhibition of the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes (de Sales et al., 2012). The inhibition of certain digestive enzymes such as those related to the absorption of carbohydrates and lipids may be beneficial, as a decrease of the levels of glucose and undesired lipids in the blood would be possible for patients with diabetes.

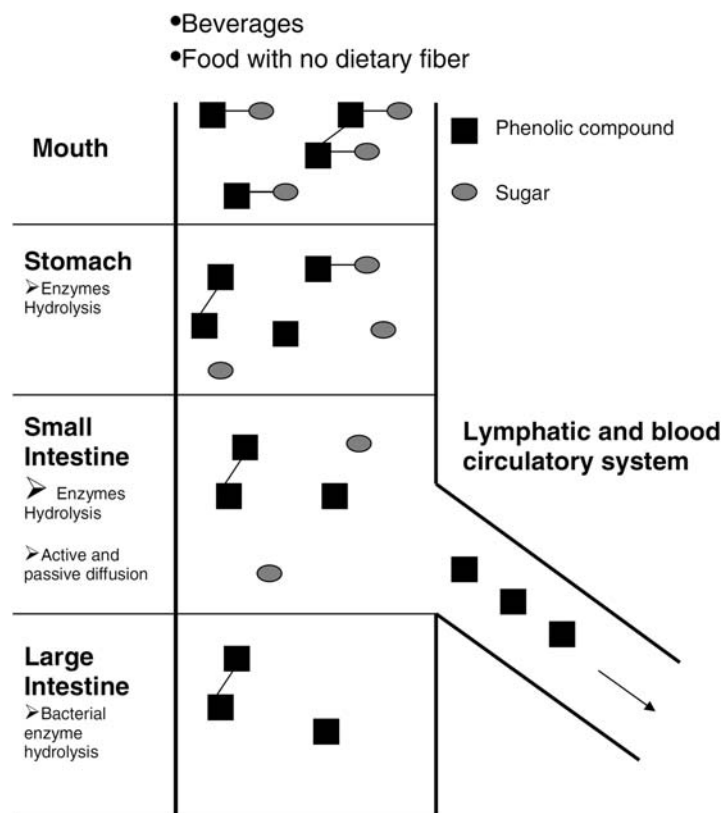
## Digestibility of Antioxidant Phytochemicals and the Influence of Their Interactions With Coexisting Food Components

Carotenoids, flavonoids, and phenolic acids are antioxidant phytochemicals that are presented in a wide range of plant-based food. They have been recognized as potential bioactive compounds that play important roles in human health and prevention or treatment of a chronic disease (Xu, 2012; Zhang et al., 2015; Palafox-Carlos et al., 2011). Moreover, food microstructure, type of macromolecules in food (carbohydrates, lipids, and protein), food source, and chemical interactions between phytochemicals and other phytochemicals or biomolecules are factors that influence the digestibility and bioavailability of the antioxidant phytochemicals (Palafox-Carlos et al., 2011; Parada and Aguilera, 2007; Chitindingu et al., 2015). Therefore, recent studies have focused on the effects of chemical interactions on the digestibility of carotenoids, flavonoids, and phenolic acids.

### Flavonoids

Generally, most flavonoids in food are found in glycoside form that is attached with a sugar moiety (Palafox-Carlos et al., 2011). Flavonoid glycosides can be digested into monomer molecules via mastication and enzymatic actions during the oral and stomach processes. From this step, the sugar residue attached with flavonoids can be absorbed in the small intestine first, resulting in the release of individual flavonoids (aglycones). The flavonoids (aglycones) are unstable under the neutral small intestine conditions, so a significant portion of aglycones cannot be digested and absorbed in the small intestine (Palafox-Carlos et al., 2011; Pinto et al., 2017).

The presence of dietary fiber could be an influencing factor that affects the digestibility and bioavailability of flavonoids in the small intestine. Dietary fibers can interact with flavonoids via electrostatic and dipolar interactions, van der Waals attraction, and hydrogen bonding, resulting in restricted flavonoid digestion and absorption in the small intestine (Eastwood and Morris, 1992). Hence, undigested flavonoids would be passed through to the large intestine where they are cleaved by colonic microflora and are either absorbed into the circulatory system or utilized to exert the antioxidant activity in the intestine environment (Del Rio et al., 2009). The consumption of foods and/or beverage containing both flavonoids and dietary fiber has been found causing restricted flavonoid digestion and absorption in the small intestine, as flavonoids' interaction with dietary fibers and their subsequent entrapment by fibers would retard their hydrolysis by digestive enzymes in the small intestine, as shown in Fig. 1 (Palafox-Carlos et al., 2011). Saura-Calixto and Díaz-Rubio (2007) reported that dietary fiber presented in wine could obstruct the bioavailability of polyphenols during the upper part of digestive tract; therefore, these polyphenols could be entrapped within the dietary fibers until reaching the large intestine. The molecular interactions such as electrostatic interactions or polar-polar interactions between apple-sourced flavonoids and dietary fiber (like cellulose membrane) might decrease the digestibility of these phytochemicals in the digestive tract (Bouayed et al., 2011).



**Figure 1** The digestibility and bioavailability of polyphenols contained in foods rich in dietary fibers. Reproduced from Palafox-Carlos, H., Ayala-Zavala, J.F., González-Aguilar, G.A., 2011. The role of dietary fiber in the bioaccessibility and bioavailability of fruit and vegetable antioxidants. *J. Food Sci.* 76 (1), 6–15.

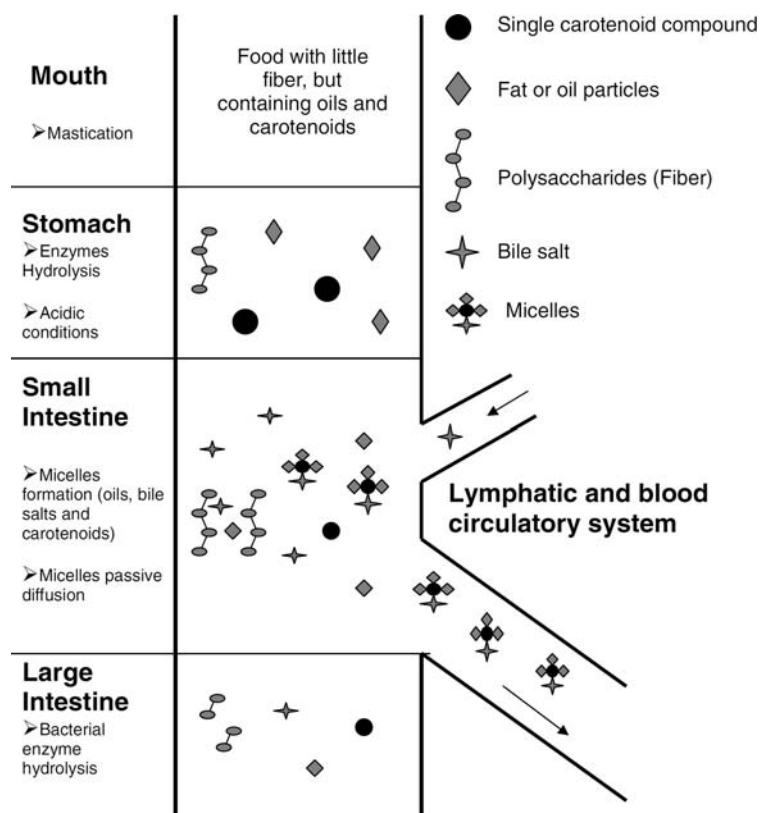
### Phenolic Acids

Phenolic acids are mostly presented in bound form and/or attached to the plant cell walls. Generally, phenolic acids can be digested and absorbed in the upper part of gastrointestinal tract in aglycone form (Konishi et al., 2006; Lafay and Gil-Izquierdo, 2008). Hydroxybenzoic acids, such as gallic acid, are easily digested and absorbed through gastrointestinal tract. Hydroxycinnamic acids in free form such as ferulic acid, caffeic acid, and chlorogenic acid can also be rapidly digested and absorbed in the gastrointestinal tract by conjugating with the intestinal and/or hepatic detoxification enzymes (Lafay and Gil-Izquierdo, 2008).

Most of phenolic acids can be found in cereal grains, and the cell wall polysaccharides in grains such as arabinoxylans could interact with the phenolic acids in bran and aleurone layers, resulting in retarded digestibility of phenolic acids in the upper part of gastrointestinal tract. This might be due to the esterification of phenolic acids with one or more arabinose and xylose, which are mostly not absorbed in the small intestine; instead, pass through to the large intestine where they can be hydrolyzed by colonic bacterial enzymes (Palafox-Carlos et al., 2011). However, some phenolic acid esters can be cleaved by the esterase in the gastrointestinal tract and converted to the free form of phenolic acids that may be digestible in the gut depending on their molecular size (Chandrasekara and Shahidi, 2012; Palafox-Carlos et al., 2011). Furthermore, the binding of some phenolic acids such as hydroxycinnamic acids to macromolecules such as proteins via hydrophobic and electrostatic interactions also decrease the bioavailability of phenolic acids (Budryn et al., 2016). The decreased starch digestibility associated with increased phenolic acids and proteins in the bread made with sprouted wheat flour as compared with that of normal wheat flour was proposed to result from the phenolic acid–bread matrix interaction (Świeca et al., 2017).

### Carotenoids

Carotenoids are predominantly hydrophobic and lipophilic compounds and are associated with lipids in chloroplasts; thus their digestion would mainly take place in the small intestine where significant amounts of lipases occur (Kiokias et al., 2016; Palafox-Carlos et al., 2011). Certain carotenoids are also able to attach to some water molecules in forms of conjugates with bile salts, forming micelles and solubilized carotenoids (Palafox-Carlos et al., 2011). Their bioavailability is associated with the degree of digestion to release carotenoids from the food matrix, formation of lipid micelles in the small intestine, absorption of carotenoids in the intestinal mucosal cells, and transport of carotenoids to the lymphatic system (Berni et al., 2015).



**Figure 2** The digestibility and bioavailability of carotenoids contained in foods rich in dietary fibers. Reproduced from Palafox-Carlos, H., Ayala-Zavala, J.F., González-Aguilar, G.A., 2011. The role of dietary fiber in the bioaccessibility and bioavailability of fruit and vegetable antioxidants. *J. Food Sci.* 76 (1), 6–15.

Carotenoids are usually digested and absorbed along with dietary lipid in the intestinal tract, which depends on the type, molecular size, and isomeric configuration (*cis* or *trans*) of carotenoids (Britton, 1995). Dietary fibers are well known for their abilities to retard the digestion and absorption of certain macronutrients such as some carbohydrates, proteins, and lipids. Dietary fibers can interact with carotenoids dissolved in oil or in a crystalline form through noncovalent interactions, resulting in unavailability of carotenoids for digestion and absorption (Zaripheh and Erdman, 2002). Dietary fibers can influence the bioavailability of carotenoids through increasing the viscosity of the whole digestive system to restrict enzymatic actions in the pancreas and to reduce the intestinal enterocyte contacts (Fig. 2) (Palafox-Carlos et al., 2011). Carotenoids can also link to the lipid component of lipoproteins via hydrophobic interactions, which facilitate carotenoid's function and transport in an aqueous environment (Britton, 1995). Interactions between carotenoids and proteins via hydrophobic effects were also found in citrus fruits that promote the bioavailability of carotenoids (Turner and Burri, 2013). Therefore, the bioavailability of carotenoids from foods depends on both their respective chemical structures and food matrix characteristics.

## Conclusion

Many studies reported the chemical interactions between antioxidant phytochemicals and other coexisting substances such as dietary fibers, lipids, and proteins and the effects of these interactions on the digestibility and bioavailability of phytochemicals in the digestive system. The interactions include electrostatic interactions, van der Waals attractions, hydrogen bonding, and hydrophobic interactions, as well as covalent interactions; e.g., those in esterification. Dietary fibers would likely present an antagonistic effect on polyphenols and carotenoids, whereas carotenoid and lipoprotein might work in a synergistic way.

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## Relevant websites

- <http://www.phytochemicals.info/>– Phytochemicals.
- <http://www.carrotmuseum.co.uk/phyto.html> – The power of phytochemicals-carrots.
- [http://www.aicr.org/reduce-your-cancer-risk/diet/elements\\_phytochemicals.html?referrer=https://www.google.co.jp/](http://www.aicr.org/reduce-your-cancer-risk/diet/elements_phytochemicals.html?referrer=https://www.google.co.jp/)– Phytochemicals: The cancer fighters in your foods.



# Bioactive Delivery Systems Based on Stimuli-Sensitive Biopolymer Stacks: Chitosan-Alginate Systems

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## Glossary

**Biopolymers** Are biomacromolecules that possess biological functions owing to their hierarchical structures (primary, secondary, tertiary or quaternary structure) and chemical composition of their building blocks (i.e. monomeric units), and include carbohydrates (mostly polysaccharides), proteins and nucleic acids. Their structures and functional properties depend on environmental conditions such as temperature, pH, surrounding media and co-existing substances (including salts and other additives).

**Biopolymeric nanocomposites** Refer to a type of deagglomerated, well-dispersed and multiphased biopolymer material with beyond usual chemical, physical, mechanical, thermal, optical or electrical properties, in which nanostructures (structures containing nano-scale repeating distances between different phases) and/or nanomaterials (matrices with at least one phase having one, two or three dimensions at a nanoscale, including those containing nanoparticles) are present.

**Self-emulsifying nano-/micro-emulsions** Are a thermodynamically stable colloid system of two or more immiscible liquids (emulsions) in which droplets within the nano-/micro-meter range are formed using hydrophilic biopolymers capable of gelling in the continuous minority emulsion phase, thereby simultaneously emulsifying and stabilizing the colloidal system in the absence of additives such as surfactant, co-surfactant or stabilizer.

## Introduction

Advances in layer-by-layer (LbL) assembly methods for polyelectrolyte complexes (PECs) over the past three decades (Decher, 1997) has resulted in the widespread utilization of multilayered polyelectrolyte assemblies for various applications, in particular bioactive delivery. Biocompatible PECs in the form of biopolymeric stacks formed via the interactions between polyanions and polycations have recently gained special attention. These multilayered systems can be fabricated using a multitude of natural biopolymers (e.g. chitosan, sodium alginate, carboxymethyl cellulose and certain active peptides) and their individual properties tuned during fabrication to deliver intricate composite architectures for the encapsulation and delivery of bioactive substance(s). Since these composites consist of biocompatible polysaccharides and proteins, the polymer composites also possess high biocompatibility as bioactive carriers. Chitosan (Park et al., 2006) and alginate (Zhou et al., 2013) are two biopolymers that chemically resemble extracellular matrix components.

Polysaccharides are commonly used as gelling agents and exhibit varying solubility and viscoelasticity in water. Under appropriate conditions, they can be dissolved or dispersed in a solution owing to the disruption of hydrogen bonding networks that exist in the solid state. Subsequently, their polymer chains cross-link via covalent or noncovalent interactions, eventually yielding a three-dimensional (3-D) polymeric network in solution that occupies the whole volume of the liquid to which they were added. Intermolecular interactions also contribute to these gelling processes, and include ionic or ion dipole bonding (electrostatic attraction and polar covalent bonding), hydrogen bonding, van der Waals attraction, hydrophobic interaction and covalent bonding. Intermolecular association of polysaccharides is stable only when the chain length exceeds a minimum critical value (mostly 15–20 residues) required for the cooperative nature of interaction (Bertoft, 1993). Factors that influence the solubility and gelation of polysaccharides include intrinsic parameters such as molecular weight (MW) (e.g. large MW polysaccharides typically have low dissolution rates), molecular structure (e.g. degree of regularity in conformation, crystallinity, branching or packing density), charge (e.g. charged polysaccharides associated typically have a higher solubility than neutral polysaccharides), type of polysaccharide, as well as external environmental conditions such as temperature, pH, pressure and the presence of co-factors (e.g. counterions or cosolutes) that affect the interactions between polysaccharides and water. Certain counterions with appropriate radius and charge form junction zones in polymeric networks promoting the associations between polysaccharide molecules thus leading to the formation of unique structures, whereas co-solutes with low MW (e.g. sugars) compete with the polysaccharides for water and impact the gelling properties.

Chitosan is a linear positively charged polysaccharide composed of  $\beta(1\rightarrow4)$  linked D-glucosamine residues with varying numbers of randomly located N-acetyl-glucosamine units. It is obtained by alkaline hydrolysis or enzymatic N-deacetylation of the abundant natural biopolymer chitin. Chitin serves a structural function in insect and crustacean cuticles in a similar way to the cellulose in plants and collagen in animals. Chitosan as a raw material exhibits excellent nontoxicity, biocompatibility, biodegradability, and antimicrobial and wound healing properties (Nguyen et al., 2013). The  $-\text{OH}$  and  $-\text{NH}_2$  ( $-\text{NH}_3^+$ ) groups of chitosan can induce strong intramolecular and intermolecular interactions, resulting in the complexation of various negatively charged substrates such as carbohydrates, proteins, lipids, cells and microorganisms (associated with antimicrobial properties of

chitosan) (Kenawy et al., 2007) as well as hydrophobic interactions with substances like neutral lipids (Tao et al., 2013). Chitosan's N-acetylglucosamine residues can interact with and form conjugates with certain specialty proteins including growth factors or receptors thus enabling the development of carriers with additional advantages that retain the properties of these special proteins (Prabaharan and Mano, 2006). The degree of deacetylation (DD) greatly affects its stability, crystallinity, swelling degree, mechanical strength, bioactivity and (bio)-degradation behavior (Cao et al., 2005). Naturally sourced chitosans are neither 100% acetylated nor 100% deacetylated but instead, exist as a copolymer composed of two types of monomer building blocks with different acetyl contents. Commercial chitosans typically have a DD of 70%–95% and MWs ranging from 50 to 2000 kDa. The MW, DD, intermolecular hydrogen bonding degree, distribution of acetyl groups, time for deacetylation, rigidity of D-glucosamine structure, degree of crystallinity, concentration, viscosity, temperature and pH all influence chitosan's solubility. To prepare chitosan solutions free of agglomeration, it is common to mix a plasticizer (e.g. water or glycerol) with the chitosan, followed by the slow addition of a weak acetic acid solution (at pH < its pKa (6.3)) under agitation. Under these conditions, the amino groups (NH<sub>2</sub>) of chitosan become protonated (–NH<sub>3</sub><sup>+</sup>) forming a polycation (i.e. chitosan is completely protonated at pH 4.5 and below) (Pillai et al., 2009). The critical concentration (C\*) for chitosan chain entanglement in acetic acid is around 0.1% (w/w) (Montembault et al., 2005).

Alginate is a negatively charged polysaccharide composed of two uronate sugars,  $\alpha$ -L-guluronic acid (G) and  $\beta$ -D-mannuronic acid (M) and obtained mainly from brown seaweed. Alginates occur as copolymers with different weight fractions, length and distribution of G and M blocks, which endows alginate gels with varying physicochemical properties (e.g. different gel-forming capacities due to varying flexibility of GG, MM or MG units). Alginates are versatile encapsulants for intestinal delivery systems in functional food and biomedical applications (Shinde et al., 2014) due to their pH sensitivity, chemical and mechanical stability, gel barrier function, good cell-affinity and biological functions (e.g. muco-adhesive and appetite-regulating properties) (Georg et al., 2012). In particular, alginate oligomers, which are broken down via acidic/enzymatic hydrolysis or depolymerization from parent alginate molecules, exhibit excellent prebiotic effects (Wang et al., 2006) and offer increased antioxidant activity (Kelishomi et al., 2016). Commercial alginates typically have an average MW of 200,000 (although up to 500,000 is possible), degree of polymerization (DP) of 180–930 and pKa of 3.4–4.4. The stability of alginate depends on intrinsic factors (e.g. G/M ratio, DP and MW) and external conditions (e.g. pH, temperature and presence of contaminants). Cleavage of the glycosidic linkages between alginate's sugar monomers is possible under acidic (especially at pH 3–4) and alkaline conditions. Acid hydrolysis is due to the tendency to form six-membered rings via intramolecular catalysis involving non-dissociated carboxyl groups. A change in alginate's polymeric structure can lead to different gelation mechanisms and gels with varying properties. For example, chains rich in L-alginate form transparent and brittle gels whilst chains rich in D-alginates yield turbid gels with low elastic moduli. Alginate is compatible with many other biopolymers including polysaccharides like pectin, starch and chitosan in many formulations (Sun-Waterhouse et al., 2014; Wang et al., 2013).

Both chitosan and alginate can assume interconnected porous microstructures in water to encapsulate/immobilize bioactive substance(s) whilst also allowing controlled diffusion of nutrients, soluble factors and metabolic wastes. At appropriate pHs, they are ionized in aqueous-based media and carry opposite electric charges allowing their interactions to form PECs through the positively charged amino groups of chitosan and the negatively charged carboxylic acid groups of alginate. Various types of chitosan-alginate PECs can be prepared in solutions depending on the exact conditions, including random composites, LbL assembled multilayers and precipitates.

### Creation of Polyelectrolyte Stacks With Different Structures and Geometries

Polyelectrolyte multilayers (PEMs) with different internal architectures can be fabricated to encapsulate efficiently and release precisely various bioactive substances (including small or big molecular compounds and active substances like enzymes and cells), through selecting chitosan and alginate biopolymers with specific MW, DD/DP, G/M ratio, concentration ratio, rheological properties and existing form (charged or neutral forms), and tailoring the assembly of two polymers (e.g. substrate, starting layer, outermost/terminal layer and layer thickness). Understanding the specific molecular and polymeric interactions is important for producing stimuli-responsive delivery systems with effectively incorporated bioactives for subsequent release after consumption.

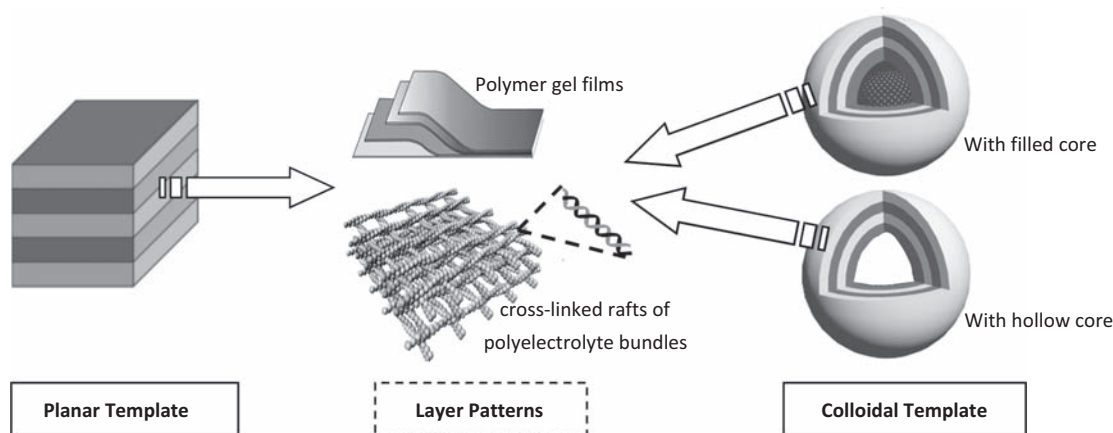
### Fabrication Methods or Processes

Electrospinning is an established technique for producing 3-D porous nanofibrous structures from biopolymer solutions, and can also be used to create multi-layered fibrous structures. During a typical electrospinning process, a high electric potential is applied between a spinneret and a collector to create an electrically charged jet of a polymer solution, and the polymer jet is solidified and deposited on the collector. The electrospun nanofibers are then fused together to form layers of nonwoven meshes or scaffolds by using different collection methods or set-ups (e.g. electro-centrifuge spinning, rotary jet-spinning or rotating mandrel), by adjusting the gap between two collectors or by stretching the mesh post electrospinning. Among various electrospinning processes, the co-axial electrospinning approach is generally preferred for the construction of multilayered PECs as it does not require an additional crosslinking step (Waterhouse et al., 2017). To prepare chitosan-alginate multilayer fibres, chitosan and alginate polymer solutions are pumped through a two capillary spinneret and the polymer jet is ejected in the form of core-sheath fibers and collected as core-shell fibers. Bioactive substance(s) can be incorporated into the core or even the shell. Manipulating the electrospinning set-up and the properties of polymer solutions (e.g. concentration, ionic strength, pH and viscosity) allows precise tuning of the characteristics of electrospun multi-layered PEC products.

Polymerized high internal phase emulsions (PolyHIPEs) represent another approach to form multilayers of highly porous chitosan and alginate PolyHIPE matrices. A typical PolyHIPE fabrication process involves the dropwise addition of an internal emulsion phase into the continuous phase containing polymerizable monomers and surfactant(s) until the total volume ratio of internal to continuous phase exceeds 0.74, followed by polymerization or solidification of HIPE via mild chemical and/or physical crosslinking and final removal of liquid via freeze-drying or Soxhlet extraction (Hayman et al., 2005). Polysaccharides contain many functional groups, thus allowing mutual interactions between different types of polymers and specific modifications of individual polymers prior to polyHIPE fabrication, in order to tailor the attributes of polyHIPEs (e.g. increased pore size and pore interconnectivity as well as improved mechanical strength and biodegradation post-delivery). Grafting a designed peptide sequence on the surface or adding to the backbones of a natural polymer can make the polymer an amphiphilic entity which can function as the matrix for polyHIPEs (thereby avoiding the use of stabilizers like surfactants which are harmful to living cells), whilst acting simultaneously as the emulsifier for emulsion templating and as the stabilizer to maintain large droplet/pore size (i.e. self-emulsifying HIPEs for polyHIPEs). For example, short peptide-chitosan copolymers with thermoresponsive properties can be created through grafting oligo(amino acid) side chains onto chitosan. The oligo(amino acid) side chains including oligo-/polyproline with hydrophilic and hydrophobic domains, negatively charged oligo-glutamic acid, positively charged oligolysine and oligoarginine, can disrupt the intermolecular hydrogen bonding between the  $-\text{NH}_2$  and  $-\text{OH}$  groups of chitosan and thereby improve emulsion stability and solubility at physiological pH and temperatures. An additional coating of the polyHIPEs may be required to improve adhesiveness between layers of alginate-based polyHIPEs and chitosan-based polyHIPEs for freestanding stacked assemblies, depending on the influence of the grafted peptides.

Layer by layer (LbL) processes involving the stepwise deposition of building blocks onto planar surfaces or core materials are commonly used to construct multilayers of PECs (Iler, 1966). Stacks of chitosan-alginate biopolymer layers can readily be deposited on planar substrates via successive dip coating cycles in chitosan and alginate solutions. The LbL technique also provides the possibility to create complex layer geometries e.g. planar template built onto differently patterned flat surfaces, and colloidal template built onto core materials with appropriate 3-D structures (Fig. 1) under very mild conditions for incorporating bioactive substances. For multilayers on a planar template, cleaned flat hydrophilic surfaces (e.g. glass, silica or mica) are typically used as substrates. The deposition of the first layer is accomplished by dipping the charged substrate into a polymer solution with opposite charge (e.g. positively charged chitosan solution for negatively charged silica glass) for a specified time period, following by rinsing to remove non-bound free polymers and air drying. The coated substrate is then immersed in a solution of the oppositely charged biopolymer (e.g. alginate), rinsed and then air dried. The process is repeated until a predetermined PEM thickness is achieved (e.g. 50 or 100 layers). For layers deposited over a colloidal template (including spherical, quasi-spherical or cylindrical geometries), the layers of PECs (e.g. chitosan and alginate) are deposited by alternating addition of polycations and polyanions to particle dispersions (including nano-biopolymer particles or bioactive molecules e.g. chitosan particles), followed by washing and centrifugation to remove excess polymers. During the LbL process for colloidal PEMs, the competition of the coagulation process of partly covered particles with the formation of a defined monolayer around the particle (the so-called “supersaturation”) needs to be monitored, which is typically achieved through reducing the MW of coating polymer particles (e.g. tens of nanometres to several micrometres) or controlling the adsorption rate of polymers to be greater than that of particle collision. The PEM deposition process can be monitored by electrophoresis. The properties of LbL assemblies derive from their preparation history: planar layers are often dried after each deposition step whilst colloids are not (to reduce unwanted aggregation), as well as variations in parameters used during fabrication including concentrations and pH of the polymer solutions, type of supporting medium and washing steps.

In addition, it is possible to increase the complexity of multilayered architectures (Fig. 1) via (1) complexation/condensation of polyelectrolytes in the presence of nonspecific ion linkers such as biological electrolytes (e.g.  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ ) prior to or during the deposition of each layer to form close-packed raft-like networks (Wong et al., 2003); (2) removal of the colloidal cores of PEMs



**Figure 1** Scheme showing polyelectrolyte multilayers built on different templates (planar or colloidal; hollow or filled) and with different layer patterns.

using a core sacrificial method to create hollow core structures following the formation of the multi-layered coatings (Zhao et al., 2007); (3) creation of nano-sized particles with encapsulated insoluble core bioactives inside the PEMs with controlled ultrasonication, distributive or dispersive mixing (Ariga et al., 2011). Stacking PEMs at different angles can also create complicated carrier architectures such as hexagonal arrays, moiré or grid patterns.

### Local Molecular Interactions and Structural Complexity

The kinetics of PEM deposition impacts PEM final properties. The PEM architecture is created mostly by electrostatic interactions (Waterhouse et al., 2017) and/or hydrogen bonding (Kharlampieva et al., 2009), with significant contributions from repulsive forces, entropic forces, hydrophobic interactions and van der Waals interactions (Song et al., 2009; Kotov, 1999). The electrostatic build-up may cooperate with other processes e.g. Langmuir–Blodgett transfer (Ruths et al., 2000) or specific binding mechanisms (Cassier et al., 1998). The formation of multilayers is governed by the absolute charge density of the component polymer chains, and also affected by the type and concentration of salts (i.e. ionic strength), dielectric constant of solvent, pH or temperature of deposition solutions, deposition time, presence or absence of counterions, ratio of different the polymers, and type, concentration, MW, stoichiometry, degree of ionization, outer surface properties, internal interfaces and entanglements of each polymer. Charge neutrality and charge reversal are essential for the subsequent deposition of multilayers and continuous layer growth, and can be achieved via intrinsic charge compensation and/or extrinsic charge compensation for strong or weak PECs. Charge renormalization may occur in PEMs owing to the strong coupling between electrostatics and chemical dissociation equilibria at the growth surface as well as the adsorption and physical binding of counterions at the surface. Strong electrostatic forces can decelerate equilibration causing very slow growth dynamics. The desorption of a full PEC chain and consequently deconstruction of PEMs may also be possible, due to local molecular interactions (e.g. under conditions where electrostatic interactions are weakened sufficiently) (Kovacevic et al., 2002). This is especially true for weak PECs where dissociation equilibria exist in multilayers. Secondary interactions could dominate under conditions when electrostatic interactions are strongly screened (e.g. at high salt concentrations) or when solvent molecules are released from the hydration shell layers (Büscher et al., 2002).

Polyelectrolyte multilayers on substrates generally comprise three distinct zones: A “precursor zone” at the substrate interface (which is influenced by substrate/polymer interactions), a “core zone” (in which the regular layer-by-layer growth occurs and component polymer layers are mostly compensated and balanced), and finally an “outer zone” (where the chains mostly assume a loop-like conformation with the last/outermost layer possessing excess charge and determining the stimuli-response). Polymer chains in the terminating layer are much more mobile than those in the “core zone” owing to their charge imbalance and lesser steric restriction. This outermost layer greatly governs the properties of PEM surfaces (e.g.  $\zeta$  –potential) and even the whole PEM). The attributes of the substrate affect the first few layers in the “precursor zone” e.g. the amount of adsorbed polymer is smaller on a flat and hard surface (owing to stratification of polymer flexible chains) compared to a rough and soft surface with the same surface charge density ( $\sigma$ ). Significant overlap between the segments of adjacent layers (“interpenetration” or “interdigitation”) can occur driven by complexation of positive and negative charges. The thickness of the “precursor zone” is generally smaller for strong PECs than for weak PECs. An approximate linear growth of layer thickness with layer number is usually found in the “core zone” while a significantly smaller thickness is observed for the “precursor zone”, although factors such as the density and total layer number of the PEM structure, pH and ionic strength affect the thicknesses of these zones (Lavallo et al., 2002).

The biopolymers in each layer of PEMs are mainly in the form of hydrophilic hydrogels created by absorbing water to approximately 20% of their dry weight which results in swelling and the formation of the 3-D gel structure. When the polymer concentration increases, electrostatic repulsion between oppositely charged monomers is eventually screened. At sufficiently high concentrations, PECs behave like charge-neutral polymers and interpenetrate at a critical overlap concentration  $c^*$ , leading to progressive overlap of their chains with the dynamics of each chain restricted/hindered by co-existing chains (as a result of entangled and non-entangled inter-chain interactions). The dual swelling and responsivity characteristics enable hydrogels to be bioactive carriers with high sensitivity to various stimuli including pH, temperature, ionic type and strength. The pH responsivity of PEMs derives from ionized groups on the PEC polymers, which is closely associated with their pKa values and the pH of medium (e.g. anionic alginate is ionized at pH above its pKa whilst cationic chitosan is ionized below its pKa). For PEMs in contact with solutions of a defined pH value, the difference between the external pH and the internal pH controls the dissociation of weak polymers in the layers by regulating the distribution of free protons between the solution and the layer interior (Rmaile and Schlenoff, 2002). In particular, the dissociation of weak PECs in PEMs can be varied by changing the external conditions even after deposition (Rmaile and Schlenoff, 2002).

In the absence of salt, the individual polymers used to construct PECs will be flexible and exist in a stretched configuration (rod-like chains) in dilute solutions owing to long-ranged unscreened electrostatic repulsions. In the presence of salt, these PECs adopt a coil-shaped structure with a lower extent of interpenetration. When present in excess, the PECs behave like neutral polymer chains and generally assume a swollen coiled configuration (isotropic conformation) owing to the electrostatic exclusion volume effect which causes a decreased accessibility of the outer layer segments to the next adsorbing layer. If PEMs fabricated in salt-free solutions are transferred to a salt solution, the PEM structure will inevitably swell due to counterion incorporation. Small salt ions entering the layers will compete for the polymer charges and occupy particular binding sites, causing a decrease in extent of complexation between polymer chains of opposing charge and thereby a looser layer arrangement (facilitating extra water uptake). A decrease of film roughness due to segmental rearrangements is expected in concentrated salt solutions. Further, the response of PEMs to ion gradients is of relevance for ion exchange and ion transport, which will vary with ion concentration, solution pH and specific layer chemistry. Charge overcompensation can occur irrespective of ionic strength. Slight charge overcompensation often

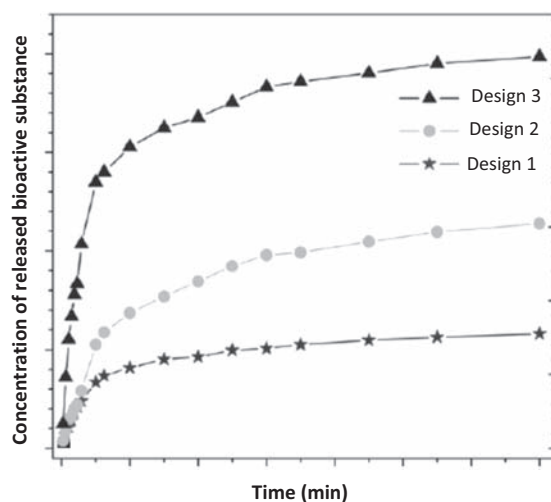


occurs to flat adsorbing chains at low ionic strength. Strong overcompensation is possible for polymer chains with a large fraction of loops and tails on the surface at high ionic strength, as adsorption is no longer dominated by electrostatics (both self-repulsion and attractive interactions are greatly screened), instead, by short-range attractive potentials (Joanny, 1999). At low surface charges, minimal ion adsorption takes place.

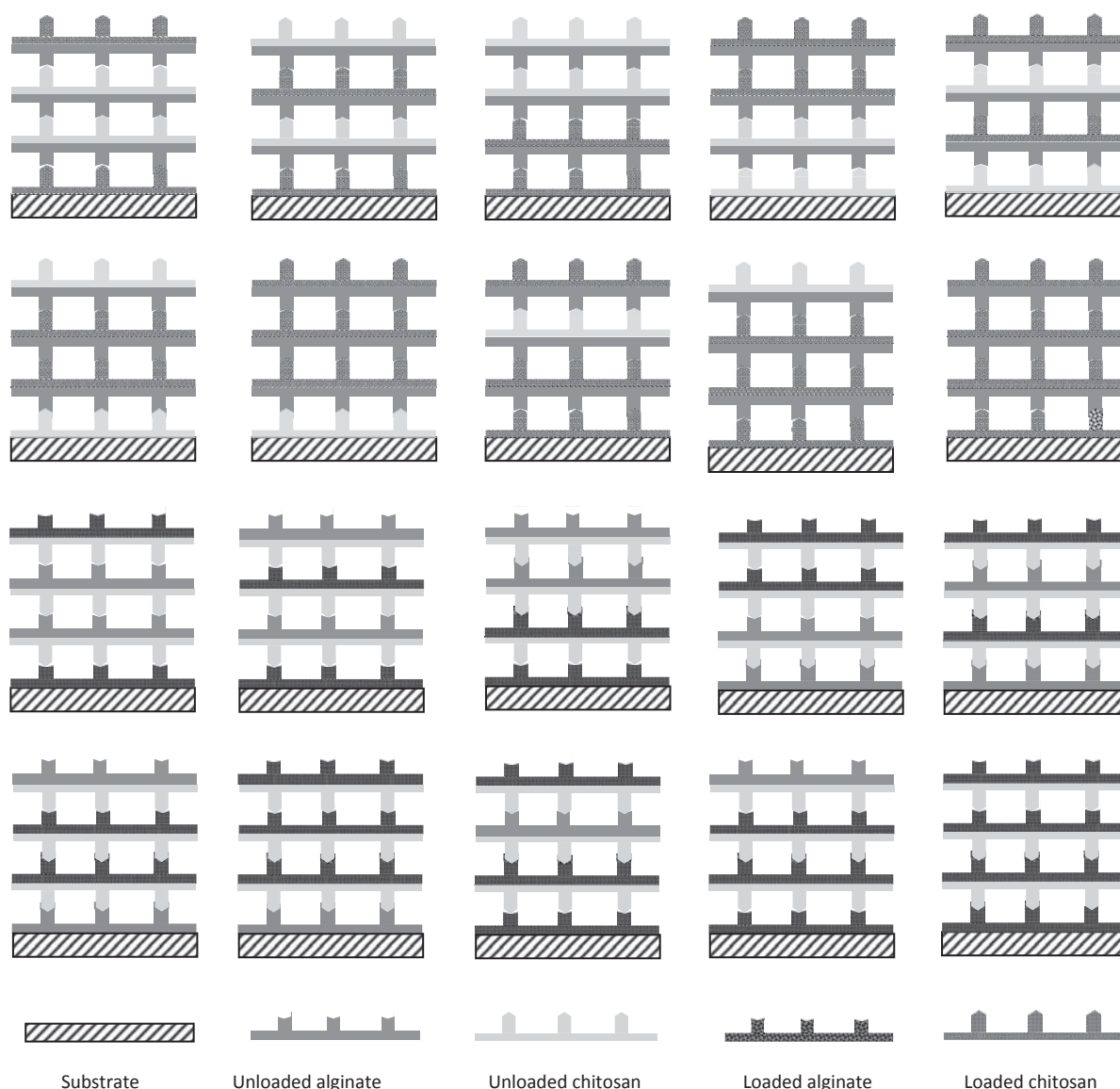
### Bioactive Release From Biopolymeric Stacks in Response to Stimuli

Different release mechanisms are employed to achieve tailored delivery of encapsulated bioactive(s). Modified release, as compared with immediate passive release, refers to a delayed, extended and/or targeted release of the desirable amount of bioactive to the intended body site at a specified time. Controlled release and sustained release are the two major mechanisms of extended release (although similarity is found between them), and can be realized through judicious choice of the carrier constituents and their properties and interactions. Controlled release targets liberation of bioactive(s) at a predetermined and nearly constant rate over a specific period (often following the zero-order release kinetics). Sustained release systems are designed to liberate continuously the bioactive(s) over a specific period at a predetermined rate (but not a constant rate, typically not less than two-fold decrease in dosing frequency over the release period) to achieve reduced side effects (often following first-order release kinetics). A wide range of delivery systems can be fabricated based on different modes of action for releasing the encapsulated bioactive substance(s). Amongst these, matrix-based or reservoir diffusion mechanisms can be utilized according to the spreading action of the encapsulated bioactive substance as it migrates from a confined region upon environmental changes (i.e. exposure to stimuli). Whilst both mechanisms are based on the changing physicochemical properties (e.g. swelling) of the encapsulation matrix surrounding the bioactive(s) and the osmotic differences between the solvent media inside and outside the encapsulation matrix, the matrix-based systems utilize pore opening of the encapsulation matrix whereas the reservoir systems depend on a permeable barrier. Moreover, degradation-controlled release mechanisms including bulk erosion, surface erosion or combinations thereof are also used in bioactive release systems. These approaches involve the inclusion of a polymer with a predetermined rate of degradation, dissolution and/or deformation (e.g. a single or multiple, early or late, short or long, and weak or severe erosion). The bioactive release profile can be tailored through monitoring not only the releasing rate, but also the starting time, high/low dosage, or "pause-restart" ("ON"/"OFF") of release, according to the chemical reactions and subsequent physical changes among the encapsulation system components (e.g. triggered evaporation or penetration) in response to one or more imposed stimuli. Fig. 2 illustrates the possible release profiles of three differently designed PEMs systems based on chitosan-alginate stacks for delivering the same bioactive substance. For a specific amount of the same bioactive, a range of loading methods can be developed based on its amount and distribution throughout the layers (Fig. 3), which would further complicate the bioactive release profile. All these release mechanisms can be implemented in PEM delivery systems including those for chitosan-alginate PECs.

Surface functionalization is critical to endowing bioactive delivery systems with specifically designed stimuli-responsive switches and/or targetable ligands as gatekeepers for achieving on-demand controlled release or exerting improved therapeutic properties while minimizing adverse effects. The surface properties of multi-layered carriers are especially important. The surface represents the point where exogenous substances make contact (e.g. living cells prefer hydrophilic surfaces in the native aqueous environment). Often the surfaces for bioactive delivery systems are designed to have timely degradation kinetics to harmonize with the metabolism or local physiological environment. For PEMs, the surface charge is mostly determined by the ionization and dissociation of functional groups (such as the carboxylic or sulphate groups) and depend on the chemical structures of PECs and local ionic



**Figure 2** Bioactive release behaviors of chitosan-alginate multi-layered films with tailored architectures. Multilayers with a low (Design 1), medium (Design 2) or high (Design 3) loading of bioactives uniformly distributed throughout all the layers of film.



**Figure 3** Schematic representation of the structures formed by chitosan-alginate polyelectrolyte stacks with different bioactive loading locations.

environment. Thus, any variation of local environment would alter the surface charge and relative motion of neighboring polymers, as well as the architecture and properties of PEMs. Accordingly, PEMs can readily be created to possess responsive properties to exogenous stimuli e.g. Magneto-stimulus (Wang et al., 2009), photo-stimulus (Angelatos et al., 2005), ultrasound-stimulus (Mourad and Webster, 1999), electro-stimulus (Yabutani et al., 2014), thermo-stimulus (Richardson et al., 2016), as well as endogenous stimuli (e.g. enzymes) (Constantine et al., 2003), pH (Zhao and Li, 2008), redox potential (Luo et al., 2011), charge (e.g. a tumor protease triggered charge switch) (Gjetting et al., 2014), and certain substances including glucose (Qi et al., 2009), glutathione (Kim et al., 2010), antigen (Caruso et al., 1997), lectin (Zhang et al., 2008), folate receptor (Rieger et al., 2007) and biotin-conjugates (Endo et al., 2011).

Specific processing steps may change the structure of PEM systems, e.g. different agitation methods may lead to different positioning of either polymer chains within the PEC and/or PEM network. For a particular PEM carrier with a specific bioactive loading, there may exist batch-to-batch variations associated with the encapsulation effectiveness, actual location and distribution of loaded bioactive(s), maximal biocompatibility and specific release profile. The controllable responses of the inner layer stacks and the outer zone of PEMs to the surrounding environments afford PEMs with different dynamic properties e.g. nanocarriers can be tuned to have a long circulation time in the blood (Yang et al., 2010). Further, the two types of polymer molecules in PEM shells (e.g. alginate and chitosan) can be tuned to impart directly a desirable impact on the activities of encapsulated bioactive through interacting with the active molecules present in their environment, leading to a beneficial synergy of the whole bioactive-loaded PEM carrier, for example, a stable chitosan-alginate PEM carrier with multiresponsive attributes and multifunctional ligands.



With the incorporation of functional modules into hierarchical PEM structures, an auto-modulated bioactive delivery with ON/OFF responses based on a number of cooperative mechanisms can be constructed.

## Conclusion

The encapsulating and release properties of polymer electrolyte multilayers are governed by the nature of component polymers, the concentration ratio of the component polymers and the physics and chemistry of the interactions between the individual polymer chains and environmental factors. The PEM fabrication history (including substrate and processing methods), along with the location and concentration of incorporated bioactive substance(s) are also important. The self-assembly of PEM carriers requires a case-by-case approach, wherein the end goal drives PEM development. In the design of PECs stacks, the nature (e.g. internal stoichiometry and conformation) and pharmacokinetics of the target bioactive substance as well as its intended time-related release amount should be considered. The number, thickness of layers, terminal layer and carrier configuration of PEMs, along with the viscoelastic properties (e.g. viscosity, shear or elastic modulus), surface composition and charge of the outermost layer as affected by environmental conditions (pH, temperature, ion type and strength), all determine the stability, bioactive release behavior and biodegradation of the delivery system and even the functionality of intended bioactive substance. In all cases, irreversible changes in the bioactive substance of interest and its pharmacokinetics as a therapeutic should be avoided.

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# Interactions Between Food Ingredients and Nanocomponents Used for Composite Packaging

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## Introduction

Global sales of nanomaterial based products to the food and beverage packaging sector has been significantly rising in recent years (Ravichandran, 2010). Nanotechnology has offered wide range of alternatives with improved performance in food packaging. The typical components of packaging formulation include barrier-forming polymers, oxygen scavenger, moisture control agents, anti-microbials, and UV protective agents. The development or selection of individual packaging formulation depends on the nature of the food to be packed; for example, dry powder and crispy foods would need a strict control on moisture, otherwise caking of dry powder or softening of crisp food may take place. On the other hand, excess moisture loss from some food items can lead to desiccation. The moisture, if not regulated, can also cause microbial growth or increase the rate of oxidation in combination with other factors. Similarly, oxygen can trigger the oxidative reactions and promote the growth of certain microbes, which finally results in spoilage or deterioration of food quality causing foul odors, unacceptable color change, change in flavor, reduction in nutritional values, etc. Bionanocomposites have offered greener alternatives to traditional packaging materials i.e. providing similar packaging function while being environmentally benign. These bionanocomposites can then be tuned into active and smart packaging with additional functions (as shown in Table 1). Polymers commonly used in nanoparticle composites include low density polyethylene (LDPE), gelatin, isotactic polypropylene and polylactic acid polymers. Examples of nanoparticle composites include Ag/LDPE, CuO/LDPE, TiO<sub>2</sub>/LDPE and ZnO/LDPE composites, as well as ZnO/gelatin, Ag/OMt-LDPE, Ag/poly(3-hydroxybutyrate-co-18 mol%-3-hydroxyvalerate), ZnO/PLA, ZnO/graphene oxide/PLA. Foods are often made up of a variety of components such as water, carbohydrate, fats, protein and minerals which are arranged in different structural patterns/forms. To understand the interactions between packaging (nanocomponents) and a food, the migration of particles from packaging composite into the food should be considered. In literature, there are quite a lot of reports on the occurrence of particle migration, but most of them haven't found strong evidence to the possibility of the human exposure. Also, the synergistic or antagonistic effect of food components caused by their action deserve attention.

## Nanoparticles and Migration Issues

### Silver (Ag) Nanoparticles

Silver usually shows action against those bacteria which, otherwise, have developed resistance against antibiotics (Emamifar, 2011). The proposed mechanisms underlying the antimicrobial activity of Ag nanoparticles include the generation of reactive oxygen species (ROS) (which induces oxidative stress and causes degradation of cell membrane structure), and the release of ions from the surface of nanoparticles resulting in death of the microbes (due to their binding to the cell membrane) (Emamifar et al., 2011; Sharma et al., 2015). Silver ions that are released from the surface of nanoparticles are also found to interact with the thiol groups present in cell proteins, inducing bacterial inactivation, condensation of DNA molecules, and loss of their replication ability (Emamifar et al., 2011; Sharma et al., 2015). A recent study concluded that the total amount of silver released from the food

**Table 1** Classification of nanocomponents used in food packaging

Classification of nanocomponents of food packaging based on function		
<b>Sensors (in active and smart packaging)</b>		
Detection of time/temperature abuse over storage		
Detection on micro-organisms and deterioration of food quality		
Microwave doneness		
Moisture control		
Gas indicators		
Freshness indicators		
<b>Active components based on functions</b>		
Food storage	Ag	Anti-bacterial action
Packaging film	Ag, SiO <sub>2</sub> , Mg, ZnO	Barrier properties
Nano screens	Colloidal precious metals	Removal of pathogens
UV protection	TiO <sub>2</sub>	UV absorption
Prevention of oxidative damage	ZnO	Free radical scavenging
Nano reinforcement	Nanoclays, Nanocellulose,carbon nanotubes, silica	Reinforcement of composite properties
Antimicrobial properties	Modified clays, Ag, ZnO	Microbial-Growth inhibition

containers to which consumer would expose is low, in comparison to the background silver exposure of general population. Since natural background concentrations are only known for ionic silver, the exposure to silver nanoparticles can not directly be compared with a safe background level (von Goetz et al., 2013). Another study observed that the amount of migrated nanosilver increased with storage time and temperature (Huang et al., 2011). Bott et al. studied the potential of silver nanoparticles (Ag-nanoparticles) that may migrate from food contact polyolefins into food, and then suggested that such migration would not lead to significant exposure to consumer (Bott et al., 2014a). In another study conducted by Jokar and Abdul Rahman, it was found that a higher silver concentration in the nanocomposite, higher temperature, and higher acidity of contact liquid would promote the silver ion release from the nanocomposite films. This migration of silver ions from nanocomposites followed the first order diffusion kinetics (Jokar and Abdul Rahman, 2014). Another sensitivity analysis showed that silver migration from the nanocomposite to the food surface was influenced most by the percentage fill, followed by the storage time and storage temperature of composite packaging (Cushen et al., 2013). A comprehensive study on marketed packaging revealed silver migration for all samples studied, with the total silver migration values ranging between 1.66 and 31.46 ng/cm<sup>2</sup> (lower than the permitted limits). The size and morphology of the silver nanoparticles changed among different samples, and migration of other nanosized materials was also observed (Echegoyen and Nerín, 2013). An investigation was carried out at 40 °C for 10 days to examine four brands of commercially available plastic food packaging, the total amount, particle size and concentration of silver, and the migration rates into three different food simulants (Milli-Q grade water, 10% ethanol, and 3% acetic acid). In this study, the total content of silver in the containers was found in the range of 13–42 µg/g. The highest migration was shown by the 3% acetic acid food simulant for all four brands of packages, with total silver released up to 3.1 ng/cm<sup>2</sup> after 10 days. This study concluded that silver has the potential to migrate into food, especially when in contact with more acidic substances (Mackevica et al., 2016). Some negative results about the migration issue of Ag nanoparticles under abnormal conditions (no migration in normal conditions) were observed by Addo Ntim et al. (2015).

### Cupric (CuO) Nanoparticles

CuO nanoparticles are used in products used for human care and in the household because of their bactericidal properties. CuO nanoparticles release copper (Cu) ions into solution; high toxicity of ions is associated with their high redox activity. Free Cu ions can produce hydroxyl radicals on the external surface of the cells, destroying the cell membrane. Excessive heavy metal(s) can also lead to breakage of protein chains. In a study on migration of Cu nanoparticles, significant migration of nanoparticles from polyethylene composite packaging to a food material was found. Simulation models for accurate assessment of the extent of such migration was feasible, although the varied background levels of copper in the food matrices would make the prediction of trace nanocopper migration more difficult (Cushen et al., 2014).

### Silica (SiO<sub>2</sub>) Nanoparticles

Silica nanoparticles (unmodified) are commonly used for reinforcement in packaging composites. Some studies reported that the presence of grafted polymers on the surface of silicon dioxide improved the tailorability of the composites by affecting the tensile properties and interfacial interactions (Wu et al., 2002; Zhang and Rong, 2003). In the study conducted by Wu et al. (2002), silica was found to improve tensile properties of the composite material, when added to polypropylene matrix. Xiong et al. (2008) also obtained similar results that tensile properties of a starch-based composite were improved after the addition of silica nanoparticles. Vladimirov et al. incorporated silicon dioxide in an isotactic polypropylene (iPP) matrix using maleic anhydride grafted polypropylene (PP-g-MA) as a compatibilizer. Silicon dioxide was also reported to increase storage modulus of the iPP matrix, thus making the material stiffer while improving oxygen barrier properties (Vladimirov et al., 2006). Jia et al. prepared nanocomposites of polyvinyl alcohol and silica by radical copolymerization of vinyl silicon dioxide nanoparticles and vinyl acetate. The nanocomposites could improve the thermal and mechanical properties as compared to the pure polyvinyl alcohol, probably due to strong covalent interactions between silicon dioxide and the polymer matrix (Jia et al., 2007). Further, Tang et al. prepared biodegradable films composed of starch, polyvinyl alcohol, and silicon dioxide, and the relative amount of silica was found to affect directly the tensile properties and water resistance of the films (Tang et al., 2008). Due to their high affinity, SiO<sub>2</sub> nanoparticles can be hazardous to health. nanoparticles can enter the human body via three routes: products containing nanoparticles (16%), skin (58%) and the respiratory system (25%). The safety of nanoparticles-containing systems used in the food industry must be tested thoroughly and considerable work in this aspect has been conducted. However, the interaction between nanoparticles and food has not yet been well investigated. On the other hand, food ingredients can also affect the absorption and toxicity of nanoparticles. SiO<sub>2</sub> can react with saccharides, proteins, fatty acids and minerals. Maximum 4.6% of saccharides, 1.7% of fatty acids, and 1.6% of minerals were found absorbed on nanoparticles. Weak interaction of SiO<sub>2</sub> with the proteins was found. In a European Food Safety Authority (EFSA) study on silica, it was observed that the substance silicon dioxide does not raise a safety concern for the consumer in the currently authorized conditions of use (EFSA, 2014). A study demonstrated an approach to track nanomaterials from source-to-sink and establish a baseline occurrence of nano-scale SiO<sub>2</sub> in foods and wastewater treatment plants (Yang et al., 2016). In a study by Go et al. (2017), the interactions between food additive silica nanoparticles and food matrix components such as saccharides, proteins, lipids, and minerals, were investigated. The results showed that zeta potential and hydrodynamic radius of silica nanoparticles were affected by all the food matrices, although their solubility was not affected. However, quantitative analysis of the interactions revealed that a small portion of food matrices interacted with silica nanoparticles and such interactions were

dependent on the type/nature of food component. Additionally, minor nutrients could also affect the interactions, as shown by greater nanoparticles interaction with honey as compared to that with a mixture of simple sugars with equivalent amounts of fructose, glucose, sucrose, and maltose (Go et al., 2017). Further study on the effect of the presence of food components on the oral absorption of nanoparticles along with their interactions, revealed that oral absorption of nanoparticles ( $3.94 \pm 0.38\%$ ) was greater than that of bulk materials ( $2.95 \pm 0.37\%$ ), possibly due to intestinal transport by microfold (M) cells. Oral absorption profile of silica nanoparticles was highly affected by the presence of sugar or protein, resulting in a high glucose absorption rate, probably due to their surface interaction on nanoparticles (Lee et al., 2017).

### Zinc Oxide (ZnO) Nanoparticles

Zinc oxides in the form of nanoparticles are widely used. The antibacterial activity of zinc oxide is known to increase with a decrease in particle size and such an action can be stimulated by visible light (Mishra et al., 2017). The additional UV-absorbing property would cause reduced UV damage and thereby improved composite stability (Martirosyan and Schneider, 2014). Additionally, zinc oxide is recommended as a generally recognized as safe (GRAS) material by FDA. Zinc oxide nanoparticles can induce disorganization of the cellular membrane due to their accumulation (Sharma et al., 2015; Emamifar, 2011). Jin et al. (2009) studied several approaches for applying zinc oxide nanoparticles to food systems. A recent study investigated the combined toxicity of zinc oxide nanoparticles (ZnO nanoparticles) and vitamin C (Vc, ascorbic acid). The results showed that Vc increased the cytotoxicity significantly compared with ZnO nanoparticles alone. When the cells were exposed to ZnO nanoparticles at a concentration less than  $15 \text{ mg L}^{-1}$ , or to Vc at a concentration less than  $300 \text{ mg L}^{-1}$ , there was no significant cytotoxicity. However, when  $15 \text{ mg L}^{-1}$  of ZnO nanoparticles and  $300 \text{ mg L}^{-1}$  of Vc were introduced together to the cells, cell viability decreased sharply indicating significant cytotoxicity (Wang et al., 2014). In another study, six nanosilver-labelled products and five silver ion ( $\text{Ag}^+$ )-labelled products were used to investigate the migration of Ag from food-contact plastics, including nanosilver into various food simulants. Migrations of Ag and Zn were the highest in 4% acetic acid, and such migrations also occurred in water and 20% ethanol. Big differences were not observed in the migration ratio between nanosilver products and  $\text{Ag}^+$  products. Ultrafiltration experiments revealed that Ag migrated from nanosilver products into 4% acetic acid was in its ionic form, while Ag migrated into water and 20% ethanol was in its nanoparticle form (Ozaki et al., 2016). Emamifar et al. (2011) prepared LDPE films containing Ag and ZnO nanoparticles through melt mixing in a twin-screw extruder. The packaging made from nanosilver-containing nanocomposite film showed a more pronounced antimicrobial effect, as compared with nano-ZnO during 112 days storage of inoculated orange juice. However, LDPE + 5% P105 packages showed a significant antimicrobial activity compared with others (Emamifar et al., 2011).

### Titanium Dioxide ( $\text{TiO}_2$ ) Nanoparticles

Titanium dioxide is another compound that has been approved by FDA for uses in human food, drugs, cosmetics and food packaging. Bactericidal property of  $\text{TiO}_2$  has been reported against *Escherichia coli*, *Salmonella choleraesuis*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Diaporthe actinidiae* and *Penicillium expansum*.  $\text{TiO}_2$ -coating has also been used for food preparations or packaging equipment (Emamifar, 2011). Chawengkijwanich and Hayata developed a titania coated packaging film and tested its antimicrobial ability against *E. coli* via both *in vitro* and in actual tests, under the conditions of two different particle sizes and two types of illumination at different intensities. They showed that both UV-A alone, and titanium dioxide coated OPP (oriented polypropylene) film combined with UV-A, reduced the number of *E. coli* cells *in vitro*, although the reduction of *E. coli* cell numbers was greater for titanium dioxide coated OPP film combined with UV-A (Chawengkijwanich and Hayata, 2008). In a study on the migration into food simulants of titanium nitride (TiN) nanoparticles incorporated at three different levels in low density polyethylene (LDPE) films was carried out under severe test conditions. The study concluded that due to the usual size, shape and aggregation of nanoparticles in plastic nanocomposites, nanomaterials were likely immobilized in food contact plastics, thus the possibility of exposure to the consumer was negligible (Bott et al., 2014b). The migration of Ti from nano- $\text{TiO}_2$ -PE films used for food packaging into food simulants under different temperature and migration time conditions was further investigated. It was found that increasing the additive content in the film promoted the migration of nanoparticles, via dissolution from the surface and cut edges of the solid phase (film) into the liquid phase (food simulant) (Lin et al., 2014). Faust et al. (?) found that  $\text{TiO}_2$  isolated from the candy coating of chewing gum and commercially available  $\text{TiO}_2$  food grade additive samples were of the anatase crystal structure. Through the use of two independent techniques (by which the effects of gravity and subsequent  $\text{TiO}_2$  sedimentation were removed), disruption of the microvilli was found independent of sedimentation, and food grade  $\text{TiO}_2$  exposure resulted in the loss of microvilli from the Caco-2BBE1 cell system as a result of a biological response (not a physical artifact of *in vitro* exposure) (Faust et al., 2014). In another study, the effect of the interactions between the  $\text{TiO}_2$  and biomolecules on oral absorption was investigated and the intestinal transport pathway was also assessed using 3-dimensional culture systems. The obtained results demonstrated slightly higher oral absorption of f- $\text{TiO}_2$  (food grade) nanoparticles compared to g- $\text{TiO}_2$  (general grade) nanoparticles, which could be related to their intestinal transport mechanism by M cells. However, most of the nanoparticles were eliminated from the body through feces. Additionally, the biokinetics of f- $\text{TiO}_2$  nanoparticles was found to depend on their interaction(s) with biomolecules, and their dispersibility was affected by surface chemistry (Jo et al., 2016).



### Nanoclays

Nanoclays are commonly used in nanocomposite form with packaging polymers and usually forms intercalated or exfoliated type of composites. The exfoliated nanocomposites are known to exhibit the best properties because of the improved interactions between polymer and clay. A commonly used clay for food packaging nanocomposites production is Montmorillonite (MMT) with general formula  $M_x(Al_{4-x}Mg_x)Si_8O_{20}(OH)_4$ . MMT exhibits excellent reinforcing filler abilities in the range between 50 and 1000 (Uyama et al., 2003). According to Nielsen (1967), the improvement in barrier properties of nanocomposites is probably due to the increasing tortuosity of the diffusive path for permeants (such as moisture, oxygen, microbes etc.), presenting a longer path for them to travel for diffusion through the film. Other authors (Adame and Beall, 2009; Mirzadeh and Kokabi, 2007) further reiterated this theory. The mineral clays have also been investigated as biocide carriers for inorganic biocides such as silver, zinc, copper, magnesium, etc (Patakfalvi and Dékány, 2004; Patil et al., 2005). Nanoclays have also been functionalized to be used as antimicrobial agents (Thostenson et al., 2005). The aminopropyl functionalized magnesium phyllosilicates (AMP clay) were found to inhibit strongly the growth of microorganisms such as *E. coli*, *S. aureus* and *C. albicans* (Rhim et al., 2013). In a nanoclay migration study, a bionanocomposite consisting of polylactide (PLA) with 5% Cloisite30B (a derivatized montmorillonite clay) was used as a filler, and the particles ranging from 50 to 800 nm in radius migrated into the 95% ethanol (which was used as a food simulant). No clay mineral could not be detected in the full hyphenated AF(4)-MALS-ICP-MS system and absence of clay mineral in migrate was concluded (Schmidt et al., 2009). Farhoodi et al. (2014) prepared poly(ethylene terephthalate) (PET)/clay (Cloisite 20A) nanocomposite samples and they observed that the migration process was dependent on storage time and temperature. The molar ratio of aluminum and silicon in the acidic aqueous solution  $(Al/Si)_{aq}$  used for migration testing to the ratio in the solid phase of prepared nanocomposites  $(Al/Si)_{solid}$  was about 23% higher for the samples stored at 45 °C (Farhoodi et al., 2014).

### Nanocellulose

Two types of reinforcement can be generated from cellulose, namely CNFs (Cellulose Nano-fibrils) and CNCs (Cellulose Nano-crystals) (Azizi Samir et al., 2005). CNFs are actually the bundles of molecules, elongated and stabilized by hydrogen bonding. The diameters of microfibrils are in nanosize range, around 2–20 nm, and lengths are in the range of micrometer. The length of the cellulose nanocrystals ranges from 500 nm to 1–2 µm, and diameter from 8 nm to 20 nm or less, resulting in high aspect ratios. The CNC can be isolated from CNF by acid hydrolysis. Acid hydrolysis works by removing the amorphous parts of the fibrils by dissolving them in acid (usually sulphuric acid), leaving the crystalline regions intact (De Azeredo, 2009). De Azeredo (2009) studied the effect of cellulose nanofibers on the physical properties of mango puree edible films and found that cellulose nanofibers, when used as filler, increased effectively the tensile strength and young's modulus of mango puree films (De Azeredo, 2009). Helbert et al. (1996) explained this effect of cellulose nanofibers on the modulus: an increase in modulus is due to not only the geometry and stiffness of the nanofibers but also the formation of a fibrillary network within the polymer matrix (Helbert et al., 1996). Cellulose nanofibers seem to link through hydrogen bonds. No migration of nanocellulose has ever been reported.

### Carbon Nanotubes

Another important group of reinforcing agents are carbon nanotubes. Carbon nanotubes have extraordinarily high aspect ratios and elastic modulus (Zhou et al., 2004). According to Lau and Hui (2002), carbon nanotubes can have theoretical elastic modulus and tensile strength values up to 1 TPa and 200 GPa, respectively. In a study by Kim et al. (2008), carbon nanotubes were functionalized with carboxylic acid groups on their surfaces to enhance the intermolecular interactions between the poly(ethylene-2,6-naphthalene) matrix and carbon nanotubes. Carbon nanotubes showed an improvement in thermal stability as well as tensile strength and modulus of poly(ethylene-2,6-naphthalene), even at a concentration as low as 0.1 wt%. Polyvinyl alcohol, polyamide and polypropylene are other polymers whose tensile strength/modulus could be improved by the addition of carbon nanotubes (Bin et al., 2006; Chen et al., 2005; Manchado et al., 2005; Zeng et al., 2006). In a recent study (reference?), carbon black nanoparticles was assessed in terms of the possibility to migrate out of plastic materials used in the food packaging industry. Two types of carbon black were incorporated in low density polyethylene (LDPE) and polystyrene (PS) at 2.5% and 5.0% loading (w/w), respectively, for migration studies. The samples were exposed to different food simulants according to European Union Plastics Regulation 10/2011, simulating long-term storages for aqueous and fatty foodstuffs. At a detection limit of 12 µg kg<sup>-1</sup>, carbon black did not migrate from the packaging material into food simulants. The experimental findings are in agreement with theoretical considerations based on migration modelling. From both the experimental findings and theoretical considerations, it can be concluded that carbon black does not migrate into food once it is incorporated into a plastics food contact material (Bott et al., 2014c).

### Conclusions

The interactions between packaging nanocomponents and foods have mainly been studied on the particle migration from packaging to food. The migration of nanoparticles from packaging to food has largely been assessed in terms of the safety for human



exposure. The chemical nature of food component including surface activity and propensity to bind nanoparticle affect both the nanoparticles and food of interest. For example, flavonoids (compounds with proven antioxidant properties) and some lipids and proteins (substances with significant surface activity) show strong tendency to bind nanoparticle surface. Although many studies can be found on food additive nanoparticles and food components, inside and outside the GIT (gastrointestinal tract), the number of studies on the impact of migrated particle on food is fairly few. More investigations in this direction are required to obtain a more comprehensive understanding of this issue.

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## Use of Pectin to Formulate Antimicrobial Packaging

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### Introduction

Food packaging systems have traditionally been used to contain the food and isolate it from environmental factors that affect their quality and therefore can arrive intact to the consumer from the place where they are produced (Valdés et al., 2015). However, the food industry has been given to the search for new packaging systems in view of increasing environmental concerns for synthetic packaging; as well as the growing consumer demand for more natural foods. As an option, biopolymers have emerged as food packaging materials due to their versatility and biodegradability, which could be helpful to solve the environmental problems caused by wastage of plastic packaging. Therefore, biodegradability is not only a functional requirement but also an important environmental attribute. Among biopolymers, polysaccharides are frequently used as innovative packaging materials by their ubiquitous presence in nature. Particularly, pectin is a polymeric chain of hydrophilic nature, with excellent oxygen, aroma, and lipid barrier ability as well as good gelling properties (Espitia et al., 2014). This last characteristic gives to pectins the ability to form edible films and coatings used as biodegradable packaging. Pectin provides film integrity and can trap different antimicrobials to formulate antimicrobial packaging, which could protect foods against contamination with spoilage and/or pathogenic microorganisms, maintaining safety and quality.

### Antimicrobial Packaging

Nowadays, demands of high-quality and safety foods became the major challenge of food industry, which has been continuously explored novel food processing technologies to achieve this goal (Miranda et al., 2016). In this sense, microbial contamination became a major concern because food is more susceptible to present some kind of spoilage when it finally reaches the consumer. This represents great economic losses throughout the food chain and health sector by foodborne diseases. Some alternatives have been used to assure safety of food products; however, the main way to isolate food from deteriorating and/or contaminating conditions is through packaging (Sung et al., 2013). Antimicrobial packaging (obtained by adding antimicrobial substances to packaging materials) has emerged as a novel development in the field of food processing that can significantly reduce pathogen survival and extend shelf life by facilitating the release of antimicrobials from the packaging (Sung et al., 2013).

Among the main materials used to formulate food packaging, plastics derived from petroleum have been by far the most used. They are cheap, present good aesthetic quality, and possess excellent physicochemical properties (Rhim et al., 2013). However, due to nondegradability of plastics and the environmental impact problem that the disposal of these wastes causes, research has been focused in the use of biopolymers obtained from natural sources (Siracusa et al., 2008): proteins (gluten, collagen, wheat and milk proteins, carrageenan, etc.), lipids (fatty acids, monoglycerides, waxes, etc.), and polysaccharides (starch, cellulose derivatives, chitosan, alginates, carrageenan, pectin, etc.) (Tang et al., 2012; Galgano et al., 2015).

Polysaccharides have been widely used as food packaging materials by their ubiquitous presence in nature, as well as by their low cost, film-forming ability, and good mechanical and gas barrier properties (Valdés et al., 2015). Among them, pectin has been used to formulate edible films and coatings and have shown good gelling properties and gas permeability, in addition to carrying antimicrobial additives, even when it has poor water barrier properties (Espitia et al., 2014). Pectin in combination with antimicrobials has served as antimicrobial packaging, controlling growth of spoilage and pathogenic microorganisms such as *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella Typhimurium*, *Alttrernaria alternata*, *Salmonella Choleraesuis*, among others (Ayala-Zavala et al., 2013; Alvarez et al., 2014; Rodriguez-Garcia et al., 2016).

As mentioned before, antimicrobial packaging is created by an “activation” of packaging material using compounds with antimicrobial activity. Some antimicrobials mostly used for this purpose include organic acids, enzymes, bacteriocins, plant extracts, essential oils (EOs), and their main components (Mauriello, 2016). The purpose of incorporating antimicrobial agents in polymeric matrices such as pectin aims to reduce the necessary concentrations of antimicrobial compounds to kill or inhibit the growth of microorganisms. The main mode of action is the gradual diffusion of the active compound from the package to the food surface; in addition, this gradual release prevents inactivation of the antimicrobials (Galgano et al., 2015).

### Pectin Sources, Structure, and Properties

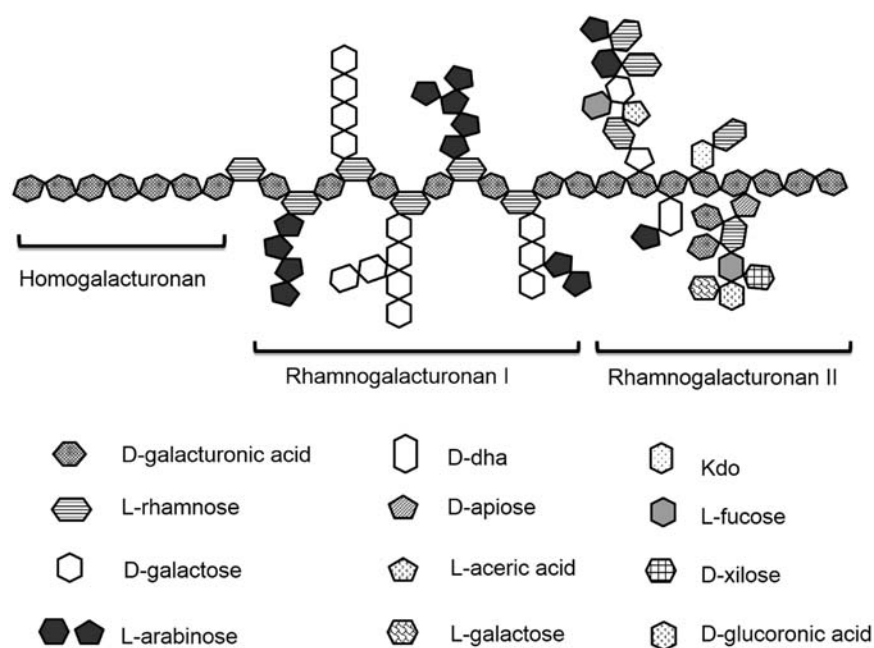
Pectins are an important water-soluble biopolymer present all around us, which constitute about one-third of the cell wall of higher plants (Srivastava and Malviya, 2011). There are a lot of fruits that are good sources of high-quality pectins, such as apples, citrus fruits, papaya, peach, among others (Faravash and Ashtiani, 2007; Guo et al., 2012; Sharma et al., 2015; Yadav et al., 2015). Although pectin is commonly found in most plant tissues, the number of sources that can be used for commercial manufacture of pectin is limited. This is due to the smaller proportions of pectin found in plants, which is related to their dilution in great

amounts of tissues and their degradation due to rapid metabolic changes (Matsumoto and Otagaki, 1990). On each plant or fruit, the content of pectins is variable and depends on the age, ripening stage, and environmental conditions (Navarro and Navarro, 1985). Pomace from apples, citrus albedo, sugar beet pulp, and sunflower rinds have been proposed as by-products from the agricultural and food industries to obtain pectic substances with contents over 15% on dry basis (Giovanetti et al., 2012). However, apple pomace (10–20% of pectin) and citrus peel (30–35% of pectin), obtained from juice industry, are the two main raw materials used for industrial extraction of pectins (Kumar and Chauhan, 2010). Other plant materials reported as sources for pectin extraction are listed in Table 1.

Structurally, pectin is a heteropolysaccharide constituted by  $\alpha$ -(1, 4)-galacturonic acids with various degrees of methylation of carboxylic acid residues and/or amidated polygalacturonic acids (White et al., 1999). Pectin structure can be divided in two main regions: a “smooth” region composed by homogalacturonan and a “hairy” region constituted by rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II) (Willats et al., 2001; Fig. 1). HG is a linear homopolymer of  $\alpha$ -(1, 4)-linked galacturonic acids that comprises about 65% of pectin. The other pectic polysaccharides are considerably more complex in structure than HG (Mohnen, 2008); for example, RG I consists of a backbone of galacturonic acids interrupted by residues of L-rhamnose attached

**Table 1** Plants materials as sources of pectins

Source	Yield (%)	References
Mulberry branch bark	31.47	Liu et al. (2010)
Sugar beet pulp	4.1–16.2	Yapo et al. (2007)
Grapefruit	27.81	Bagherian et al. (2011)
Peach pomace	7.03	Faravash and Ashtiani (2007)
Cocoa husks	7.62	Chan and Choo (2013)
Yellow passion fruit rind	6.4	Yapo (2009)
Yuza pomace	7.3	Lim et al. (2012)
Orange peel	20.44	Guo et al. (2012)
Sweet potato	7–30	Nurdjanah et al. (2013)
Pomegranate peel	6.8–10.1	Abid et al. (2017)
Apple pomace	13.3	Sharma et al. (2015)
Tomato waste	21.1	Grassino et al. (2016)
Banana peels	14.23	Oliveira et al. (2016)
Papaya peel	25.39	Maran and Prakash (2015)
Sisal waste	29.32	Maran and Priya (2015)
Pomelo peel	23.19	Methacanon et al. (2014)



**Figure 1** Structure compounds of pectin: homogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II (Gutierrez-Pacheco et al., 2016).

by  $\alpha$ -(1, 2) bonds to which a variety of different glycan chains (principally arabinan and galactan) are attached and represents about the 20–35% of pectin structure (Willats et al., 2006; Mohnen, 2008). On the other hand, RG II has a backbone of HG, with complex side chains (mainly rhamnose, arabinose, and some sugars such as apiose and methylfucose) attached to galacturonic acid residues (Ridley et al., 2001).

Depending of the degree of esterification (DE), pectin can be classified as high methoxyl pectin (HMP) and low methoxyl pectin (LMP) (Espitia et al., 2014). HMPs have over 50% of their carboxyl groups esterified ( $DE > 50$ ), while LMPs have a  $DE < 50$ . The DE depends on the source of pectin and extraction method, influencing the gelation ability of pectin. This property has gained a lot of attention because it strongly determines the physical characteristics of pectins such as their capability to form films, which can be used as food packaging material (Videcoq et al., 2011). In the next section, some studies about the use of pectin in combination with antimicrobials to formulate antimicrobial packaging are illustrated, as well as their effect on food matrices.

## Pectin as Antimicrobial Packaging Material

Naturally, pectin is a nonantimicrobial material; however, due to its functional properties, it has been used as a carrier material to inhibit the growth of spoilage and pathogenic microorganisms. Some authors have reported the use of pectin films or coatings incorporated with antimicrobials such as enzymes, metals, EOs, plant extracts, bacteriocins, among others. Some studies about the use of pectin as an antimicrobial packaging against isolated strains and tested in food matrices are considered in the following sections.

## Antimicrobial Activity of Pectin Against Isolated Food Bacteria

The antibacterial activity of edible films based on pectin and apple puree incorporated with oregano, cinnamon, or lemongrass EOs was tested against *Escherichia coli*, being oregano incorporated films the most effective (inhibition zone = 1.4 mm) followed by lemongrass (1.2 mm) and cinnamon EOs (1.1 mm) (Rojas-Graü et al., 2006). Similarly, pectin films incorporated with oregano EO also have been effective against *E. coli* O157:H7, *S. Choleraesuis*, *S. aureus*, and *L. monocytogenes* showing inhibitory zones of 16.3, 18.1, 20.8, and 21.3 mm, respectively (Alvarez et al., 2014). On the other hand, Du et al. (2009) tested pectin–tomato puree films incorporated with oregano, allspice, or garlic EOs against *E. coli*, *Salmonella enterica*, and *L. monocytogenes*. The results indicated that the antibacterial activities against the three pathogens were in the following order: oregano oil > allspice oil > garlic oil. EOs antibacterial activity has been attributed to individual chemical compounds presented in their composition; for example, the activity of oregano EO has been attributed mainly to their terpenes carvacrol and thymol. On the other hand, eugenol and cinnamaldehyde are mainly responsible for the antibacterial activity of cinnamon and clove EOs (Burt, 2004). For this reason, the major constituents of EOs have been used as antimicrobials in active packaging films made of pectin.

Edible films based on pectin and tomato puree incorporated with carvacrol (0.75% and 1.0%) showed antibacterial activity against *E. coli* O157:H7, with inhibition zones of 14.1 and 43.4 mm<sup>2</sup> (Du et al., 2008). Similarly, Otoni et al. (2014) found that cinnamaldehyde (2% wt) nanoemulsions incorporated in pectin–papaya puree films showed antibacterial activity against *E. coli*, *S. enterica*, *L. monocytogenes*, and *S. aureus* with inhibition zones up to 104.72, 257.06, 176.67, and 648.59 mm<sup>2</sup>, respectively. Pectin also has been combined with alginate and lemongrass EO (0.1%, 0.2%, 0.3%, and 0.4% v/w) and proved against *E. coli* O157:H7 showing inhibition zones of 50, 69.9, 78.9, and 94.0 mm<sup>2</sup>, respectively (Maizura et al., 2007).

The bacteriocin nisin has been extensively used as potential natural preservative in the food industry. However, the antimicrobial activity of nisin is reduced when it is applied directly in foods due to its binding with other food components and enzymatic inactivation (Aasen et al., 2003). For this reason, encapsulation of nisin into polymeric matrices such as pectin has emerged as an efficient approach to overcome this problem (Khaksar et al., 2014). Nisin-loaded pectin–inulin particles were effective against Gram-positive bacteria (*Arthrobacter* sp. and *Bacillus subtilis*), killing between  $1 \times 10^9$  and  $7 \times 10^9$  cells. A lower antibacterial activity was detected against Gram-negative bacteria (*Klebsiella* sp. and *E. coli*), where about  $2\text{--}3 \times 10^7$  cells did not survive. Overall, the effectiveness of nisin-loaded pectin–inulin particles was mostly dependent of the DE of pectin (Krivorotova et al., 2016). The particles with low DE or not esterified demonstrated the higher activity as compared to particles of high DE. Due to the high efficiency of nisin loading and similar antimicrobial activity comparing to nisin–pectin particles, combination of pectin and inulin has potential to be used in the food industry (Krivorotova et al., 2016).

Moreover, pectin–polylactic acid composite films incorporated with nisin were tested against *L. monocytogenes*. Developed composite films were tested in three different media, brain heart infusion (BHI) broth, orange juice, and liquid egg white showing reductions of *L. monocytogenes* of 2.1, 3.7, and 4.5 log CFU mL<sup>-1</sup>, respectively. The use of pectin and polylactic acid in combination with nisin has potential as antimicrobial food packaging to reduce postprocess contamination (Jin et al., 2009b). Pectin also has been chemically modified with fatty acids such as linoleate, oleate, and palmitate as antibacterial agents. Chemically modified pectins exhibited the greatest inhibitory effect against *S. aureus* and *E. coli* with inhibition zone values between 50% and 70%, compared with no-modified pectin (Calce et al., 2014). On the other hand, pectin films when combined with silver nanoparticles showed strong antibacterial activity against *E. coli* and *L. monocytogenes*, with inhibition zones of  $8.4 \pm 1.2$  and  $3.9 \pm 0.8$  mm, respectively (Shankar et al., 2016).



## Antimicrobial Activity of Pectin in Food Matrices

Edible films containing plant antimicrobials are gaining importance as potential treatment to extend products' shelf life and reduce risk of pathogen growth on contaminated food surfaces.

Cinnamaldehyde or carvacrol was incorporated within pectin–apple puree edible films and its antibacterial activity was tested against *S. enterica* and *E. coli* O157:H7 on chicken breasts and *L. monocytogenes* on ham. On chicken breasts, pectin films incorporated with carvacrol (3%, 1.5%, and 0.5%) reduced the bacterial populations by 3, 1.6–3, and 0.8–1 log CFU g<sup>-1</sup> at 4°C, respectively. On the other hand, cinnamaldehyde incorporated films (3% and 1.5%) induced 1.2–2.8 and 1.2–1.3 log CFU g<sup>-1</sup> reductions at the same temperature, respectively. On ham, carvacrol films induced greater reductions of *L. monocytogenes* than cinnamaldehyde films at all concentrations tested (0.4–2.2 log CFU g<sup>-1</sup>) (Ravishankar et al., 2009). A similar study, probed the antibacterial activity of edible films based on pectin and apple, carrot or hibiscus incorporated with carvacrol or cinnamaldehyde against *L. monocytogenes* on contaminated ham and bologna. Results showed that films with carvacrol exhibited better antimicrobial activity against *L. monocytogenes* than films with cinnamaldehyde, with developed films being more effective on ham than on bologna (Ravishankar et al., 2012).

Pectin apple–based edible films containing carvacrol and cinnamaldehyde were evaluated against antibiotic-resistant (D28a and H2a) and susceptible (A24a) *Campylobacter jejuni* strains on chicken at 4 or 23°C for 72 h. At 4°C, cinnamaldehyde-incorporated films caused variable reductions for all strains, ranging from 0.2 to 2.5 log CFU g<sup>-1</sup> and 1.8 to 6.0 log CFU g<sup>-1</sup> at 1.5% and 3.0%, respectively. Films with ≥1.5% cinnamaldehyde reduced populations of all strains to below detection at 23°C at 72 h. On the other hand, films with 3% of carvacrol caused a 0.5 log CFU g<sup>-1</sup> reduction for both A24a and D28a, and by 0.9 log CFU g<sup>-1</sup> for H2a at 4°C, while at 23°C the reductions were below detection for A24a and H2a, and by 2.4 log CFU g<sup>-1</sup> for D28a. Reductions ranged from 1.1 to 1.9 log CFU g<sup>-1</sup> and 0.4 to 1.2 log CFU g<sup>-1</sup> with 1.5% and 0.5% of carvacrol at 23°C, respectively (Mild et al., 2011). The differences in antibacterial activities between cinnamaldehyde and carvacrol may be due to the fact that both antimicrobials operate by different mechanisms. It is hypothesized that carvacrol disintegrate the bacterial outer membrane, releasing cytoplasmic constituents and consequently changing the passive permeability of the cell, whereas cinnamaldehyde reacts chemically with the membrane surface resulting in cell death (Burt, 2004; Mild et al., 2011; Rodriguez-Garcia et al., 2015).

The antimicrobial activity of pectin edible films incorporated with nisin and its combination with an ionizing radiation treatment was used against *L. monocytogenes* on a ready-to-eat turkey meat. Results showed that pectin–nisin films without irradiation reduced *L. monocytogenes* by 1.76 log CFU cm<sup>-2</sup>. Moreover, the combination of pectin–nisin films with irradiation resulted in a 3.95 and 5.35 log CFU cm<sup>-2</sup> reduction at 1 and 2 kGy, respectively; indicating a synergistic effect on *Listeria* viability on the surface of ready-to-eat turkey meat (Jin et al., 2009a). Both, in vitro and in vivo studies have showed the potential of pectin to formulate antimicrobial packaging, which despite the chemical structure and nature of aforementioned compounds showed an inhibitory effect against different food pathogens.

## Conclusion

Pectin has shown potential to be used as antimicrobial packaging material because of its excellent film-forming properties and good barrier properties, which has enabled it to serve as a carrier of many types of antimicrobials, from EOs to bacteriocins. Although some of these studies have shown promising results, more research are needed about the effect of antimicrobials on the physico-chemical characteristics of the packaging material as well as the feasibility of its application on a large scale. Moreover, since many of the studies have been carried out using commercial pectins, there are still many sources of pectin that need to be exploited for food applications.

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# Effect of Three-Component Interactions Among Starch, Lipids and Proteins on the Glycemic Response

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## Starchy Food, a Complex Food

The so-called “starchy foods” are products having a high starch fraction, where other constituents (biopolymers among others) are present forming a complex edible matrix. Some examples of starchy foods are bread, pasta, baked products, etc., and in general are a very important source of dietary energy worldwide. All these foodstuffs are matrices built mainly by starch, proteins, and lipids, but type of ingredients and processing conditions affect their microstructure (microscopic arrangement), which in turn can affect their organoleptic and nutritional properties, among other (e.g. shelf life) (Aguilera, 2005). In this way, current evidence suggests that the knowledge of binary interactions among starch, lipid, and protein is neither sufficient to understand the properties of food matrix and its actual effect on starch digestibility, since really the three constituents are present together in food. In fact, since synergistic or not lineal effects derived from ternary interaction among starch, lipid, and protein may be present, extrapolation of observations done in binary systems would be not the most suitable way to explain the physical and nutritional properties of foods (Zhang and Hamaker, 2003, 2004, 2005; Zhang et al., 2003, 2010; Jacobs et al., 2009). However, detailed studies about microstructure of ternary (or more complex systems) are scarce so far, mainly due the complexity of such systems, which result in a poor reproducibility of systems and research protocols. In addition, current evidence suggests that a linear relationship between food microstructure and digestibility or glycemic response (nutritional effect) is not always clearly found due to genetic or physiological differences among consumers, which finally determine the actual nutritional effects of foods (Cummings and Englyst, 1995; Englyst and Englyst, 2005).

## Digestibility of Starch

Starch is a complex carbohydrate produced by plants to store energy generated through photosynthesis. It is constituted by glucose units which form two types of biopolymers: amylose, which is linear (glucose with  $\alpha$ -1,4 bonds), and the amylopectin, which has branches and a much higher molecular weight (glucose units are linked by two types of bonds:  $\alpha$ -1,4 in the linear sections, and  $\alpha$ -1,6 in the branching points). Both polymers are joined forming discrete granules of different sizes, shapes, and amylose/amylopectin ratio, depending on the botanic origin (Sajilata et al., 2006; Copeland et al., 2009). Starch is especially abundant in legumes, tubercles, and cereals; all these are highly consumed by human beings worldwide, hence it is considered as their main energy source (Copeland et al., 2009).

In human being, starch consumed is digested at both the level and in the small intestine by the sequential action of  $\alpha$ -amylases (salivary and pancreatic), and the concerted action of enzymes such as the Maltase–Glucoamylase (MGAM) complex and the Sucrose–Isomaltase (SI) complex (Nichols et al., 2009; Santos et al., 2012). The final process of starch digestion into free glucose is completed in the small intestine brush border, with glucose efficiently absorbed in the small intestine and transported to the blood stream.

The starch that is not fully digested into glucose in the small intestine passes to the large intestine, where it may be fermented by the microbiota, thus producing short-chain fatty acids that have been related to beneficial health effects (Cummings and Englyst, 1995; Dona et al., 2010). The starch digested into glucose and having effect on postprandial glycaemia, is called “digestible,” whereas the starch passing to the large intestine is known as “resistant.” Digestible starch, on its turn, is divided into rapidly and slowly digestible starch. Slowly digestible starch produces more moderate changes in the postprandial glycaemia (concentration of glucose in the blood after a meal), whereas rapidly digestible starch leads to rapid changes in the glycaemia, even similar to that caused by simple sugars, characterized by a fast increase in blood glucose concentration up to maximal high levels (Englyst et al., 1992). Current classification of starch based on its *in vitro* digestibility under “small intestine conditions” is the following:

- Very rapidly digestible starch (VRDS): Starch hydrolyzed within 1 min (Chung et al., 2006).
- Rapidly digestible starch (RDS): Starch hydrolyzed within 20 min (Englyst et al., 1992).
- Slowly digestible starch (SDS): Starch digested during the period between 20 and 120 min (Englyst et al., 1992).
- Resistant starch (RS): Starch not hydrolyzed within 120 min (Englyst et al., 1992).

From some decades ago a great interest in food that when consumed leads to a lower glycemic response has been generated; that is, a lower change in blood glucose concentration after food ingestion (postprandial period). This interest is based on the growing scientific evidence, suggesting that exaggerated postprandial glycemic response would be a risk factor for several chronic non-transmissible diseases, such as diabetes and cardio-vascular diseases (FAO/WHO, 1998; Brand-Miller, 2003; Thomas and Wolever, 2003; Jakobsen et al., 2010; Larsen et al., 2010). The postprandial glycemic response (commonly referred as glycemic index or glycemic load) of a great variety of foods is known; their values may be considered as referential (Foster-Powell et al., 2002; Dona et al.,

2010). Several *in vitro* methods have been developed to estimate glycemic response; they may significantly differ in their methodology and not always (in fact, almost never) predict *in vivo* glucose homeostasis precisely since such methods do not include the high number of factors that take place in the physiological responses to carbohydrate-rich food (Priebe et al., 2008; Woolnough et al., 2008).

### **Role of Food Microstructure in Starch Digestion**

Starch digestibility and glycemic response depend on several factors that can be measured (considering that digestibility is only one of factors affecting the glycemic response), with the two main groups identified as: those related to the food characteristics (for example, composition and structure), and those related to the consumer (for example, physiological state and genetic background) (Cummings and Englyst, 1995).

Among the factors related to food, food microstructural and mechanical properties (which are affected in turn by microstructure) may act at various levels, affecting the rate of starch digestion (Parada and Aguilera, 2011a; Turgeon and Rioux, 2011). It has been observed that in the mouth the size reduction of food, its disintegration, and the rate of starch hydrolysis depend on the chewing time as well as the physical characteristics (determined by microstructure) of ingested food (Hoebler et al., 1998). Gastric emptying may also be delayed by the occurrence of microstructures (which are more difficult to be disintegrated inside the stomach, thus affecting the rate of the whole digestion process in the intestine). Furthermore, the matrix where starch is in the intestine and the compound intimately interacting with the starch may retard or even prevent its contact with the digestive enzymes, hence reducing its digestibility.

Additionally, the degree of gelatinization/retrogradation of starch, which is probably the main “structural” factor affecting digestibility, is a function of food processing and storing conditions and can highly affect the proportion of total digestible starch. When the gelatinized starch (with an amorphous structure; e.g. after cooked in water excess) is rapidly digested, the molecules in native crystalline state (present in raw foods) or retrograded (starch chains that, after to be in an amorphous state by gelatinization, interact among themselves during storage at low temperatures forming crystalline zones) are digested slowly or simply not digested (Fardet et al., 1998; Mora-Escobedo et al., 2004; Parada and Aguilera, 2011a).

Finally, it should be noted that although microstructure is well known to affect starch digestibility, appropriate characterization and quantification of interactions among different food constituents, in addition to compositional analysis, remain very challenging and are crucial for a successful food design (Aguilera, 2005; Jacobs et al., 2009).

### **Interaction Between Main Components of Starchy Food and Resultant Starch Digestibility**

Most of starchy foods, such as pasta, bread, etc., are formed mainly by starch, protein (mainly gluten if wheat flour is used), and lipids (mainly triglycerides), and these contents may be simply determined using standardized methods. Nonetheless, the information on the contents of these components is not sufficient to explain the digestibility of a product (Mourot et al., 1988; Riccardi et al., 2003). In many cases, this difference is derived from the microstructure formed during the manufacturing process; however, information about how these three ingredients interact in food, and the real effect of the structures on starch digestibility, are still scarce (Zhang and Hamaker, 2003, 2005).

Food constituents interact forming structures showing particular properties, which have been deeply studied in binary systems, such as starch–lipids (Tang and Copeland, 2007); nevertheless, in ternary or more complex systems, information is still rather limited (Zhang and Hamaker, 2003; Zhang et al., 2010). For example, the complex amylose–lipid is known to form two types of structures: I and II. The structure type I melts at a temperature, from 10 to 30 °C, lower than the melting temperature of the structure type II. Similarly, the structure type II may be detected by x-ray crystallography; whereas the structure type I is not detectable through this technique, which suggests that this type is probably in an amorphous state. Amylose may form structures of both type I and II, whereas amylopectin will probably bind to a lipid only through individual chains.

The type of starch–lipid formed structure depends on the lipid type and the conditions under which the complex is formed. Lipids with charged head groups have a lower tendency to form type II structures (Eliasson and Wahlgren, 2004). In other cases, both types I and II have been reported to be dependent on how the complexes are formed. Complexes of type I are dominant if their formation occurs rapidly and at low temperatures (between 70 °C and 80 °C), whereas, the most ordered complexes type II are favored if the complex formation happens at high temperatures (Eliasson and Wahlgren, 2004). In general, many other factors can also affect the formation of the starch–lipid complex, such as monoacyl chain length, polar head of the lipid, water content, type of starch, and food processing.

Complexes between amylose and lipids (fatty acids, lysophospholipids, and monoacylglycerides) may significantly modify the properties and functionality of starch; for example, starch solubility is reduced in water, rheological properties of pasta are altered, swelling capacity is reduced, gelatinization temperature is increased, retrogradation is retarded, and susceptibility to enzymatic hydrolysis is reduced (Copeland et al., 2009).

In a study on the effect of superficial components of starch granules (non-carbohydrate surface components), Debet and Gidley (2006) classified starch as fast swelling starch (e.g. waxy maize, potato, etc.), slow swelling starch (which may be transformed into fast swelling starch by extraction of superficial lipids and proteins e.g. wheat, maize, etc.), and limited swelling starch (which is not

affected by protein/lipid extraction e.g. high amylose maize/potato). In addition, Noisuwan et al. (2011) observed that milk proteins, located in the interface or in empty spaces (channels, holes, etc.) of starch granules (rice), may restrict water diffusion inside the granules during its gelatinization. This outcome shows us how “small” changes in non-carbohydrate components can significantly modify starch properties e.g. dependence on its origin/nature/structure.

On the other hand, there are studies about how gluten proteins may form matrices covering starch granules, thus preventing both their gelatinization and contacts with digestive enzymes; all of which make digestibility more difficult (Li et al., 2007; Auger et al., 2008; Parada and Aguilera, 2011b). Pasini et al. (2001) observed in bread that at high cooking temperatures ( $>180^{\circ}\text{C}$ ), *in vitro* digestion of wheat protein is significantly limited due to the formation of protein aggregates having high molecular weight stabilized by strong irreversible interactions, different from disulfide (SS) bonds and/or hydrophobic bonds that may be present in processes at low temperatures ( $<100^{\circ}\text{C}$ ). In addition, it has been observed that an “adequate” kneading/mixing maximizes the formation of a protein matrix (gluten) through the action of disulfide bonds. However, if an excessive kneading/mixing is carried out, the matrix loses firmness when the bonds break and glutenin particles are dissociated into smaller fragments, which would influence the accessibility of digestive enzymes, and would enhance starch digestibility (Parada and Aguilera, 2011b). Hence, the processing (mechanical, thermic, etc.) may affect the interaction level among protein molecules, affecting digestibility of the whole system.

As said above, there is little information about microstructures of the ternary systems formed by starch, protein, and fatty acids and their effect on starch digestibility. Starch and proteins are two important biopolymers whose interaction is inherently difficult; Nevertheless, the presence of free fatty acids (FFAs) has been observed to favor the formation of ternary complexes, where possibly three different structural elements may take effect: formation of complexes starch-FFA, formation of complexes protein-FFA, and the disulfide bond-linked protein aggregate (Zhang et al., 2003; Zhang and Hamaker, 2005).

Since interactions among food components mandatorily affect the rheological (and/or textural) properties of food, several studies focused on rheological behaviors of model systems have been carried out to investigate such interactions. For example, Zhang and Hamaker (2003) studied interaction between sorghum starch, serum protein, and FFAs (20:2:1, p/p/p) by means of a rapid visco-analyzer (RVA). The authors observed the presence of a noticeable peak in viscosity (during cooling phase) when the three components are present in the system, which is different from the event of the binary systems constituted only by starch and protein or FFAs (i.e. where the viscosity peak was not observed or was significantly lower). In addition, it was observed that both the addition sequence of to the components of the ternary system and the fatty acid used (probably related to the unsaturation degree) affected peaks. For clarity, it should be taken into account that when isolated starch is in an aqueous solution and heated beyond the gelatinization temperature under agitation, viscosity increase until reaching a maximum value due to granule swelling (gelatinization); after that, viscosity decreases if agitation continues due to granule fragmentation; finally, when the solution is cooled, the viscosity increases again, but now by the retrogradation (interaction between starch chains). Exact values of viscosity highly depend on starch type and other component of the system (proteins, lipid, salts, sugar, etc.).

Regarding role of proteins in the formation of complexes, it has been observed, also in model systems, that the presence of serum protein diminishes the formation of the starch-FFA complex; Nevertheless, the crystalline order of this complex (V type crystals) was better defined. These changes were related to the formation of a ternary complex of starch, FFA, and protein, since the movement of basic structural elements of the complex amylose-FFA would be restricted, thus helping the formation of more ordered complexes and diminishing the opportunity to form new complexes between amylose and FFAs (Zhang and Hamaker, 2004).

In another study, Zhang et al. (2010) concluded that FFAs may act as a bond between amylose and protein molecules, which are thermodynamically incompatible, and whose functional carboxyl groups are essential for the formation of a stable ternary complex. The authors suggest that electrostatic interactions among carboxyl groups, possessing negative charge, of FFAs and the poly-ionic protein are the basis for the self-assembly of the complex.

Concerning the effect of lipids on the digestibility and glycemic response of starch, the lower glycemic response observed when lipids are ingested with starchy food (which may be related to various factors such as delay in gastric emptying), the higher insulin response, lower glucose absorption in the small intestine, and a lower accessibility to the digestive enzymes (Latgé et al., 1994; Fardet et al., 1999; Henry, 2009). Fardet et al. (1999) studied the effect of lipids on the accessibility of  $\alpha$ -amylase in a wheat-based food. The authors observed that, depending on the food and the lipid emulsion used, the presence of lipid might not affect, decrease, or slightly enhance starch digestibility without presenting large differences in any case. These and other results suggest that lipid can act at both a molecular (interaction at molecular level) and “meso-structural” level (where probably the digestibility is more affected by resultant texture than by fine molecular architecture, although evidently the latter affects the former).

Also, lipid (via starch-lipid interaction) may prevent gelatinization by inhibiting hydration of amylopectin chains, and retard retrogradation, which affects starch digestibility (Henry, 2009).

Regarding processing, it affects the textural/physical properties of food, thus affecting starch digestibility and finally glycemic response (Lee et al., 2005). Many studies have been conducted to investigate how processing and storing conditions affect starch digestibility and glycemic response. Specifically concerning potato, it has been observed that any process involving heating above the gelatinization temperature would cause very high glycemic responses, similar to those reached by glucose; while a storing period at low temperatures significantly decrease the response due to the retrogradation process; some variations may be observed depending on the actual experimental conditions (Kingman and Englyst, 1994; Soh and Brand-Miller, 1999; Garcia-Alonso and Goñi, 2000; Foster-Powell et al., 2002; Fernandes et al., 2005; Tahvonon et al., 2006). Again, the challenge lies in the understanding of the details at both the structural and compositional levels, and the advanced analysis technologies (as modern microscopy techniques) are particularly helpful.



## Impact of Consumer Characteristics on Final Glycemic Response

In order to have a full understanding of postprandial glycemic response, knowing food characteristics appears to be insufficient. The knowledge of consumer characteristics including genetic background and metabolic status is crucial. In general, variability of glycemic response among individuals after a carbohydrate-rich meal is known to be larger than within-subjects variability (Frost and Dornhorst, 2002). This variability (among individuals) can be explained by several intrinsic factors such as health status and genetic profile (Cummings and Englyst, 1995), and some other extrinsic factors such as degree of particle breakdown resulted by mastication (which can affect significantly the outcome analysis) (Ranawana et al., 2010).

Probably, diabetes is one of diseases that is the most relevant in relation to glycemic response. As defined by the American Diabetes Association (2010), Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. In type 2 diabetes, the most common type of diabetes, insulin deficiency is caused by a combination of pancreatic  $\beta$ -cell defect coupled with insulin resistance, and frequently accompanied by inadequate glucagon suppression after meals, increased hepatic glucose output, and decreased amplification of insulin secretion mediated by gut hormones responsible for the incretin effect, such as GLP-1 or GIP (DeFronzo, 2004).

Although many genetic loci have been previously implicated in type 2 diabetes (Hiveert et al., 2011), there is no clear evidence of interaction between food microstructure (or type of food components' interaction) and genetic susceptibility to type 2 diabetes. In this context, genetic variation of salivary  $\alpha$ -amylase emerges as a possible genetic marker related to glycemic response to food microstructure. Some evidence (related with the action time) suggests that although salivary  $\alpha$ -amylase is not relevant for total starch digestibility, it could affect the final postprandial glycemic response. The action of salivary  $\alpha$ -amylase on starch digestion and glycemic response is relatively not so important compared with the effect of pancreatic  $\alpha$ -amylase or other enzymes on carbohydrate digestion in the small intestine, given its short action owing to the inhibition caused by low pH in the stomach (Frayn, 2010; Woolnough et al., 2010). The salivary  $\alpha$ -amylase is encoded by the AMY gene that shows a particular type of genetic polymorphism called copy-number variation (CNV), which means that the number of functional gene copies may range from 2 diploid copies of the AMY1 gene (1 of paternal and 1 of maternal origin) to as many as 16 copies (Perry et al., 2007). Interestingly, a positive correlation between AMY1 CNV and  $\alpha$ -amylase concentration or activity in saliva has been found, whereas CNV of AMY1 gene has been found in different populations, probably due to genetic adaptation during human groups' evolution associated with different diets (Perry et al., 2007; Santos et al., 2012).

It has been reported that high endogenous salivary  $\alpha$ -amylase activity due to high AMY1 copies would be associated with improved glycemic homeostasis (resulting in lower glycemic response) following starch ingestion in healthy adults with normal body weights. Such a theory is supported by some evidence (Mandel and Breslin, 2012) that individuals having low activity of salivary  $\alpha$ -amylase (a low CNV of AMY1 gene) may be at greater risk of glucose intolerance after ingesting starch-rich diets compared with the subjects with high levels of endogenous salivary  $\alpha$ -amylase. It is suggested that the early release of starch digestion products in the oral cavity may provide signals to the body to prepare for incoming starch and ensuing glucose through vagal activation, resulting in an early insulin release that can be termed "pre-absorptive insulin release" (PIR, also known as cephalic-phase insulin release) (Mandel and Breslin, 2012). This type of pre-absorptive secretion has been clearly characterized in the cephalic release of other hormones such as pancreatic polypeptide (Teff, 2010). It has been described that, although PIR is weak and probably represents only a minor proportion of secreted insulin, PIR may have an important influence on glycemic response (Åhrén and Holst, 2001). Butterworth et al. (2011) suggest that sweet receptors in the upper sections of the gastrointestinal tract could detect any maltose formed from starch by salivary amylase, and if coupled with the release of a number of signaling peptides, such as GLP-1 and PYY, influence CNS-mediated control of gastric emptying, insulin secretion, and appetite. In the case of maltose generated in the mouth, there could also be a feed-forward effect in such a way that after its detection by receptors in the gastrointestinal tract, the signals could be interpreted as a warning that a starch-containing meal has been consumed, leading to increased secretion of pancreatic amylase for digesting the unexpected starch load. Moreover, it has been observed that starch hydrolysis in the mouth also depends on the initial food structure as well as the breakdown of solid food (Hoebler et al., 1998), suggesting that food microstructure could also affect PIR.

It has been observed that the consumption of slowly digestible starch does not always result in a lower glycemic response, and does not lead to the widely accepted consensus on such phenomenon (i.e. a low starch digestion should generate a lower glycemic response). The explanation is probably that if glucose enters the circulation at a slower rate, less insulin is secreted to keep glucose concentrations beneath an acceptable limit, resulting in a slower uptake of glucose into tissues, and a high glycemic response (Eelderink et al., 2012a,b). This fact, together with the possible effects of PIR described above, supports the idea that the food having a small fraction of very rapidly digestible starch could help to achieve a lower glycemic response than the food without this fraction due to the early secretion of insulin. Evidence obtained by our research group suggests that, although the role of early starch digestion in the mouth in glycemic response is a plausible possibility, the differences between consumers could most likely be found only among very different persons (high difference in CNV of AMY1 gene) and/or foods with clearly different digestibility, due the high intrinsic variability of the whole system and the capacity of the body to regulate the glycemic homeostasis (Mandel and Breslin, 2012; unpublished results of our group).



## Non-starch Components of Food and Satiety

The structural effect of proteins not only affects the stomach emptying rate and starch digestion, but also helps to regulate postprandial glycaemia through the regulation of food intake and satiety. Several mechanisms have been proposed to explain the satiety effect of proteins: increase in the concentrations of hormones involved in satiety signals, increase in energy expenditure, variation in amino acid concentrations, and increase in the process of gluconeogenesis. It has been found that high-protein foods cause high sensory-specific satiety and decreased feeling of hunger than the same type of foods with low protein contents (Lang et al., 1999; Turgeon and Rioux, 2011). Hence, this structural effect of proteins should also be considered when the nutritional properties of food related to the control of glycaemia are studied.

Fat is a major contributor to overall energy intake, however fat with satiating properties could potentially reduce energy intake. In this line, several lipids have been found to exert satiating effects. Maher and Clegg (2018) have recently reviewed the potential mechanisms whereby some lipids show this property. The authors state that the lipid with the highest potential to enhance satiety is Medium-Chain Triglycerides (MCT) and short-chain fatty acids (SCFA) can also promote satiety, but oral administration has been linked to poor tolerability rather than satiety. The authors also indicated that data on the appetite effects of conjugated linoleic acid (CLA) are limited but do suggest potential. Others lipids with satiating effects are diacylglycerol (DAG), *n*-3 PUFA, and small particle lipids.

## Conclusions

Basic interactions of food constituents are known and have been studied, however, information about ternary or more complex systems, as well as their effect(s) on starch digestibility, is still limited and the obtained results are not easy to be analyzed and understood due to intrinsic complexity of the entire system: "components and food"-*"human metabolism"*-*"actual nutritional effect"*. In addition, there is evidence to suggest that the presence of fatty acids (along with their type) and processing conditions, may affect the physical properties of protein and starch-based systems. Further, intrinsic differences among individuals need to be considered *in vivo* to determine the actual effect of food microstructure and components on the glycemic response of starch-rich foods when consumed by different individuals (affecting glycemic homeostasis and satiety, for example).

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# Encapsulation Systems Containing Multi-Nutrients/Bioactives: From Molecular Scale to Industrial Scale

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## Glossary

**Bioactive substances** herein refer to non-nutrient constituents including compounds (e.g. carotenoids, flavonoids and phytosterols), enzymes and microorganisms that occur in small quantities in foods but can interact with one or more component(s) of a living organism, tissue or cell by imparting probable biological effects (including beneficial physiological, behavioral, and immunological functions beyond the basic nutritional value of the product). Certain nutrients capable of promoting health or preventing diseases are not included in this category.

**In situ matrix encapsulation** is a delivery approach, in which encapsulation is integrated as a part of normal food processing, and a finished food matrix with tailored structure is used as a “natural” encapsulation system for target substance(s).

## Introduction

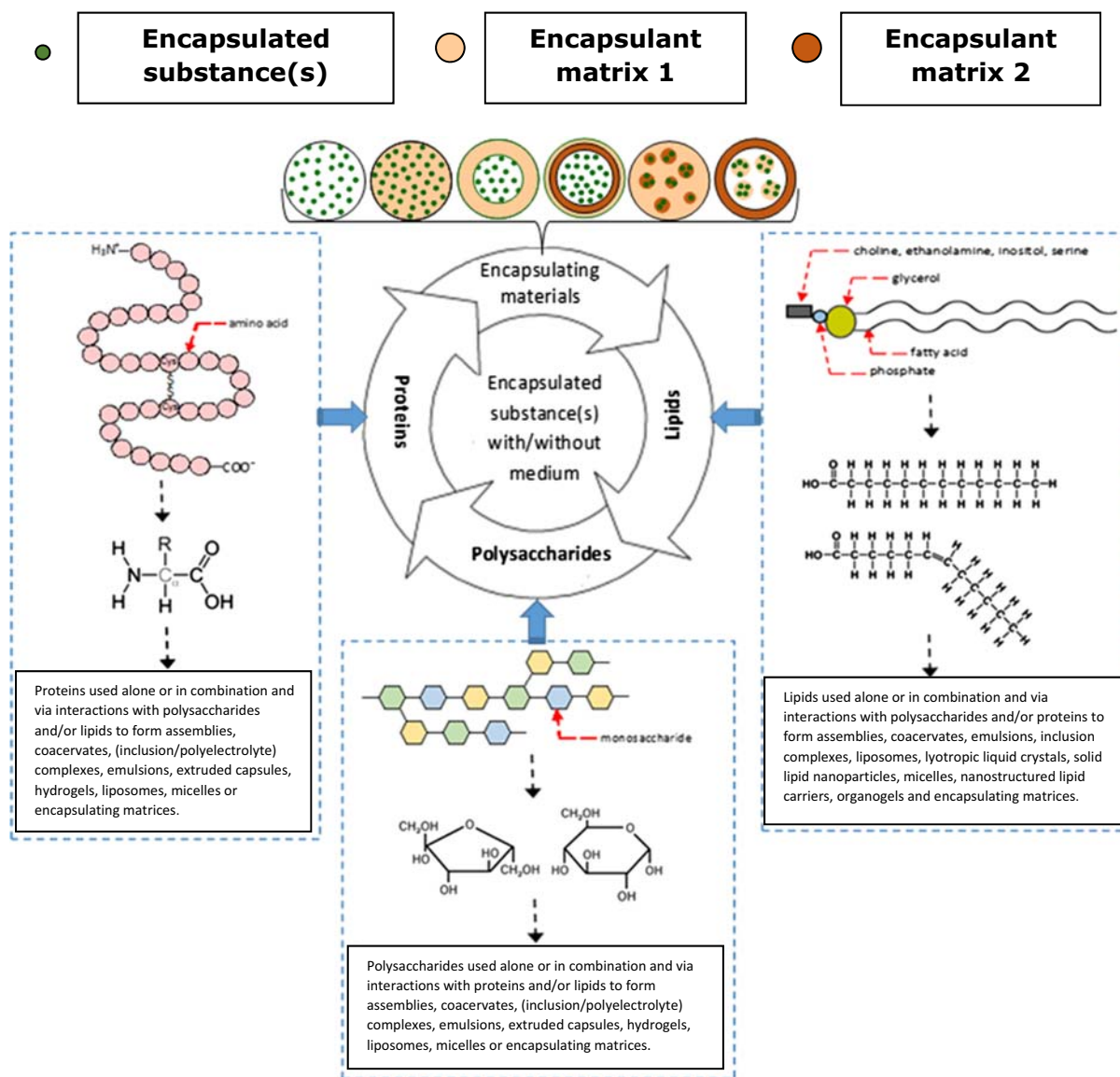
Increased consumer awareness of the close relationship between diet and good health, together with growing recognition of the specific health benefits of individual nutrients and bioactive substances, motivate attempts to increase the stability, bioaccessibility and bioavailability of these bioactives in foods and various dietary supplements. Consumer foods are complex matrices that differ enormously in chemical composition, molecular arrangements and physical state, and contain diverse components including natural food ingredients, additives and different water/lipid contents. Direct fortification of foods with highly demanded nutrients or bioactive substances (including “functional ingredients” with positive effects on overall well-being and/or specific health benefits) presents huge challenges associated with maintaining product quality and food acceptance from a technical perspective, as well as potentially reduced bioavailability of target bioactives due to unwanted interactions or degradation during processing. This has led to the search for efficient delivery systems for highly demanded substances in both academic and industrial research settings.

Nutrients and bioactive substances encompass micro- or macro-molecules such as proteins (including enzymes), carbohydrates, lipids, phenolics, carotenoids, vitamins and minerals. Besides their nutritional significance, many of these substances play dominant or contributing roles in food molecular dynamics and structure. However, many have low solubility in aqueous-based or oil-based foods and poor transmembrane permeability through gastric or intestinal membranes, whilst being very susceptible to deterioration under particular external influences. They can induce desirable or undesirable food component interactions via chemical and physical mechanisms under the stresses imposed during food processing, handling, ingestion and digestion. Hence, the utilization efficiency of fortifying food ingredients is important for preserving biological functionality of desired nutrients and bioactive substances, whilst maintaining overall food quality and sensory acceptability for consumers (Sun-Waterhouse and Wadhwa, 2013).

Encapsulation is a physicochemical or mechanical process to entrap/enclose one or more substances within a protective barrier (i.e. a shell or a matrix), “temporarily isolating” the substance(s) from external environments for a pre-determined period or until the target site of action inside the human body is reached. Encapsulation can serve as an independent process to produce “encapsulated ingredients” or can be integrated into normal food processing. Accordingly, encapsulation allows immobilization, stabilization, protection, dose management, structuration, release and functionalization of nutrient/bioactive substance(s) for a more precise delivery with greater palatability, efficacy and safety at the point of use. Encapsulation systems are also called “delivery systems” or “carriers” interchangeably, and can be grouped based on their scale i.e. macroencapsulation (>1 mm), microencapsulation (100 nm–1 mm) and nanoencapsulation (1–100 nm). Components in a delivery system or encapsulation matrix can be selected from various validated nutrients and bioactives from plants, animals and microbes e.g. carbohydrates, proteins, lipids, phytochemicals, minerals, enzymes, cells and microorganisms, to maximize their respective amounts for human consumption. The determination of how, when and where these substances are included into the delivery systems and final fortified foods requires smart design and state-of-the-art technologies to optimize the interactions among co-existing formulation components in response to changing environments.

## Carrier Materials (“Encapsulants”)

Encapsulation systems should be designed to engage nutrients or bioactive substances as components of the carrier’s shell or matrix barrier to maximize the nutritional advantages and health benefits of the whole encapsulation system. Carbohydrates, proteins, lipids, water and surfactant(s) are the common ingredients used to create carriers with different structures, conformations,



**Figure 1** Schematic representation of molecular interactions and carrier dynamics in various encapsulation systems involving the three major food components.

topologies, shapes and sizes (Fig. 1) e.g. multi-layered planar sheets, fiber webs, microclusters, hydrogel beads, nano-/micro-particles, emulsions, liposomes, colloidomes and micelles are all commonly employed carrier systems.

Carbohydrates are mono-, di-, oligo- and poly-saccharides and encompass a wide range of compounds with different molecular structures. Polysaccharides are important nutrients (e.g. dietary fibers) and versatile biopolymers that can adopt shapes of varying sizes, including neutral polysaccharides (e.g. cellulose, starch, dextran, agarose and guar gum) or ionic polysaccharides (e.g. anionic alginate, pectin, xanthan and carrageenan, and cationic chitin and chitosan). Their HCOH repeating segments make them highly hydrophilic, but their CH residues give rise to some hydrophobic features. The hydrophilic groups result in desirable solubility in polar media and confer temperature stability (compared to proteins and lipids). When the molecular weight is high (i.e. from monomers to dimers, oligomers or polymers), the influence of hydrophobic domains increases forming three-dimensional (3-D) hydrophobic cavities e.g. toroid structure with hydrophilic exterior and almost hydrophobic interior (cyclodextrins) or helical structures (e.g. modified species with amphiphilic characteristics) to entrap hydrophobic guest molecules. In practice, cellulose or starches and their derivatives, plant exudates and extracts, and microbial and animal polysaccharides are often used to construct emulsions, inclusion complexes, coacervates and polymeric extruded capsules. Some oligo-/poly-saccharides exhibit unique favorable properties e.g. Gum Arabic, sugar beet pectin and galactomannans naturally possess high emulsifying and film-forming properties, and inulin or alginate (after depolymerization) exhibit good prebiotic effects.

Proteins and derived peptides are food components with high nutritional value and sourced from meat, fish, egg, milk, wheat, soy, corn and pea. They possess high reactivity and surface activity thus are superior encapsulants for the development of carriers such as emulsions, coacervates and polymeric extruded capsules. The building blocks of peptide chains include hydrophobic amino acids (e.g. alanine, glycine, leucine, isoleucine, phenylalanine, proline, methionine, tryptophan and valine) and polar amino acids (asparagine, cysteine, glutamine, serine, threonine and tyrosine). Proteins in native state or after modification (e.g. via aggregation or denaturation) are biopolymeric amphiphiles and can self-organize and co-assemble with other molecules. Intrinsic block-wise distribution (i.e. long hydrophobic domains with hydrophilic patches) and large polymer size (especially the open structure of proline-rich proteins) promote protein amphiphilic behaviors. At a pH below or above protein's isoelectric point (pI), protein solubility increases due to positive or negative surface charges, respectively. This makes protein microstructures (e.g. filamentous or particulate gels) and properties essential for encapsulation (e.g. surface charge, particle size and hydrophobicity) sensitive to changes in pH, temperature, pressure, shear and/or ionic strength, which opens opportunities for tailoring protein self-assembly to create suitable polymer architectures for binding to/co-assembling target encapsulated substances. Certain native or modified oligopeptides are amphiphilic and can self-assemble via driving forces including  $\pi$ - $\pi$  stacking, electronic interactions and disulphide bonding with additional contributions from hydrogen bonding, hydrophobic effect and van der Waals interactions.

Food lipids mainly comprise triacylglycerols, phospholipids and sterols and take the form of either a liquid oil or solid fat at room temperature. As macro-molecules or matrix media for heat or mass transfer, lipids exhibit distinct physico-chemical characteristics that impact enormously on food's physiological, nutritional and sensory properties. Fats, fractionated or hardened fats, polar lipids, fatty acids and fatty alcohols, mono-/di-glycerides, waxes (insect and plant waxes e.g. beeswax, candelilla wax and carnauba wax), and lipid-polymer conjugates are versatile materials for encapsulation. The carboxylic acid group ( $pK_a = 4.5$ ) of a fatty acid afford a range of chemical reactions e.g. linking to an alcohol via an ester linkage or forming a complex via ionic interaction making lipids more water-soluble and promoting self-assembly. Unique physical properties of certain lipids such as melting over a wide range of temperatures and crystallizing in different polymorphic forms including the most important  $\alpha$  and  $\beta$  forms, make them excellent encapsulants e.g. lipids with melting points well above the human body temperature are desired for lipid nanoparticle carriers targeting sustained release. Phospholipids and monoglycerides are commonly used emulsifiers and can form self-assembled structures comprising submicrometre regions (diameter as 10–50 Å) with various polarities that can host hydrophilic, amphiphilic and lipophilic molecules in the form of liquid or solid carriers for controlled release. The use of more unsaturated phospholipids (e.g. phosphatidylcholine from egg or soybean) or more saturated phospholipids (e.g. dipalmitoylphosphatidylcholine with long acyl chains) to form lipid membranes results in differences in degrees of fluidity, charge, stability and permeability.

Water is a critical food component that provide a hydrophilic matrix medium, act as a reactant and plasticizer, and participate in substance transportation, chemical reactions, and textural, rheological or thermomechanical alterations during food manufacturing and digestion. Bioactive substances for encapsulation including polyphenols, carotenoids, organosulfur compounds and vitamins have vastly different chemical structures and play crucial roles in human health promotion and disease prevention or treatment. Polyphenols comprise molecules from simple compounds (e.g. phenolic acids) to polyphenolic species. Carotenoids are oxygenated or non-oxygenated hydrocarbons with extensive conjugated double bonds and different polarities (e.g. nonpolar  $\beta$ -carotene and lycopene, or polar lutein). These bioactives can also interact with the biomolecules from encapsulant(s) or surrounding environment. The polarity of polyphenols facilitates their activities at water-lipid interfaces and interactions with medium components (e.g. water and triglycerides) depending on polyphenol partition coefficient and applied processing conditions. Furthermore, polyphenols can influence the conformation of proteins and starches in a favorable way e.g. form hydrophobic domains/pockets for encapsulation via inclusion mechanisms.

## Molecular Interactions

The processing properties and biological functions of foods depend on the events at molecular level, as well as the structural arrangement of large molecules at different hierarchical levels e.g. secondary conformation and 3-D networks. Multicomponent systems are sensitive to the delicate balance of intra- and inter-molecular forces i.e. covalent bonds (via which the atoms bound by an electron pair such as C–C, C–N, C=O, C–H, C–N–C and S–S), and non-covalent bonds (which exist within and between molecules e.g. electrostatic forces, hydrogen bonds, hydrophobic interactions, steric repulsive forces, van der Waals interactions). Although individual non-covalent bonds are weak, their cumulative effect can be substantial leading to various structural and functional properties. Ionic bonding is the attraction between opposite charges e.g.  $-\text{NH}_3^+$  and  $-\text{COO}^-$ , which is closely associated with solvent and salt effects in a matrix. Electrostatic repulsion occurs between functional groups with the same type of electric charges (both positive or both negative from polar residues e.g. carboxyl, amino, imidazole and guanido groups, which can be disrupted by pH changes and salts). Hydrogen bonding typically involves a hydrogen shared between two electronegative atoms (e.g.  $\text{N}-\text{H}\cdots\text{O}=\text{C}$  of hydroxyl, amide and phenol groups), which can be affected by dimethylformamide, guanidine hydrochloride or urea. Hydrophobic interactions involve apolar groups (e.g. long aliphatic chains and aromatic groups) and can be disrupted by detergents or organic solvents. Van der Waals interactions are relatively weak attractions between molecules and driven by induced electrical interactions between two or more atoms/molecules in the vicinity. Molecular interactions and organization largely account for macroscopic properties while non-covalent interactions likely dictate microstructure. The balance of forces is such that self-assembly occurs enabling the amplification of small segmental interactive effects into large-scale intermolecular effects to form supramolecular structures (e.g. aggregates and arrays). Further, interfacial effects play an important role for many complex condensed structures with a high surface-area-to-volume ratio. At the nanoscale, the influence of gravity is considerably reduced



while surface interactions play dominant roles and endow the material with distinct physicochemical characteristics for encapsulation (compared with the corresponding bulk material).

The nature of a food component determines its behavior in response to its environment (Sun-Waterhouse et al., 2012). Naturally occurring carbohydrates, proteins and lipids present limitations in terms of their processing, encapsulation and release properties. Besides chemical/physical/enzymatic modification of these materials, the combined use of these biomolecules is an effective approach to compensate individual weakness whilst improving a specific property (e.g. low resistance to acidic media) or imparting additional benefits (e.g. the use of Hi-maize starch to overcome the low stability of alginate gel in the presence of high affinity ions like phosphate or citrate while enhancing alginate's microbial viability-promoting properties) (Iyer and Kailasapathy, 2005). Almost all proteins are affected by acid or base and can react with many different substances including polysaccharides, sugars, oil, water, polyphenols and carotenoids. Their carboxylic acid and amine groups play major roles in inter-molecular or inter-peptide interactions, solubilization, hydration and surface events, while their apolar residues also contribute. Protein hydration is affected by hydrophilic polysaccharides, lipids and salts, along with environmental conditions (e.g. pH and temperature). Hydrophobic proteins can effectively decrease surface tension and bind lipophilic substances like lipids and emulsifiers. Polysaccharides are well known for their high hydrophilicity (owing to the large number of hydroxyl groups), although they also exhibit hydrophobic character. Such amphiphilic properties are desirable for encapsulation and cell adhesion, especially for certain oligo-/poly-saccharides e.g. cyclodextrins. Like proteins, polysaccharides are excellent gelling agents and can bind up to 100 times their own weight of water to form solid-like 3-D network to adsorb substances for encapsulation. The combined use of polysaccharides and proteins can create chemically or physically cross-linked hydrogels in water e.g. polysaccharide mixed gels and pectin-casein gels. Disulfide and hydrogen bonds, ionic or hydrophobic interactions, or their combinations are involved in gelation and the resulting properties depend on the nature of biopolymer(s) and applied thermodynamic conditions. These gel structures (hydrogels) are appropriate barrier matrices for encapsulation while offering additional benefits. Protein-soluble fiber conjugates in a heated food system could lead to increased antioxidant activity via the Maillard reaction between proteins and fiber polysaccharides. Starch can interact with protein to form complexes during gelatinization, with protein stability greatly affected by hydrophobic forces. Carbohydrate-lipid complexation is also possible. Starch tends to associate with polar lipids, saturated monoglycerides and free fatty acids (FFAs), whilst protein binds unsaturated monoglycerides. Interestingly, the incorporation of protein into an amylose-lipid structure can yield a water-soluble amylose-FFA-protein ternary complex, a nanoscaled self-assembly supramolecule with a molecular weight of  $10^6$ – $10^7$  Da. These complexes are carriers for lipophilic molecules, in which the amphiphilic FFA acts as the bridge via hydrophobic interactions between amylose's helical structure and FFA's aliphatic tail, and ionic interactions between FFA's charged negatively carboxyl group and polyionic proteins at an appropriate pH and salt concentration (Zhang and Hamaker, 2003). The so-called "co-factors" also play important roles e.g. cross-linkers like phenolics are required for some biopolymer architectures; calcium ions induce the gelation of pre-denatured whey proteins, alginate and pectin; activators and inhibitors are associated with encapsulant's emulsifying and film-forming properties; surfactants (e.g. phospholipids), co-surfactants (e.g. sugar esters) and co-solvents (e.g. alcohols and polyols) are used to optimize the emulsification of emulsions. Processing aids are sometimes needed for encapsulation such as solvents, plasticizers, drying aids, density modifiers and anticaking agents. The type, amount, timing of addition, duration of performance of co-factors depend on the encapsulant used, encapsulation technology, production approaches and production capacity (batch or continuous production), and the intended food application for the encapsulation system.

Multicomponent barriers are created using proper combinations of the aforementioned substances to deliver specific release kinetics e.g. immediate, sustained or triggered release. The underlying modes of action include simple or tailored diffusion (based on the differences in concentration gradients and translation diffusion coefficient between the system interior and surrounding environment), swelling or shrinking (via manipulating the pore size of biopolymer matrix with a diffusion process often involved), fragmentation (based on the fracture properties of selected encapsulant(s) and the applied stress, which is often followed by quick diffusion), and erosion (via chemical, physical, mechanical or enzymatic degradation of the outer layer of the matrix). The encapsulant molecules can undergo self-alteration (e.g. swelling, bulk or surface erosion) or interact with other molecule(s) in the vicinity when a specific environmental change is applied e.g. a change in a material's physicochemical properties or controllable degradation induced by a change in enzymatic activity, light intensity/wavelength, magnetic/ultrasonic/electric/electronic signal, pH, pressure, redox potential, solvent composition, temperature, and osmotic/mechanical force. Hence, the encapsulation systems are fabricated with single, dual or multiple responses to external physical, chemical and biological stimuli, and the encapsulated substances are delivered following different release kinetics such as zero-order release (by which encapsulated substances are released the moment the encapsulation system arrives at its destination i.e. a particular site of human body or a particular step of human metabolism), first-order release (by which the encapsulated substances are released at a pre-defined time or condition and at an exponentially decreasing rate with time), or triggered release (by which encapsulated substances are released upon a change of environment).

## Encapsulation Methods

Recent advancements in material science, processing and biotechnology enable various encapsulation systems to be created in both research and commercial settings. Their fabrication may be through mechanical processes (mostly based on a top-down approach, in which larger systems are broken down to homogeneously small sized particles or droplets), physicochemical processes (mostly based on a bottom-up approach, in which molecular building blocks are assembled into structured systems e.g. microemulsions,



micelles and certain polymeric nanoparticles), or a mixed approach (in which molecular assembly and comminution processes are integrated together to form multilayer emulsions, liposomes and biopolymeric nanoparticles). The carriers are tailored to possess essential encapsulating and release properties as well as other desirable characteristics e.g. biocompatibility, biodegradability, taste masking, extended shelf life, convenient handling and intake.

A range of techniques are available to create encapsulation systems. Typically these involve physicochemical approaches or processing approaches. A successful commercial encapsulation process often requires the integration of these two types of approaches. The physicochemical approaches include assemblies, biopolymeric inclusion complexes, coacervates (simple/complex), co-extrusion, co-crystallization, emulsions, granulates, hydrogels, liposomes, matrix encapsulation, micelles, polyelectrolyte complexes, polymerization (dispersion, interfacial, emulsification or *in situ*), lyotropic liquid crystals (LLCs), solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs) and structured lipids (organogels). The processing approaches include centrifugal extrusion, centrifugal suspension-separation coating, co-extrusion, coacervation, dripping and jet breakup, electrospinning, electrospray, electrostatic extrusion, emulsification, fluid bed coating, freeze drying, hot melt extrusion, layer-by-layer deposition, melt extrusion, melt injection, microwave drying, molecular inclusion, pressure extrusion, spinning-disk coating, spray-bed drying, spray chilling, spray cooling, spray congealing (spray melt dispersion), spray-freeze-drying, spray drying and vacuum drying. Various processing approaches can be used throughout the carrier fabrication while physicochemical-based inputs are often required (e.g. during polymerization, coacervation and solidification). Stabilization is usually the last step of encapsulation involving chemical (polymerization), physical (evaporation, solidification or coalescence) and/or physicochemical (gelification) mechanisms. Based on structural characteristics (Fig. 1), the encapsulation systems are grouped: (1) reservoir type (like those created by co-extrusion or spray coating technology), (2) matrix type (like those created by emulsification, spray drying or extrusion technology), and (3) coated matrix type (having the combined features of reservoir and matrix types). The selection of an encapsulation method depends on the type and nature of bioactive substance of interest, its intended functionality and end use (e.g. its delivery site in humans, and dietary format as a functional food ingredient or dietary supplement for direct intake). The size of carriers ranges from tens of nanometers to tens of millimeters, but generally a specific size range is common for each encapsulation method. Physical processes generally lead to larger sized units. Emulsification allows a particle size ranging from 0.2 to 5000  $\mu\text{m}$ . Spray drying and spray coating generate similar particle sizes (10–3000  $\mu\text{m}$ ). Extrusion gives particles sized 200–8000  $\mu\text{m}$  and co-extrusion offers a narrower range (150–5000  $\mu\text{m}$ ). Nano-scale carriers are available in the forms of (bio)polymeric nanoparticles, nano-complexes, liposomes, micelles, nanotubes and particulates (Sun-Waterhouse *et al.*, 2015).

Co-extrusion is a physical technique used to produce core-shell microcapsules/microbeads, by which a liquid, dispersion, slurry or emulsion is extruded through a stationary, centrifugal, vibrating or submerged coaxial inner nozzle while the wall materials are passed through the outer nozzle. This technology allows continuous carrier fabrication (production rates as high as several thousand pounds per hour) and creation of multiple coating layers (using extrusion nozzles with multiple concentricity for slow and controlled release). The physicochemical properties of shell and core solutions are critical to forming particular core-shell morphologies and minimizing droplet agglomeration and shell breakage. Typical requirements include surface tension and viscosity in the range of 10–72 dyne/cm and 1–1000 cP (2000 cP in some cases), respectively, along with a matching density (0.7–1.3 g/cm<sup>3</sup>) between the shell and core. Microbeads containing multi-nutrients/bioactives have been developed to deliver bioactive compounds and probiotic bacteria in water- or oil-based media such as those using alginate as the key encapsulant (Sun-Waterhouse *et al.*, 2011; Shinde *et al.*, 2014). The microbead shell can be created using a polysaccharide or combination of polysaccharides (alginate-pectin or alginate-starch) (Sun-Waterhouse *et al.*, 2014; Wang *et al.*, 2013). Some bioactives can be incorporated in the core or shell although the resulting carrier properties and end applications are usually quite different (Waterhouse *et al.*, 2014).

Spray drying is a widely used as an encapsulation process in the food industry due to its continuous operation and cost-effectiveness (typically 15–30 s drying time). The resulting bioactive carriers in powder form possess advantages in terms of storage stability and convenience. Smart pretreatments of the feed solution containing conventional or non-conventional encapsulants prior to spray drying can impart important benefits e.g. pre-warming briefly at 50 °C, or tailoring the feed formulation by monitoring the functions imparted by enzyme species, dietary fibers, proteins and antioxidants naturally occurring in the plant extract and dairy ingredients (Sun-Waterhouse and Waterhouse, 2015; Waterhouse *et al.*, 2017). *In situ* one-step encapsulation by spray drying under mild conditions for large-scale production of “nanosized core coated with micron-sized outer network” powders is feasible, through utilizing the polymerization of biopolymer encapsulants and agglomeration of powder particles during spray drying process (Sun-Waterhouse *et al.*, 2013, 2015). This technical breakthrough is of high significance to the food industry as it minimizes the potential hazards associated with nanoparticles e.g. accidental inhalation of powder into the lung during manufacturing, and mistransit or premature absorption of nanosized substances along the gastrointestinal tract before reaching the target absorption site.

Lipid-containing encapsulation systems include emulsion, micelles, liposome, LLCs, SLNs, NLCs and oleogels. Unlike solid particles, emulsion droplets and liposomes have mobile and deformable surfaces. An emulsion is a mixture of two or more immiscible liquids in which one is present as minute droplets distributed throughout another. Micro-/nano-emulsions have very high surface area to volume ratios thus are useful ‘liquid transport vehicles’ for almost any substance irrespective of their solubilities in any food systems. Emulsions with different arrangements (e.g. water-in-oil in water (w/o/w) versus oil-in-water in oil (o/w/o), or Pickering emulsions versus inverse Pickering emulsions) have immiscible multiphases to encapsulate large quantities of target substances (e.g. prebiotics, phytochemicals or proteins) mainly in the inner phase with some at the interface. Micelles and microemulsions are often grouped into the same carrier category due to their nature as surfactant aggregates containing

water, oil, surfactant, co-surfactant and salt, although they differ in droplet dimensions and complexity of aggregates. Micelles generally refer to aggregates made of surfactant only and dispersed in water, whilst microemulsions are much bigger aggregates with large oil cores (for oil-in-water systems) or water cores (for water-in-oil systems) surrounded by a surfactant monolayer. Different types of micelles can be created using surfactants or amphiphilic materials including amphiphilic copolymers to encapsulate almost any kind of bioactive substance: Normal/regular micelles are used in aqueous media to encapsulate substances with low water-solubility, reverse micelles are used in a nonaqueous medium for delivering hydrophilic species, and unimolecular micelles containing hydrophilic and hydrophobic regions in the same amphiphilic block copolymer (i.e. self-assembly of one molecule into a micelle) can be used for both hydrophobic or hydrophilic substances in either media. Micelles made with reverse thermo-responsive polymers undergo phase change from the solid state (enabling encapsulation of target substance) to a gel structure (enabling sustained release of encapsulated substance) when the environmental temperature rises from room temperature (for micelle fabrication) to the body temperature (after ingestion). A liposome is a spherical vesicle with at least one phospholipid bilayer and the same aqueous phase on both sides of the lipid bilayer. Liposomes can be modified to possess varying chemical compositions and physical properties for encapsulating substances with different solubilities. Polyphenols like those in green tea can be well protected in liposomes before fortification into foods (otherwise causing severe bioactive loss e.g. almost all unencapsulated green tea polyphenols lost in the whey fraction during functional cheese making) (Rashidinejad et al., 2014). Single- or multi-layered emulsions and unilamellar or multilamellar liposomes can be constructed involving combinations of lipids, surfactants, carbohydrate or protein polymers (e.g. phospholipids, glycolipids, mikfat, chitosan, xanthan, caseins and whey). Surface-active proteins influence the interfaces of emulsions and liposomes during formation, disintegration or aggregation of system units. Carbohydrates like starch, microcrystalline cellulose and xanthan gum can act as rheological modifiers or impart additional coating(s), thereby reducing and slowing the diffusion of encapsulated substances inside these carriers. Thus, the type and mass ratio of polar lipids, proteins and carbohydrates, as well as the type, intensity and input method of energy (e.g. microfluidization and sonication), all influence the characteristics of emulsions or liposomes and encapsulation efficiency. High energy input often leads to large numbers of tiny vesicles with increased surface area.

Besides self-assembled liquid lipid systems, lipid solid-containing carriers (e.g. LLCs, SLNs, NLCs and oleogels) deserve attention. The particle growth process during carrier fabrication is critical to final carrier's properties. In LLC systems, amphiphilic lipids such as glycerol dioleate, glycerol monooleate, oleyl glycerate and phytanyl glycerate can form semi-permeable crystal structures with cubic, hexagonal or lamellar mesophases, equipped with different controlled release mechanisms to improve bioactive bioavailability e.g. 20–100 times greater bioavailability for water-soluble peptides (Kim et al., 2015). SLN and NLC systems are constructed using physiological lipids or lipids with “generally recognized as safe” (GRAS) status by water-based technologies (no organic solvents involved) to encapsulate both lipophilic and hydrophilic substances at high concentrations (as compared to polymeric nanoparticle carriers). Unlike SLN carriers, NLC systems are colloidal carriers with a solid lipid core comprising a nanostructured mixture of solid and liquid lipids, thus overcoming some of the drawbacks of SLNs e.g. low payload and potential expulsion during storage. NLC systems with specially required properties can be created by altering the composition of the lipid blend and processing variables e.g. Mixing solid lipids with liquid lipids (oils) endows NLC carriers a lower melting point but still a solid matrix at body temperature (compared to the systems with solid lipids alone). The drawbacks of SLN carriers are an inherently low incorporation rate of substances in the lipid crystalline structure, unpredictable gelation tendency and complex polymorphic transition dynamics. NLC systems face the same challenges as all other colloidal nanoparticle carriers i.e. their potential interaction with the reticulo-endothelial system (which may be mitigated by increasing their hydrophilicity and/or controlling their size to be not larger than 200 nm). Oleogels are unique carriers where solid lipids (oleogelators) at lower concentrations (<10% weight/weight) are dispersed in bulk liquid oil and form 3-D network through molecular-level self-organization driven by van der Waals forces, hydrogen bonding, steric entanglements, ionic interactions or covalent bonding (Das-sanayake et al., 2011). High-melting lipids are used as oleogelators e.g. small molecules saturated fatty acids (which crystallize to form colloidal or fibrillar networks) and hydrophobic polymers like ethylcellulose (which self-assembles under appropriate processing conditions). The type and concentration of gelators, along with the nature of bulk liquid oil and processing variables (heating, stirring and cooling conditions), determine oleogel structure and mechanical strength and subsequent susceptibility to lipolysis and release of encapsulated molecules. For all these discussed lipid-based carriers, oxidation of unsaturated acyl chains and hydrolysis of ester bonds, along with unpredictable change in physical properties related to gelation and polymorphic transition, are the major considerations to stable product development. The degree of unsaturation of unsaturated lipids determines their susceptibility to oxidation and phase transition temperature, while their fatty acid chain lengths influence barrier thickness and diffusion rate of solutes. Saturated fatty acids, although less likely causing membrane degradation, tend to be more rigid compared to their unsaturated counterparts. The fatty acid composition, position and chain length of phospholipids affect both the stability and barrier permeability of derived carriers. Accordingly, a combined use of various lipid materials (e.g. inclusion of saturated fatty acids, phytosterols, cholesterol and hydrogenated lipids), incorporation of antioxidants like tocopherols, and/or addition of biopolymeric coatings and Pickering particles at the interface/surface of lipid carriers, can improve carrier structural integrity and stability.

### **Challenges for Industrial-Scale Encapsulation: Monitoring Factors Beyond Molecular Interactions**

Despite the proliferation in scientific publications about encapsulation over the past few decades, only a few encapsulation systems have been successfully applied industrially, with spray drying and extrusion accounting for over 80% of market success stories. Safety considerations in line with legal requirements should be addressed throughout the industrial manufacturing of encapsulation systems either for direct consumption or further incorporation into foods to eliminate toxicity and minimize side

effects on non-targeted body sites, in addition to other criteria such as delivery efficiency, bioefficacy, cost, application restrictions and environmental sustainability. The materials, ingredients and additives used must be biodegradable and food grade (with GRAS approval). Many substances used in academic research may be not allowed for food applications. Biopolymers that appear on the GRAS list of the Food and Drug Administration (FDA) of the United States of America (USA) include agar, alginate, carob bean gum, carrageenan, cellulose, cornsilk, dextran, dextrans, guar gum, methylcellulose, pectin and starch. Some GRAS approved materials may not be suitable for products intended for frequent intake e.g. concern has recently been raised regarding the healthiness of maltodextrin (a conventional drying aid for spray drying) owing to its high glycemic index ([Brand-Miller et al., 2013](#)). Further, the whole carrier fabrication process, as with any other normal food processing step, must be monitored according to good manufacturing practices (GMPs), and under processing conditions approved by the regulations of intended countries for sale e.g. those of the US FDA, Health Canada, European Food Safety Authority (EFSA) and Food Standards Australia New Zealand (FSANZ), or processed in a way that allows classification as Kosher or halal.

During scale up of carrier fabrication and the production of carrier-containing foods, predictable or unpredictable technical issues may occur (as with any other processing scale-up). It is not uncommon that an encapsulation system successfully developed on a laboratory scale fails when scaled up to industrial scale. Industrial scale encapsulation systems mainly involve spray drying, spray chilling, fluid-bed coating, melt injection, particularization, crystallization, emulsion, liposome, coacervation, melt infusion and extrusion technologies. Some of these are “in-situ matrix encapsulation” methods (in which encapsulation is integrated as part of the normal food processing, and a finished food matrix with tailored structure is used as a “natural” encapsulation system for target substance(s)). Understanding the advantages and disadvantages of each encapsulation technology as well as their limitations in industrial settings is the key to achieving a successful lab-to-industry scale up. e.g. Low production capacity along with large but inhomogeneous particle size occur with extrusion, whereas inconsistent and large droplet size was found in industry scale emulsions. Large scale layer-by-layer (LbL) assembly of multilayered carriers especially those weak polyelectrolyte stacks could be challenging, as the processes established in laboratories tend to be time consuming and not practical for high volume production expected in large scale industrial operations. Maintaining a constant size distribution for encapsulated substances can be easily achieved using laboratory-scale droplet devices, but quite often it is difficult or impossible to achieve a similar size distribution and emulsification outcome by applying the same mixing method to the large volume of carrier matrix in industrial manufacturing which involves many additional variables. From the industrial perspective, fully enclosed systems such as capsules, spheres or lipidic droplets (e.g. emulsions or liposomes) with a complete physical barrier (such as membranes, films or coatings) may not be favorable when used as food ingredients for further consumer food applications. Instead, “in-situ matrix encapsulation” is more desirable considering cost-effectiveness and operational feasibility resulting from less processing steps, high utilization of existing equipment, increased safety and ingredient compatibility, acceptance and preference by a wider range of consumers, legal and cultural/religion constraints. The nature of the food matrix, existing form of food components (including the substance for encapsulation), stage of food processing at which the target substance is added, and food processing conditions all influence the ultimate success of “in-situ matrix encapsulation” approaches.

Molecule behaviors and polymer physics play important roles in scaling up of encapsulation systems. Intra-/inter-molecular interactions, charge distribution, molecular dynamics, molecule or particle interactions, macromolecular crowding, polymer chain dynamics and statistics in large-scale systems all differ when compared to small laboratory-scale encapsulation systems. Comparing laboratory-scale to industry-scale production, at least four aspects would be changed: (1) distance-dependent molecular interactions and matrix effects (e.g. charge screening/shielding and steric hindrance effects); (2) geometry effects and space confinement; (3) magnitude and degree of inhomogeneity for applied energy and stresses (e.g. those associated with temperature, pH, shear, pressure, air/O<sub>2</sub> and light distribution); (4) undefined and unquantified salts introduced (e.g. those associated with water source, tap water or reverse osmosis water used in industries versus distilled or Milli-Q water used in laboratories). Difference in the applied conditions would affect food components differently. There exist unspecific dilution effects along with intricate reptation dynamics and long-range behavior of unscreened electrostatic interactions encountered for large volumes involved in commercial manufacturing. Also, different container/pipe confinement effects on biopolymers may be present in industrial tanks compared with laboratory vessels. High shear stresses may be imposed during industrial fabrication considering the need of pumping products long distances through pipes (as compared to the transfer of small amounts of mass using small spatulas or short glass/rubber tubing in laboratories). Some local microenvironments around the force applying spot may be overpowered by mixing and shearing stress, causing unpredictable perturbation (under small amplitude of stress), disruption and cracking (under large amplitude of stress) or even breakdown (under excessive stress) of carrier architectures. At the interface of two phases of self-assemblies, co-occurrence of lipophilic, amphiphilic and aqueous domains along with concentration effects could arise, which may initiate or enhance certain chemical and physical interactions causing an unusual outcome. Many biopolymers carry different types and densities of electric charges. The altered carrier architecture may lead to new opportunities for neighboring counterions to interact, thereby causing counterion condensation (due to the tendency of macroscopic electro-neutrality) and subsequent development of structures very different from the initially created architecture. Further, processing speed is a critical factor in industrial scale-ups. Higher processing rates are expected for industrial applications compared to those commonly used in laboratory settings. It remains unclear how to estimate the speed of certain processes involving complicated kinetics e.g. self-assembly as a process moving down a free energy gradient. The free energy landscapes along the paths towards self-assembled products are not fully explored. All these challenges encountered during scaling up carrier fabrication would also be encountered when the carriers are incorporated as ingredients into consumer finished foods. The lack of technologies and means for in-situ or real-time monitoring the compositional and structural changes on nano- and micro-scales create further challenges to optimizing scale up processes.

## Conclusion

Nutrients and bioactive substances with different physico-chemical and nutritional properties can be protected or delivered via various encapsulation systems, with choice of an encapsulation system influencing the release and bioefficacy of the carrier product or final consumer food product. Encapsulation is feasible and beneficial in terms of meeting modern consumers' demands for convenient dietary products with multiple nutritional advantages and specific biological function(s). The ultimate aim for using encapsulation in commercial manufacturing is regulatory approval and consumer acceptance. The bottleneck in the development of complex carrier systems and their use as ingredients in consumer food applications relates to the availability and optimization of matrix materials and specialty equipment for carrier fabrication (especially in an industrial context). Detailed knowledge about molecular interactions and biopolymer dynamics in response to food processing conditions, as well as pharmacokinetics of biological active compounds (both target encapsulated substance and other nutrients and bioactives co-existing in the same carrier), are all essential for developing industrial-scale encapsulation technologies for nutrients and bioactives. Particular care needs to be taken when attempting to upscale laboratory encapsulation research to industrial scale production.

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# Nanoparticle-Based Encapsulation of Green Tea Polyphenols: An Approach to Enhance Their Bioavailability and Therapeutic Efficacy

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## Chemical Composition and Structure of Green Tea Polyphenols

Tea, made from the leaves of the *Camellia senensis* plant in the family Theaceae, is now the second most consumed beverage world-wide after water. The whole tea processing includes plucking, wilting, disruption, fermentation, fixation, yellowing, shaping, drying and aging steps. Shortly after tea leaves are picked, the wilting process starts involving enzymatic oxidation or browning. There are six common tea varieties: white, yellow, green, oolong, black and post fermented teas. Green tea (unfermented), black tea (fermented) and oolong tea (semi-fermented) that account for about 20%, 78% and 2% of global tea consumption, respectively, and are produced after different extents of fermentation (Yang and Pan, 2012). Fresh tea leaves subject to only plucking, fixation, shaping and drying steps yields green tea, involving almost immediately steaming or panning to prevent fermentation via inactivating intrinsic oxidase activities. An extra yellowing step to the green tea product is needed for the production of yellow tea thus yellow tea is commonly considered as a semi-fermented tea. Black tea is obtained by allowing the oxidation and polymerization of tea catechins during fermentation by the endogenous polyphenol oxidase and peroxidase to form oligomeric and polymeric derivatives. Oolong tea is considered one type of tea between green tea and black tea. Its production involves some basic steps resembling those in black tea production such as withering, rolling, shaping and firing, although unique timing, temperature and physical stress are required throughout the manufacturing process, e.g. freshly picked tea leaves are subject to withering under strong sun, intentional “bruising” by shaking, and partial oxidation/semi-fermentation (often terminated via steaming or pan-frying). White tea is also categorized as a semi-fermented tea. Its production is somewhat similar to that for oolong tea but does not require bruising, oxidation and curing steps. Epidemiological, laboratory and human intervention studies have revealed that regular consumption of tea is linked to many health benefits including antioxidant, anti-inflammatory, hepatoprotective, anti-mutagenic, anti-cancer, anti-neurodegenerative, anti-diabetic and anti-obesity effects (Adhami and Mukhtar, 2007; Chantre and Lairon, 2002; Ibrahim et al., 2015; Pan et al., 2016; Zou et al., 2010).

Green tea has a complex chemical composition and constituents typically include: proteins (15%–20% dry weight), consisting of enzymes and amino acids (e.g. aspartic acid, arginine, glutamic acid, glycine, leucine, lysine, serine, theanine, threonine, tryptophan, tyrosine and valine); carbohydrates (5%–7% dry weight), consisting of dietary fibers (e.g. cellulose and pectins) and free sugars (e.g. glucose, fructose and sucrose); minerals and trace elements (17 species, 5% dry weight, e.g. aluminium, calcium, copper, iron, magnesium, manganese, phosphorus, potassium, selenium, sodium and zinc); lipids (trace amounts) e.g. linoleic acid,  $\alpha$ -linolenic acids and sterols (stigmasterol); vitamins (small or trace amounts A, B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, B<sub>6</sub>, C, E and folate); alkaloids (caffeine, theophylline and theobromine); pigments (chlorophyll and carotenoids); volatile substances (up to 38 species such as aliphatic/aromatic aldehydes, alcohols, esters, lactones and hydrocarbons). Green tea contains polyphenols (up to 30% of the dry weight) including flavanols, flavandiol and phenolic acids, with the major monomeric flavanols (also called catechins) as: (+)-catechin (C), (–)-catechin gallate (CG), (–)-epicatechin (EC), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), (–)-gallocatechin (GC), (–)-gallocatechin gallate (GCG) and (–)-epigallocatechin gallate (EGCG). The catechins share the same basic structural feature, i.e. two close parallel aromatic rings and a third aromatic ring vertical to the two parallel rings, but differ in the number and distribution of hydroxyl groups on the B-ring, the type of configuration (*cis*- or *trans*-isomers), and the presence or absence of a galloyl moiety. In addition to variables such as origin, variety, growing conditions and collection timing, the handling history and preparation method also affect the composition of GTPPs in the final dietary product. A typical cup of brewed green tea contains 20%–45% (dry weight) catechins. EGCG is the most abundant catechin in green tea, accounting for 38%~40% dry weight of tea and about 16.5 wt% in the water extractable fraction of tea. Commercial products derived from green tea are mainly green tea extracts in a liquid or powder form, with a standardized green tea extract containing about 90% total polyphenols and one green tea capsule equivalent to approximately five cups of green tea.

## Metabolism, Biotransformation and Bioavailability of Green Tea polyphenols

Proven biotransformation of tea polyphenols in the intestinal, hepatic and microbial metabolism has heterogeneous and complex influences on their bioavailability and bioactivities. Metabolic transformation of GTPPs affects their absorption, distribution, metabolism, excretion and toxicity, which is a process based on personalized responses. The metabolism of GTPPs has been



investigated using both animal and human models, and its pathways include methylation, glucuronidation, sulfation and ring-fission in the liver, kidneys and gastrointestinal tract. The major metabolism pathways of GTPPs are illustrated in **Fig. 1**. EGC and EGCG may be readily methylated by catechol-O-methyltransferase (COMT) to form 4'-O-methyl-(–)-EGC and 4''-O-methyl-(–)-EGCG or 4',4''-O-dimethyl-(–)-EGCG, respectively. Although variations were found between animals and between animal and human models, the influencing role of EGCG concentration in final type of methylated metabolite has been confirmed: the dimethylated species predominate at low EGCG concentrations whilst the monomethylated form could prevail at high EGCG concentrations (Lu et al., 2003). A number of products result from glucuronidation catalyzed by a group of UDP-glucuronosyltransferases (UGT) (including at least six EGCG and EGC glucuronides such as EGCG-4''-O-glucuronide and EGC-3'-O-glucuronide). The type and concentration of obtained glucuronides depend on the types of parent catechins, *in vivo* models and tissues collected for analysis. The glucuronidation of EGCG was much greater than that of EGC. The efficiency of enzymatic catalysis in microsomes follows these orders: mouse intestine > mouse liver > human liver > rat liver > rat small intestine for EGCG-4''-O-glucuronide, whereas, mouse liver > human liver > rat liver > rat or mouse small intestine for EGC-3'-O-glucuronide. Enzymatic-catalyzed sulfation of GTPPs takes place with sulfotransferase family (SULTs) being the major catalysts (such as SULT1A1 in human liver and intestine and SULT1A3 in human intestine). SULTs can distinguish and respond to small molecules, e.g. the most abundant isoform SULT1A1 can bind catechins like EGCG via one specific noninteracting allosteric-binding site that is physically separated from the other binding site for nonsteroidal anti-inflammatory agents (Cook et al., 2016). The sulfation of EC likely takes place in cytosol of human liver and intestine whilst EGCG mostly in human liver cytosol. Microbial actions greatly account for the gastrointestinal metabolism of GTPPs. This is especially true for the human colon where microbial catalytic and hydrolytic activities are very high and GTPPs can be broken down via ring fission into simple compounds such as 5-(3',4',5'-trihydroxyphenyl)- $\gamma$ -valerolactone, 5-(3',4'-dihydroxyphenyl)- $\gamma$ -valerolactone, 5-(3',5'-dihydroxyphenyl)- $\gamma$ -valerolactone, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-methoxy-4-hydroxybenzoic acid and 3-methoxy-4-hydroxy-hippuric acid (Wan, 2013). Depending on the biotransformative pathways (which vary on a case-by-case basis), the biotransformation process may inactivate or break down some polyphenols while activating or generating more active metabolites than parent compounds. It is worth noting the possible interplay between the different steps and pathways e.g. glucuronidation on the B-ring or D-ring of EGCG may suppress the methylation on the same ring, whilst glucuronidation on the A-ring of EGCG or EGC likely has no influence on methylation. Therefore, understanding of biotransformation of GTPPs will help optimize the design of future intervention and epidemiological studies and associated result interpretation.

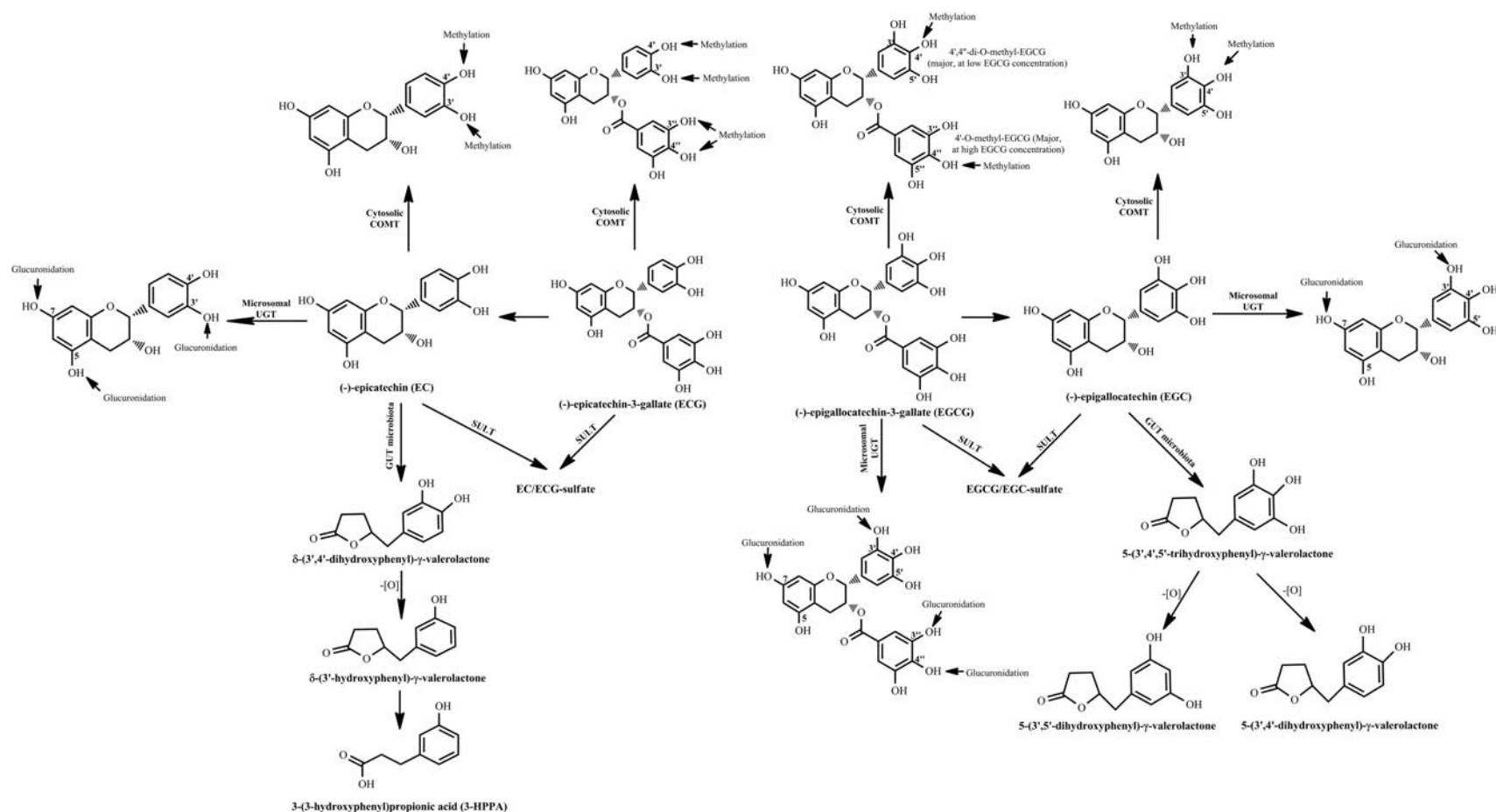
### Health Benefits of Green Tea Polyphenols and the Challenge in Determination and Utilization

Epidemiological, cellular, animal and human intervention studies have indicated many potential health benefits of tea polyphenols and its major constituent EGCG. However, these different *in vitro* and *in vivo* studies have not yet achieved consistent and conclusive results in terms of the efficacy and dose for all the illness and diseases of interest. In 2004–06, the US Food and Drug Administration (FDA) concluded that “there is no credible evidence to support qualified health claims for green tea or green tea extract reducing the risk of heart disease” and “it is highly unlikely that green tea reduces the risk of breast cancer or prostate cancer” (FDA, 2005, 2006). For example, an animal trial revealed the potential of high green tea consumption to cause oxidative DNA damage of hamster pancreas and liver (Takabayashi et al., 2004). An epidemiological study on the protective effect of green tea against breast cancer has been inconsistent (Zhang et al., 2007). Human studies in China reported a significant low incidence of pancreatic cancer upon high green tea consumption, whilst the studies in Japan implied an opposite trend (Zeng et al., 2014). Not all the reasons for the inconsistency are well defined. While the composition, dose, times, preparation and consumption patterns of the treatment, individual and local bias, or even lifestyle are possible contributors, stability and bioavailability of tea polyphenols could be critical factors.

GTPPs are very unstable and reactive owing to their chemical structures, thus, highly susceptible to chemical, physical and biological stress factors during food processing, distribution, storage, ingestion and digestion. Temperature, oxygen, pH, light and metal ions are the significant influencing factors. In aqueous solutions, GTPPs are readily oxidized to form quinone products via temporary semiquinone intermediates. GTPPs are especially unstable in alkaline or neutral pH solutions, and an increased oxidation rate corresponds an elevated pH. Green tea would lose approximately 10%–15% of catechins when brewed in boiling water for 15 min. EGCG in tea or in a purified form would undergo epimerization upon autoclaving at 120 °C for 20 min (Chen et al., 2001).

Low bioavailability of GTPPs and certain adverse effects associated with very high GTPP concentration have been unresolved issues. After oral administration of 20 mg green tea solids/kg body weight, the maximum plasma concentrations ( $C_{\max}$ ) of human subjects were 0.17, 0.73 and 0.43  $\mu$ M, respectively, for EGCG, EGC and EC (Renouf et al., 2011). This finding indicates the irrelevance of quite a lot *in vitro* results associated with tea polyphenols such as bioactivities like antioxidant capacity, as their *in vivo* concentrations would never be so high irrespective of plasma or tissue. Molecular and cellular mechanisms associated with poor





**Figure 1** The metabolism pathways of green tea catechins. COMT, catechol-O-methyltransferase; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase.

bioavailability of GTPPs have been proposed including loss or inactivation owing to metabolic transformation or degradation, low cellular uptake due to poor hydrophobicity to cross cellular membranes, and active efflux by the multidrug resistance-associated protein 2. The chemical structures of GTPPs lead to poor membrane permeability but highly reactive nature, thus, both auto-oxidation and *in vivo* modifications (e.g. severe and unspecific degradation by gut microorganisms, and increased hydrophobicity due to acetylation of their phenolic alcohol groups) as well as significant efflux from cells would take place. When GTPPs are consumed at very high concentrations, adverse impacts would be possible, e.g. an oral dose of EGCG ranging in 750–500 mg/kg body weight led to hepatotoxic effects in CF-1 mice (Lambert et al., 2010). At high doses, the naturally antimicrobial GTPPs could become undesirable for certain vulnerable populations due to their non-specific inhibition or even killing of desired microbiome in the human gastrointestinal tract (Vossoughinia et al., 2014). Prolonged supplementation of tea polyphenols may cause altered bile acid synthesis, increased hepatic oxidative stress and inflammatory hepatic injury in mice fed high cholesterol diets (Hirsch et al., 2016). Accordingly, optimizing the interplay of dose and bioavailability of GTPPs is probably the most critical aspect for maximizing the health benefits of GTPPs.

## Nanoparticle-Based Encapsulation of Green Tea Polyphenols

Strategies aiming at improving GTPP bioavailability, preserving their structural integrity and masking their undesired tastes have been developed. Among which, encapsulation is a promising approach.

Nanoencapsulation is defined as a technology to pack solids, liquids or gaseous materials in miniature, sealed particles with diameters ranging from 1 to 1000 nm. This technology has recently been used for encapsulating polyphenols. Compared with free polyphenols and microencapsulated polyphenols, polyphenol nanoparticles appear to have higher stability, greater bioavailability and more precise site-specific delivery. Colloidal complexes of methylcellulose and EGCG would exhibit a sustained EGCG release, i.e. 60% liberation at the intestinal pH after 2 h (Patel et al., 2011). Electrospray-enabled nanoencapsulation of catechins could improve the release and permeability of catechins, especially at lower core-to-wall ratios (1:50 or 1:10) compared to catechins in free form (Bhushani et al., 2017). In addition, some pH-responsive and magnetic-responsive nanoparticle systems have been developed by incorporating pH/magnetic-responsive polymers in nanoparticles. Self-assembled nanoparticles composed of chitosan (CS) and an edible polypeptide, poly( $\gamma$ -glutamic acid) ( $\gamma$ -PGA) were prepared for the delivery of tea catechins, which were pH-responsive and demonstrated different tea catechins release profiles in simulated gastrointestinal tract (GI tract) media (Tang et al., 2013). The release of green tea polyphenols from nanoparticles may be based on time specific, site specific and/or stimulus specific.

Modification of the surface of coating material (e.g. via functionalizing with ligands such as albumin, hyaluronic acid, biotin, folate, transferrin, monoclonal antibodies) can further improve the delivery efficiency of encapsulated GTPPs to specific organs/tissues for intended treatments of many cancers. EGCG-loaded PLGA-PEG nanoparticles functionalized with small molecules like the prostate-specific membrane antigen (PSMA) inhibitor were found to exhibit *in vitro* efficacy against PSMA-expressing prostate cancer cells (Sanna et al., 2011). Some nanoparticle-based delivery systems for delivering GTPPs are discussed briefly as follows.

Chitosan-based solid nanoparticles are commonly used carriers for nanoencapsulation of GTPPs. This is mainly attributed to its versatile functionalities, such as biodegradability, biocompatibility, non-toxicity, low allergenicity, simple nanoparticle preparation process, good mucosal surface adhesive properties, and bacteriostatic properties. Moreover, the positive charge carried by chitosan enables interaction with negatively charged polymers, macromolecules and polyanions resulting in sol-gel transition stages. The reactive hydroxyl and amine groups on the backbone of chitosan facilitate chemical modifications to form a variety of chitosan-based nanoparticles. In the gastrointestinal tract, oxidation/degradation of GTPPs encapsulated in chitosan nanoparticles was significantly reduced, and such chitosan nanoparticles can penetrate across small intestinal epithelium via either paracellular or transcellular route thereby improving *in vitro* intestinal absorption of GTPPs. Grafting specific ligands onto chitosan not only retains the original properties of chitosan but also endows with tumor-targeting activity. GTPPs-loaded chitosan nanoparticles have been produced via ionotropic gelation with chitosan and sodium tripolyphosphate. It would likely require fourfold longer time for EGCG encapsulated in chitosan-tripolyphosphate nanoparticles to degrade 50% of its initial amount, compared with EGCG in a free form (Dube et al., 2010). Folate conjugated EGCG-loaded chitosan nanoparticles were also prepared via ionic gelation using the folic acid modified carboxymethyl chitosan and chitosan hydrochloride as carriers (Liang et al., 2014). In addition to chitosan, other food grade natural and synthetic biopolymers can be used to fabricate biopolymer-based nanoparticles including carbohydrates (i.e. maltodextrins, starches, corn syrup solids and pectin), proteins (i.e. milk and/or whey proteins and gelatin), and glycoprotein-polysaccharide complexes (i.e. gum arabic).

Liposomes are small, well rounded structures with the bilayer amphiphilic lipid membrane and an aqueous core, and created using biodegradable, biocompatible, nontoxic, low immunogenic phospholipids such as phosphatidylcholines. They can be grouped into 1) unilamellar liposome vesicles with a single bilayer or multilamellar liposome vesicles with two or more bilayers, based on the number of bilayers within the single liposome; 2) small (20–100 nm) or large unilamellar liposome vesicles (10  $\mu$ m) based on size. Liposomes can encapsulate hydrophilic compounds in their internal water core and hydrophobic compounds into the membrane, thereby are used for deliver GTPPs at higher bioactive loading (higher loading efficiency) compared to other

lipid-based delivery systems such as solid lipid nanoparticles and emulsions. Catechin-loaded liposomes can be prepared using egg phosphatidylcholine, cholesterol or anionic surfactant in the presence of 15% ethanol (Fang et al., 2006). Attention should be paid to the drawbacks of liposomes associated with physical and chemical instability especially in acidic conditions (e.g. those of gastrointestinal tract). They would undergo degradation via oxidation of unsaturated acyl chains and hydrolysis of ester bonds causing leakage of encapsulated bioactive substance(s). Also, differences in the number of hydroxyl groups on the B-ring and the stereochemical structure of the C-ring cause different degrees of hydrophobicity and affinity for lipid bilayers, e.g. more *cis*-type than corresponding *trans*-type GTPPs would be incorporated into liposomes (Kajiya et al., 2001).

Solid lipid nanoparticles (SLNs) are considered as an alternative carrier to colloidal systems such as liposomes, emulsions and polymeric nanoparticles, and combine the advantages of these traditional carriers. SLNs consist of spherical outer solid lipid particles in the range of 10–1000 nm and a monolayer coating of phospholipids. The solid lipid core contains dissolved or dispersed bioactive substances such as GTPPs and the surface of SLN consists of emulsifiers. The crystallinity of the lipid matrix is very important. The difference in polymorphic transition of SLN's solid lipid matrix would affect the release or activity of encapsulated compounds, and polymorphic transitions may occur right after SLN preparation and during SLN storage. SLNs have high long-term stability and are commonly prepared through high shear, ultrasonication or high speed homogenization, solvent emulsification/evaporation, supercritical fluid technology and spray drying process, EGCG-loaded solid lipid nanoparticles (EGCG-SLNs) can be prepared using the emulsion-solvent evaporation method and physiological lipid matrix, resulting in additional structural reinforcement of the nanoparticle assemblies, along with advantages of low acute and chronic toxicity but good biocompatibility (Radhakrishnan et al., 2016). EGCG-SLN can exhibit 8.1 times greater inhibitory effect on the proliferation of MDA-MB 231 human breast cancer cells and 3.8 times greater inhibition on DU-145 human prostate cancer cells than EGCG in free form (Radhakrishnan et al., 2016).

## Conclusion

Green tea is consumed globally in different dietary forms. Regular consumption of GTPPs exhibit diverse and significant benefits to general or specific health. Nanoparticle-based encapsulation techniques hold great promise for addressing issues related to GTPPs' poor stability, solubility and bioavailability encountered in food, cosmetic and medical applications. However, there is still much more to do in terms of optimizing the performance of nanoparticle-based delivery systems for GTPPs. Despite the well-recognized high bioavailability and efficacy of nanoencapsulated phytochemicals, there is a need to determine the actual magnitude and pharmacological mechanisms of GTPPs encapsulated in nanoparticles as compared to GTPPs in free form in order to address newly emerging safety issues associated with the potential "local overdose" effect for nanotechnology-enabled encapsulation systems. Also, the success of GTPPs as therapeutic agents depends on the optimized synergies with other co-existing compounds in the tea extracts (e.g. caffeine and certain minerals like zinc and chromium), other dietary components present in the same treatment regime or accompany meals (e.g. proteins, sugars and ascorbic acid, which may introduce additional molecular interactions with GTPPs and make contribution to energy metabolism), and even other lifestyle habits (unadjusted confounding factors).

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# Food Soft Nano-Dispersions for Bioactive Delivery: General Concepts and Applications

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## Overview

Nanotechnology focuses on the engineering, characterization, modification and development of biological and non-biological structures at the scale of 1 to 100 nm. These biological and non-biological compounds have revealed some unique properties and the research on the field is thus gaining momentum rapidly. Since the beginning of the 20th century, food industry has shown an increasing interest to nano-technological products focusing on improving safety, shelf-life and nutrient delivery of the food products (Weiss et al., 2006; Chen et al., 2006).

The possibility to mimic the nature towards engineered food nano-structures with enhanced properties has offered numerous potential applications in the food industry. Examples of nano-technological advances proposed by researchers in the food sector include:

- **Food processing.** Nanotechnology provides the aid of improving product shelf-life, texture and taste preservation during food processing. These nano-entities are used as food additives and are suggested to enhance food quality for a longer period of time (Singh et al., 2017).
- **Food Safety.** One of the most promising sectors of food industry involves the introduction of nanotechnology for food preservation and safety *via* packaging. Several nano-materials have been proposed from pathogens' nano-sensors to polymer nano-composites to combat product contamination, degradation of nutritional value, environmental and other risks (Duncan, 2011).
- **Bioactives' delivery.** The interest of the researchers as well as the food industry focuses on the fabrication of the so called "functional foods" using nano-carriers to promote targeted delivery and enhanced bioavailability of bioactive compounds. Following the consumers' demand for a healthier diet, scientists are providing solutions towards healthier "smart" foods *via* nanotechnology (Singh, 2016).

In summary, during the last decades scientists have introduced nanotechnology to improve many aspects of a food product. In this chapter we will focus on the general concepts, challenges and potential applications of food nano-dispersions proposed as vehicles of bioactive compounds. The most potent advantages of the aforementioned carriers of bioactive molecules include the increased solubility of therein molecules which subsequently leads to their enhanced bioavailability. Moreover, soft nano-dispersions mimic biological membranes already existing in the human body and are able to protect the encapsulated molecules from environmental stress such as pH alterations or proteolytic degradation. Finally, entrapped bioactives may be more efficient as food additives as they are protected from thermal or mechanical stress occurring during the processing of the final food product.

## Types of Soft Nano-Dispersions

In the present chapter attention will be paid to the most common nano-structures proposed in the food industry. These colloidal nano-dispersions are formed from water, oil and emulsifiers and are the most promising carriers of bioactive molecules.

It is well known that water and oil are two immiscible liquids with a high surface tension of 30–60 mN/m gradually leading to phase separation. When these liquids are mixed using mechanical means, a so-called "emulsion" is formed that will eventually break down into two phases post mixing. To overcome the aforementioned problem, scientists have proposed the addition of surface active agents (surfactants) that decrease the surface tension between the two phases leading to a slower collapse of the system. For the food industry these surfactants are food grade amphiphilic molecules called "emulsifiers" and will be analyzed below.

## Nanoemulsions

Conventional emulsions are optically opaque metastable colloids containing droplets from a few nanometers to 100  $\mu\text{m}$  (Leal-Calderon et al., 2007). These systems, as mentioned above, have a high surface tension and need external energy to induce their formation. In the last decades, food scientists have proposed nanoemulsions as a means to replace conventional emulsions for food applications. There has been much of a debate for the size limits of the nano-droplets ranging from 100 nm (McClements and Rao, 2011), 200 nm (Huang et al., 2010) to 500 nm (Anton et al., 2008). Nevertheless, these systems are in most cases almost transparent, a characteristic that is beneficial compared to conventional emulsions due to their slight alteration of food color

and organoleptic characteristics. One of the most important advantages of nanoemulsions is the relatively high shelf-life due to the nano-droplets' resistance to gravitational separation (Rao and McClements, 2011). Depending on the composition of the immiscible liquids, nanoemulsions are distinguished in water-in-oil (W/O) and oil-in-water (O/W) ones. In the first case, the continuous phase is oil with water droplets dispersed in it while the polar heads of the emulsifiers are facing the water core. In the second case, the reverse situation is taking place with the aqueous medium being the continuous phase and the non-polar part of the emulsifier laying towards the inside "entrapping" the oil molecules.

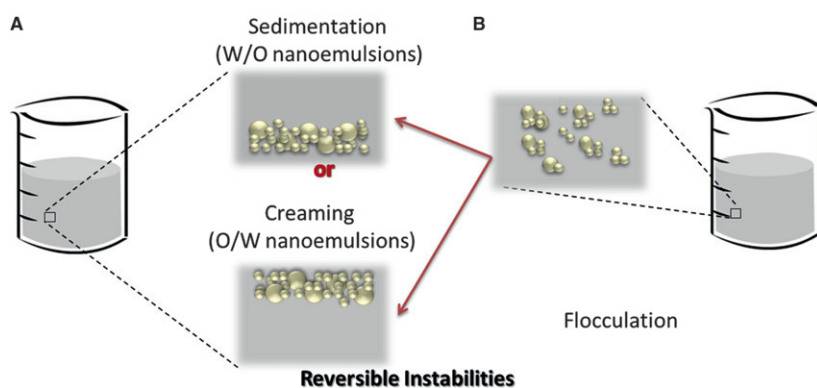
In order for nanoemulsions to be formed, an energetic barrier has to be overcome thus energy input is required. There are two types of methods proposed for the emulsification involving either high or low amounts of energy (Acosta, 2009). High energy input refers to techniques like microfluidization, high pressure homogenization and sonication. These methodologies provoke disruption of the droplets under homogenization thus decreasing their size (McClements and Rao, 2011; Jafari et al., 2008). The second type of methodologies is based on a spontaneous formation of smaller droplets due to alterations in the composition of the nanoemulsion. Most frequently, the emulsifier is solubilized in the solvent (water or oil). Spontaneous emulsification is based on the diffusion of one phase to another when these phases are brought into contact. Another low energy emulsification technique includes the alteration of the emulsifiers' molecular geometry towards phase inversion by altering the composition (Phase inversion composition) or the temperature (Phase inversion temperature) of the nanoemulsion. In the recent years, even though the low energy emulsification methodologies are gaining momentum the vast majority of applications involve the former type of emulsification due to ease of preparation and handling at an industrial scale.

Due to the fact that nanoemulsions are only kinetically stable, they are susceptible to gradual phase separation. These destabilization phenomena have been extensively studied in order to reverse or delay the nanoemulsions' collapse and are distinguished in reversible and irreversible instabilities (Figs. 1 and 2). The first case includes creaming or sedimentation depending on the type of nanoemulsion. For both phenomena, the nanoemulsions' droplets aggregate *via* flocculation retaining their initial size and shape (McClements, 2015). On the other hand, irreversible instabilities include coalescence and Ostwald ripening and refer to phenomena that lead to gradual phase separation (Leal-Calderon et al., 2007). Coalescence is caused due to collision of the nanoemulsion droplets when an energy barrier is overcome. Coalescence is the most "violent" disruption mechanism as size evolution is self-accelerated leading to a fast collapse of the system (Leal-Calderon and Cansell, 2012). Ostwald ripening is caused when the droplets are not highly monodispersed. Differences in the size lead to differences in the Laplace pressure that eventually cause a diffuse transfer gradient from smaller to larger droplets. In 1962, Higuchi and Misra proposed the addition of a solute to the droplets in order to cause osmotic pressure mismatch (Higuchi and Misra, 1962). In that way, they were able to compensate the effect of Ostwald ripening thus retarding phase separation.

### Microemulsions

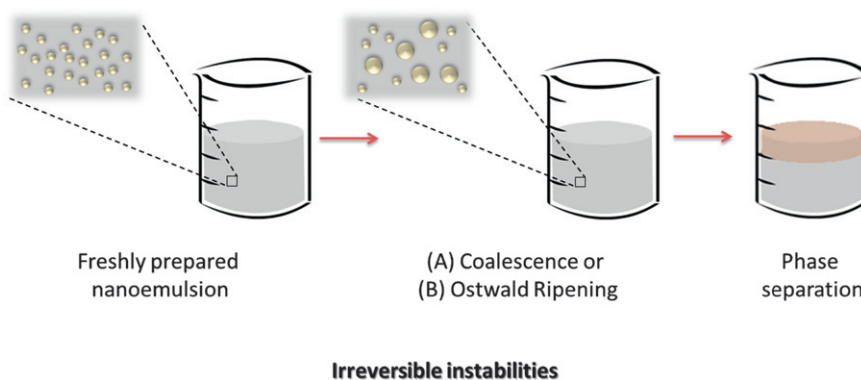
Microemulsions, also known as swollen micelles, are nano-structures that self-aggregate due to micellar formation, which is a thermodynamically favorable state (Shinoda and Kunieda, 1977). Despite their name, microemulsions droplets range from 5–50 nm and opposite to nanoemulsions no external energy is required for their formation. This is a major difference between these soft nano-dispersions that will be discussed extensively below. Even though microemulsions were reported from the 1940s, the most widely accepted definition was given almost forty years later from Danielsson and Lindman. According to this definition: "a microemulsion is a system of water, oil and an amphiphile, which is a single optically isotropic and thermodynamically stable liquid solution" (Danielsson and Lindman, 1981). Microemulsions are macroscopically transparent liquids due to the fact that the dispersed droplets are smaller than the wavelength of light. Microemulsions are also mainly distinguished in O/W and W/O depending on the continuous phase. A system containing oil droplets dispersed in an aqueous phase is called O/W microemulsion whereas the reverse composition is leading to a W/O microemulsion. For the case of microemulsions, a third case that almost equal amounts of water and oil are solubilized in a macroscopically transparent liquid could occur and is called "bicontinuous microemulsion".

In order to determine the limits of the monophasic region of a microemulsion, a ternary phase diagram is usually utilized. The phase behavior of water-oil-emulsifier is visually assessed at different composition points eventually constructing an illustration of

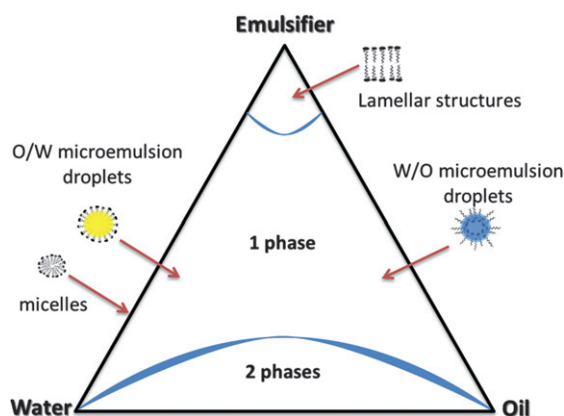


**Figure 1** Reversible instabilities occurring in nanoemulsions.





**Figure 2** Irreversible instabilities occurring in nanoemulsions.



**Figure 3** Typical ternary phase diagram representation at constant temperature and pressure.

the phase regions at constant pressure and temperature (Lawrence and Rees, 2000). A typical phase diagram is shown in Fig. 3. Briefly, every corner of the triangle represents the pure component whereas every axis is the binary mixture of the relative components. Every point inside the equilateral triangle represents a specific composition of water, oil and emulsifier and thus different phases can be optically distinguished. For compositions approaching the water–oil axis, phase separation is induced due to the lack of sufficient amounts of emulsifier. On the other hand, for compositions close to the emulsifier edge, lamellar structures are favored due to the high ratio of emulsifier compared to water or oil.

As mentioned above, the microemulsion region represents a one-phase area where systems are macroscopically transparent. The construction of the ternary phase diagram is frequently a time-consuming tool as the equilibration becomes slower with approaching to the multi-phase region (Garti, 2003). The typical protocol followed for the construction of a phase diagram includes the formation of binary mixtures (usually in the ratios of 1:9 to 9:1) followed by the gradual titration of the third component at constant temperature and pressure conditions (Kalaitzaki et al., 2013).

The addition of one or more components has been proposed in order to increase the monophasic region and decrease the amount of emulsifier needed. There systems are represented with the so-called “pseudo-ternary” phase diagrams. In these cases, one or more triangle edges represent a mixture of components that has been shown to enhance microemulsion formation. Garti and his team proposed the addition of alcohols and/or food-grade short polyols for the increase of the monophasic ( $L_2$ ) region due to the destabilization of liquid crystals (Garti et al., 2001; Yagmur et al., 2002).

### Nanoemulsions versus Microemulsions

In order to distinguish between the two aforementioned soft nano-dispersions mainly proposed for the food industry, one should consider the fundamental differences concerning systems’ thermodynamics. For the case of nanoemulsions, an energetic barrier needs to be overcome for the formation of the nanodroplets. A nanoemulsion could therefore be kinetically stable after the energy input in the sense that the energy barrier between droplet formation and phase separation is high enough. On the other hand, for the case of microemulsions, the formation of micellar droplets is the thermodynamically favorable state, thus occurring spontaneously. Consequently, the shelf-life of a microemulsion concerning the structural characteristics of therein droplets is higher than those of a nanoemulsion.

Another important difference between the two systems concerns the emulsifiers typically used for the droplets formation. Often, the amphiphilic molecules used for the formation of micelles tend to form different shapes depending on the molecules geometry and packing parameters. On the other hand, nanoemulsion droplets have the tendency to be spherical due to the relatively large Laplace pressure (McClements, 2012).

Finally, the two types of soft nano-dispersions differ in the amount of emulsifier needed for the formation. Because of the external energy input in the case of nanoemulsions, a relatively small amount of emulsifier is required for the droplet formation. On the other hand, microemulsion formation requires a larger amount of emulsifier which has to exceed the Critical Micellar Concentration (CMC) for spontaneous formation to occur.

For the food industry, the cost, the legislation restrictions on the amount of emulsifier used, the possible off-taste effect and the shelf-life of the final product, are the parameters taken into consideration for the selection of the appropriate bioactives' nano-carrier.

The most commonly used emulsifiers in the food industry are low cost and food-grade amphiphilic molecules derived from foods such as proteins, phospholipids, and polysaccharides. Synthetic emulsifiers are also being used but in most cases the legislation restrictions are strict, thus synthetic molecules are often used in mixtures with naturally occurring amphiphiles (Leal-Calderon et al., 2007).

Other types of soft nano-dispersions such as complex formulations of nanostructured multilayer emulsions or nano-liposomes have been also proposed as efficient vehicles for bioactive nutrient delivery (Weiss et al., 2006; Reza Mozafari et al., 2008)

## **Nano-Dispersions and Bioactive Molecules**

Numerous studies have proposed nano- and/or microemulsions as soft carriers for nutrient or drug delivery applications. Due to their aforementioned unique physicochemical properties as well as the use of naturally occurring emulsifiers these systems are ideal for encapsulation of bioactive ingredients as they tend to protect therein molecules from environmental stress (pH, oxidation, proteolytic degradation etc.) (Leal-Calderon and Cansell, 2012; Flanagan and Singh, 2006). They are also able to enhance their solubility and, in some cases, their bioavailability under gastrointestinal conditions.

In the recent years, another cutting-edge perspective in the food chemistry and engineering is gaining momentum concerning the design of novel foods with health benefits. The tendency of the consumers as well as the increasing demand of today's way of life for healthier foods has lead food scientists to the design of "functional foods". A definition given by the Institute of Medicine's Food and Nutrition Board (IOM/FNB) refers to functional foods as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains. Nevertheless, the European Commission's Concerned Action on Functional Food Science in Europe (FuFoSE) along with the International Life Science Institute (ILSI) of Europe give the following definition: "a food product can be considered functional if together with the basic nutritional impact it has beneficial effect on one or more functions of the human organism thus either improving the general and physical conditions or/and decreasing the risk of evolution of diseases".

Numerous bioactive molecules of natural origin have been proposed for encapsulation in delivery matrices in order to increase their effective bioavailability in the gastrointestinal tract. For instance, lipophilic molecules, such as omega-3 fatty acids have been claimed to protect from cardiovascular, immune, mental and other disorders thus providing high nutritional value. Due to their hydrophobic character, omega-3 fatty acids show low-solubility in water leading to precipitation and low bioavailability. To this respect, nano-carriers have been proposed as ideal matrices for the incorporation and delivery of these fatty acids as they are able to solubilize them at higher amounts and increase thus, their bioavailability under gastrointestinal conditions (Garg et al., 2006).

Another type of naturally occurring molecules that have been proven to function more efficiently in a delivery system rather than in "free" state, are antioxidants. By definition, antioxidants are "substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation" (Shahidi et al., 1992). On the other hand, due to the fact that oxidation occurs, also, in the human body being involved in aging, cancer, degenerative diseases and others, these molecules are crucial for human health. Interestingly, Porter and his team observed that hydrophilic antioxidants are more effective when present in lipophilic media, whereas the opposite behavior occurred for hydrophobic antioxidants which were more efficient in emulsions rather than oils (Porter, 1993). The so called "polar paradox" has been extensively studied ever since, in order to understand the mechanism of the antioxidants' activity under various conditions (Laguerre et al., 2015; Shahidi and Zhong, 2011). Nowadays, the most acceptable theory for naturally occurring polar scavengers is that they tend to migrate to oil-water interfaces. Consequently, the protection of the molecules from oxidation under storage conditions as well as the increased molecules' efficacy, make nano-formulations a very promising solution to the aforementioned challenges. Indeed, it is currently known that in nature self-organization of monomers in nano-associations take place in bulk oils entrapping moisture and solubilized polar scavengers. More specifically, it has been proven that the minor amphiphilic components of vegetable oils such as phospholipids, sterols etc. self-form nano-structures in bulk oils entrapping hydrophilic bioactives (Sotiroudis et al., 2005; Chaiyasit et al., 2007; Xenakis et al., 2010; Papadimitriou et al., 2013). Even though the mechanism of radical scavenging is not yet fully understood, scientists, in an attempt to mimic nature, have extensively studied the beneficial effect of nano-dispersions as antioxidants' carriers either experimentally, or using molecular dynamics approaches (Chatzidaki et al., 2015, 2016a; Laguerre et al., 2008; Chatzidaki et al., 2017)

Proteins or peptide molecules are another type of compounds that could be beneficial to human health, nevertheless they are susceptible to proteolytic degradation when incorporated into foods and/or human gastrointestinal tract. Depending on the structural characteristics of a peptide (molecular mass, characteristic amino acid sequence etc), scientists could determine their functionality. Peptides or amino acids are also very sensitive to thermal processes or pH instabilities and could lose their effectiveness during food processing, storage or transportation (Chatterton et al., 2006). Some peptides are also being used from

the food industry as naturally occurring antimicrobial agents and have been studied for their efficacy under encapsulation conditions with promising results (Chatzidaki et al., 2016b).

## Food Nano-Dispersions and Final Product Issues

Nowadays, it is evident that ingested nanoparticles increase the permeability limits thus enhancing the absorption of therein bioactive molecules (Fröhlich and Roblegg, 2012; Constantinides, 1995). Nevertheless, due to the complexity of these systems, their potential toxicity should be thoroughly investigated. On the other hand, formulation of soft materials at the nano-scale is a practice mimicking natural processes to minimize the free energy needed. To this respect, self-assemblies resembling the naturally occurring aggregates of phospholipids, proteins etc. should not pose a high risk to the body compared to inorganic nanoparticles. Unfortunately, there are no specific differentiation regulations between soft nano-dispersions and metal or polymeric nano-particles. Moreover, there are no reported limitations concerning the uptake of these nano-materials or their potential toxicity levels. Although the FDA suggests that "size is not an issue", the Institute of Food Science and Technology (IFST), an independent authority for food quality is rather critical on food nanomaterials declaring that they should be considered as potentially harmful unless specified testing proves otherwise. Also, it suggests the classification of nano-materials to the E-numbering using the subscript "n" for their use at the labeling of the final product (Weiss et al., 2006). On the other hand, the European Food Safety Authority (EFSA) has issued a guidance on risk assessment of nanomaterials in the food and feed chain (EFSA, 2011). The European Commission (EC) is planning modifications to the current regulations to include nano-foods in a case-by-case basis. To this respect, EFSA elaborates an update of the guidance of 2011, to help the EC in its final decisions.

Although there is a scientific evidence that nanotechnology could strongly enhance the beneficial characteristics of our nutrition while suppressing possible weaknesses, consumers' opinion remain skeptical. While public perception on science and nanotechnology is positive for other industrial fields such as biomedicine or pharmacy, public acceptance of nano-engineered foods remains at its infancy (Macoubrie, 2004). Since there are no specified regulations for nanofoods, a database for these products currently released in the European market has been established since 2012 (Hansen et al., 2016). To date, the database contains information about 3038 products, 128 of which refer to nano-engineered food and beverages currently in the European food market (www.nanodb.dk). Some food products containing nanomaterials that are already in the market or patented are summarized in Table 1.

On the other hand, the rising consumers' demand for healthier foods with respect to their increased nutritional characteristics, is an emerging driving force for industrial research and development towards nano-structured food products (Singh et al., 2017; Tiju and Morrison, 2006).

## Conclusions

Food grade soft nano-dispersions are currently being used as a cutting-edge technology to overcome solubility and bioavailability of bioactive molecules. Moreover, they serve as effective carriers to protect specific molecules from environmental stress during food processing and storage. In an attempt to mimic naturally occurring self-assemblies at the nanoscale, scientists are currently using these nano-dispersions as vehicles to encapsulate bioactive molecules that are crucial for human health. The two major formulations of soft nano-carriers are nanoemulsions and microemulsions with the latter containing smaller droplets than the former type. More specifically, nanoemulsions are only kinetically stable and need external energy for the droplet formation whereas

**Table 1** Nano-materials currently patented or already released in the food market

Name	Patent/Product	Type of nanoformulation	Description	Source
Solgar Nutri Nano	Product	Microemulsion	Encapsulation of CoQ-10 and Alpha Lipoic Acid	<a href="http://nanodb.dk/en/product/?pid=3009#prettyPhoto">http://nanodb.dk/en/product/?pid=3009#prettyPhoto</a>
Nano Melatonin	Product	Nanoemulsion	Encapsulation of Melatonin	<a href="http://www.phfsa.com/en/products/nutritional-drugs/neurology/nano-melatonin-patented/">http://www.phfsa.com/en/products/nutritional-drugs/neurology/nano-melatonin-patented/</a>
Edible microemulsions with encapsulated plant extracts as dressing type products	GR Patent GR1008863 (B)	Microemulsion	Encapsulation of plant extracts	<a href="https://worldwide.espacenet.com/publicationDetails/biblio?FT=D&amp;date=20161006&amp;DB=&amp;locale=en_EP&amp;CC=GR&amp;NR=1008863B&amp;KC=B&amp;ND=4">https://worldwide.espacenet.com/publicationDetails/biblio?FT=D&amp;date=20161006&amp;DB=&amp;locale=en_EP&amp;CC=GR&amp;NR=1008863B&amp;KC=B&amp;ND=4</a>
Mustard oil nanoemulsion and preparation method thereof	CN103315956 (A)	Nanoemulsion	Decrease of the volatilization of the mustard oil and alleviation of its pungent smell	<a href="https://worldwide.espacenet.com/publicationDetails/biblio?II=2&amp;ND=3&amp;adjacent=true&amp;locale=en_EP&amp;FT=D&amp;date=20130925&amp;CC=CN&amp;NR=103315956A&amp;KC=A">https://worldwide.espacenet.com/publicationDetails/biblio?II=2&amp;ND=3&amp;adjacent=true&amp;locale=en_EP&amp;FT=D&amp;date=20130925&amp;CC=CN&amp;NR=103315956A&amp;KC=A</a>

microemulsions are formed spontaneously and thus are thermodynamically stable. To this respect, depending on the final product and the bioactive encapsulated, food processing could involve minor energy input hence being environmentally sustainable.

On the other hand, due to the consumers' skepticism on food nanotechnology, the final products currently released in the food market are relatively limited. Since there is strong evidence on the beneficial characteristics of nano-carriers for bioactive molecules, informative campaigns on nano-foods should be adopted from food companies and stakeholders.

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# New Insights on Bio-Based Micro- and Nanosystems in Food

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## Introduction

The production of innovative functional food products has been the focus of food industry in response to the on-going trend towards the consumption of healthy foods, with additional properties (e.g., antioxidant, anti-inflammatory and anti-cancer), apart from their nutritional value (Guiné et al., 2016; Simões et al., 2017; Ramos et al., 2017; Weiss et al., 2008; Dordević et al., 2014). Such properties can be accomplished by the incorporation of bioactive compounds (i.e., nutraceuticals) that are often added to food or beverages. Bioactive compounds are functional molecules that have significant health benefits when consumed or applied. Vitamins, antioxidants, prebiotics, probiotics, antimicrobials, enzymes, among others, can be used and incorporated into food products to enhance their functional properties, or to increase their shelf-life. However, such compounds can have inadequate water solubility and/or low cellular absorption. A strategy that has been used to overcome this issue is the application of micro- and nanotechnologies, in particular, for the protection (i.e. from the adverse conditions of the surrounding environment, such as temperature, pH and ionic strength), control release and bioavailability enhancement of bioactive compounds (Simões et al., 2017; Ramos et al., 2017; Weiss et al., 2008; Robles-García et al., 2016).

At the micro ( $10^{-6}$  m) and nano ( $10^{-9}$  m) scale, materials have different behaviors and may acquire novel functional properties, when compared with those in the bulk state, due to e.g., the higher surface area-volume ratio observed at these scales (Simões et al., 2017; Madalena et al., 2016). Such feature can be explored through micro- and nanosystems that can be produced and tailored to encapsulate bioactive compounds, thus increasing their oral bioavailability (Robles-García et al., 2016). Since these micro- and nanosystems are intended to be applied to food products, they must be generally recognized as safe (GRAS). Therefore, proteins (e.g., whey, soy and egg proteins), polysaccharides (e.g., chitosan, alginate, gum arabica and pectin) and lipids (e.g., vegetable oils, phospholipids and triacylglycerol) are being used to produce micro- and nanosystems for the encapsulation and controlled release of bioactive compounds (Simões et al., 2017; Ramos et al., 2017; Aditya et al., 2017). These bio-based materials can be used to produce controlled delivery micro- and nanosystems due to their ability to form gels, particles/capsules and emulsions, as well as bonds with bioactive compounds (Simões et al., 2017). For instance, whey proteins have the ability to conjugate a large variety of bioactive compounds through either primary amino groups or ionic and hydrophobic binding. This behavior allows protection of labile compounds from degradation, while permitting a release in a specific site (e.g., small intestine for absorption) at a controlled delivery rate – i.e. by swelling behavior of gel in response to environmental stimuli (e.g., pH, temperature and/or electric fields) – thus allowing an improved bioavailability of such compounds (Ramos et al., 2017). Even though these bio-based materials can form stable controlled release systems, they are intended to be applied to food products that are further ingested and submitted to the harsh conditions of the human gastrointestinal (GI) tract. Consequently, understanding the interactions of such controlled delivery micro- and nanosystems with food components and subsequently their behavior under GI conditions is of utmost importance (Madalena et al., 2016). It is also crucial to assess the toxicity of such systems, in particular nanosystems since they are at a sub-cellular scale and may induce cellular damage or produce some unwanted phenomena (e.g., tissue bioaccumulation, inflammatory response and systemic exposure) (Simões et al., 2017; Rossi et al., 2014).

Therefore, this chapter covers the most commonly used bio-based and bioactive micro- and nanosystems (i.e., bioactive compounds encapsulated into micro- and nanosystems) for food applications, as well as of possible interactions established between different nanosystems. Moreover, some applications of such systems in commercialized food products are also depicted. Fig. 1 represents the main topics discussed in this chapter.

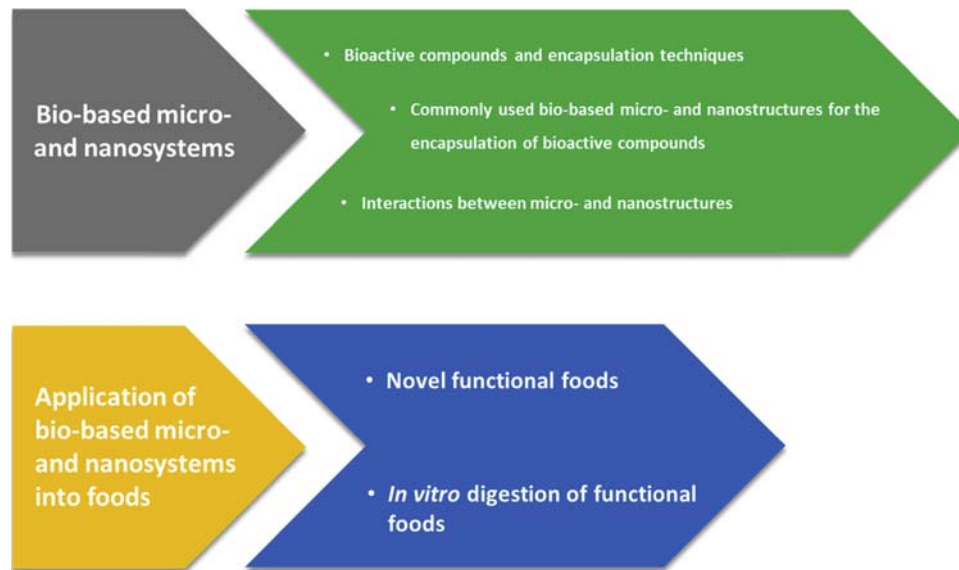
## Bio-Based Micro- and Nanosystems

### Bioactive Compounds and Encapsulation Techniques

Bioactive compounds are functional ingredients that occur in Nature, are part of the food chain, and can provide additional beneficial properties to food products (Biesalski et al., 2009). Vitamins (e.g., vitamin B<sub>2</sub> and vitamin E) (Madalena et al., 2016; Yang and McClements, 2013), polyphenols (e.g., curcumin and quercetin) (Pinheiro et al., 2016; Souza et al., 2013), probiotics (e.g., *Lactococcus lactis* ssp. *Cremoris* and *Lactobacillus rhamnosus* GG) (Ramos et al., 2016; Ying et al., 2012), minerals (e.g., iron and magnesium) (Zimmermann, 2004; Bonnet et al., 2009), fatty acids ( $\omega$ -3 and  $\omega$ -6) (Chen et al., 2017; Xu et al., 2013), among others, can be applied for this purpose. These compounds can have poor water solubility, and present low digestion stability and GI absorption, and may be influenced by external environmental conditions (e.g., temperature, light, oxygen, metallic ions, enzymes and water exposure), thus influencing their final performance and purpose. This can lead to their degradation that changes the

\* (corresponding author)





**Figure 1** Schematic representation of the chapter content structure.

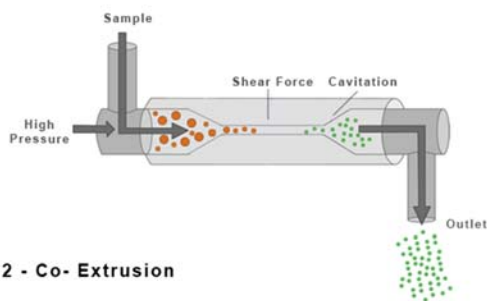
overall properties of the final food product (i.e., presence of off-color, off-flavors and toxic degradation residues) (Simões et al., 2017; Đorđević et al., 2014; Jia et al., 2016). The encapsulation of bioactive compounds can overcome these challenges and two strategies can be used to achieve this purpose – top-down and bottom-up approaches (Simões et al., 2017).

The top-down strategy implies the mechanical processing of bulk materials to reduce their size and molding their shape. Consequently, precise tools must be used to achieve the desired final material characteristics. An example of this approach is the development of micro- and nanoemulsions by high-pressure homogenization (Fig. 2-1). Sample grinding and extrusion (Fig. 2-2) are also alternative top-down strategies for the encapsulation at the micro- and nanoscale. However, this strategy has

## Top-down Strategies

## Other Techniques

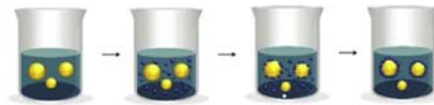
### 1 - High Pressure Homogenization



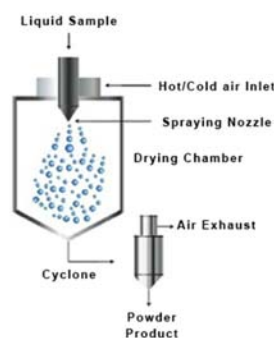
### 2 - Co- Extrusion



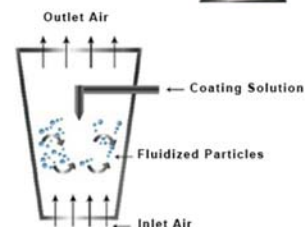
### 3 - Complex Coacervation



### 5 - Spray Drying



### 6- Fluid Bed Coating



### 4- Freeze Drying



**Figure 2** Schematic representation of the most used encapsulation techniques. Adapted from Simões, L.S., Madalena, D.A., Pinheiro, A.C., Teixeira, J.A., Vicente, A.A., Ramos, Ó.L., 2017. Micro- and nano bio-based delivery systems for food applications: in vitro behavior. Adv. Colloid Interface Sci. 243, 23–45.

some limitations from an economical point of view (i.e., expensive equipment, operability and maintenance costs) (Simões et al., 2017; Joye and McClements, 2014). The bottom-up strategy comprehends the application of self-assembly methodologies, at the molecular level, by inducing changes to the surrounding environmental conditions (e.g., temperature, pH and ionic strength). This approach allows a more accurate control over particle size and shape and is more energy efficient, when compared with top-down approaches (Joye and McClements, 2014).

Choosing an appropriate encapsulation technique depends on the nature of the bioactive compound and encapsulating structure (i.e., hydrophilic or lipophilic affinity). For instance, emulsions can be used to entrap lipophilic compounds (e.g., curcumin) (Simões et al., 2017; Jia et al., 2016). Pinheiro et al. (2016) produced curcumin nanoemulsions stabilized with lactoferrin and lactoferrin/alginate biopolymers by high pressure homogenization, which allow increasing curcumin bioactivity and bioavailability. Although, other techniques (Fig. 2-3:6) can be used to encapsulate bioactive compounds (e.g., coacervation, spray drying, freeze drying, inclusion and fluid bed coating) (Simões et al., 2017).

### Commonly Used Bio-Based Micro- and Nanosystems for the Encapsulation of Bioactive Compounds

Bio-based micro- and nanosystems are being widely used as delivery vehicles for controlled release of bioactive compounds. These structures can be produced using bio-based materials, such as proteins, polysaccharides and lipids, or their combination, to form different structures (e.g., capsules, hydrogels and emulsions) intended for the encapsulation of bioactive compounds – please see Table 1 (Simões et al., 2017; Cerqueira et al., 2014).

Capsules (Fig. 1) are colloidal structures that can be used to entrap bioactive compounds on their hollow aqueous or oil core, surrounding them with a wall material that can be made of bio-based materials. This structure can be produced at macro-, micro- and nanoscale. Despite the advantages of producing capsules at microscale (e.g., the preparation techniques employed and their scale-up), some limitations exist regarding their applicability. Capsules at microscale tend to present a heterogenous size distribution (i.e., high polydispersity) and low thermodynamic stability (Simões et al., 2017). On the other hand, capsules at the nanoscale (i.e., size below 100 nm) exhibit low polydispersity, high thermodynamic stability, high long-term kinetic stability, fast release kinetics of bioactive compounds and better mucus adsorption in the small intestine (Simões et al., 2017; Weiss et al., 2008). Although, due to their lower diameter, particles at the nanoscale can require complex techniques that may increase the cost of their production and scale-up, as well as require more sophisticated characterization methodologies (Jarzębski et al., 2017). Several techniques can be used to produce nanocapsules, which can include ionic pre-gelation/coacervation, polymerization (e.g., emulsion polymerization and dispersion polymerization) and dispersion of preformed polymers (e.g., nanoprecipitation, spontaneous emulsification, self-assembly, salting-out and supercritical fluid) (Cerqueira et al., 2014). Kamburova et al. (2017) produced nanocapsules from natural polymers as chitosan and pectin to entrap indomethacin (Table 1). Since chitosan and pectin polymers have opposite charges, the indomethacin was adsorbed to nanocapsules using a self-assembly layer-by-layer methodology. The authors established that the coating thickness was proportional to the number of deposited layers and observed a prolonged release rate of indomethacin from nanocapsules, as a result of its encapsulation.

Hydrogels are three-dimensional networks that are composed by hydrophilic or amphiphilic polymeric molecules that form intermolecular interactions through covalent and non-covalent bonds (e.g., hydrogen bonds, van der Waals interactions and physical entanglements). These structures are mainly formed by gelation processes that typically include linking of polymeric chains, thus leading to a progressively larger embranchment of molecules and to the formation of a three-dimensional network (this process is called as “sol”). Continuous networking through intermolecular interactions increases the ramification of polymeric chains, thus leading to a decrease in the network solubility. The transition from aggregation to a continuous building process is called “sol–gel transition” and the continuous building process is known as “gelation”. Hydrogels are formed when the building process terminates and the critical point where gel is achieved is called “gel point” (Simões et al., 2017; Ramos et al., 2014). These structures are mainly used due to their ability to swell and retain large amounts of water without disrupting their network. This feature is possible due to the presence of e.g., hydroxyl, amino and carboxyl groups in the network structure, which makes them suitable carriers for bioactive compounds (Ramos et al., 2017). Nanohydrogels of lactoferrin-glycomacropeptide (Lf-GMP) were

**Table 1** – Micro- and nanosystem examples for controlled delivery applications and their respective production technique

Micro- and nanosystems	Material	Bioactive compound	Production technique	References
Capsules	Chitosan and pectin	Indomethacin	Layer-by-Layer	Kamburova et al. (2017) Rivera et al. (2015)
	Chitosan and alginate	GMP		
Hydrogels	Whey protein concentrate	5-Aminosalicylic acid	Electrospraying Gelation	López-Rubio and Lagaron (2012) Bourbon et al. (2016)
	Lf-GMP	β-Carotene		
	Chitosan	Caffeine		
	Hyaluronic acid and pullulan	Curcumin		
Emulsions	Linseed oil	5- Fluorouracil	High-pressure homogenization	Liu et al. (2016) Di Meo et al. (2015) Sotomayor-Gerding et al. (2016)
		Riboflavin		
		Astaxanthin		
	Soybean oil and whey protein fibrils	Lycopene		
		–		Mantovani et al. (2017)

successfully used for the encapsulation of bioactive compounds with different chemical nature, such as caffeine (hydrophilic) and curcumin (hydrophobic) (Table 1) (Bourbon et al., 2016). Chitosan, alginate and pullulan nanohydrogels were also produced to protect 5-fluorouracil (Table 1) from *in vitro* digestion conditions (Liu et al., 2016) and stable structures (i.e., in water and physiological conditions) (Di Meo et al., 2015), respectively.

Emulsions are structures composed by two immiscible phases that can be classified as oil-in-water (O/W), water-in-oil (W/O), liquid-in-liquid or solid-in-solid emulsions. Their stability may depend on many aspects, as follows: (i) the type of emulsifier/surfactant used to stabilize the emulsion; (ii) the homogenization conditions; (iii) the solvents and co-solutes present; (iv) the surfactant-to-oil ratio; and (v) the emulsion composition. Therefore, when submitted to high temperatures, pH variations and ionic strength changes, emulsions can exhibit weak physical stability during transportation, storage or application (Simões et al., 2017; Lu et al., 2016). However, micro- and nanoemulsions currently exhibit a good coalescence stability likely due to the decrease of the van der Waals attractive forces between droplets, with a concomitant decrease in particle size. In this sense, micro- and nanoemulsions can be used as suitable functional carriers for lipophilic compounds (e.g., essential oils, polyphenols, fatty acids, antioxidants, antimicrobials and some vitamins) (Simões et al., 2017). Micro- and nanoemulsions are often produced using high-energy methodologies that require the use of specific equipment, such as high-pressure valve homogenizers, sonicators and microfluidizers. The high mechanical energy produced by the equipment enables the production of stable oil droplets. Micro- and nanoemulsions can also be produced using low energy techniques that rely on the intrinsic physicochemical characteristics of the surfactants and oily and water phases used (Simões et al., 2017; Jia et al., 2016). Soybean and linseed oil were previously used to produce O/W micro- and nanoemulsions, using stabilizers (e.g., whey protein fibrils and Tween 20, respectively) and high-pressure homogenization techniques to entrap bioactive compounds (e.g., astaxanthin and lycopene) (Table 1), thus protecting them from the harsh conditions of the *in vitro* GI conditions (Sotomayor-Gerding et al., 2016), and maintaining their stability during storage (Mantovani et al., 2017).

### Interactions Between Micro- and Nanosystems

Micro- and nanosystems can establish covalent and non-covalent bonds (e.g., electrostatic, steric, van der Waals, hydration and intermolecular hydrophobic interactions, and hydrogen bonds) with other systems, bioactive compounds and/or food components, thus allowing the improvement of the existent properties of food ingredients or even the creation of innovative ones. For instance, protein molecules can be tailored to form different food structures, depending on the extent of protein association. This allows developing protein-protein, polysaccharide-protein and lipid-biopolymer complexes in order to take advantage of the synergistic effects between both materials (Ramos et al., 2017; Liu et al., 2017).

Protein-protein non-covalent interactions (e.g.,  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin interaction) can be influenced by pH (electrostatic interaction), temperature (intermolecular hydrophobic interactions by exposing apolar residues), hydration (repulsive forces due to high hydration levels), protein conformation (steric interactions) and protein molecular weight and size (van der Waals interactions increase with the increase in protein size). Protein covalent interactions are formed when a reaction between free sulphydryl and disulfide groups occurs, which is responsible for protein aggregation and further gelation. This kind of complexation enables the functionalization of protein matrices by modulating their rheological properties. Therefore, it is possible to control the release rates of bioactive compounds that can be entrapped in this kind of structures (Ramos et al., 2017). Oancea et al. (2017) developed  $\beta$ -lactoglobulin ( $\beta$ -lg) microparticles to encapsulate anthocyanins from sour cherries. The protein matrix was developed by dissolving pure  $\beta$ -lg in Tris-HCl buffer with a pH of 7.7, far from the isoelectric point of  $\beta$ -lg (i.e., pH of 5.2) to promote repulsive electrostatic intermolecular interactions. The protein mixture suffered a heating treatment in order to promote a protein destabilization/denaturation, thus exposing the  $\beta$ -sheets present in the  $\beta$ -lg secondary structure and permitting the establishment of electrostatic interaction with the anthocyanins and its encapsulation (Liu et al., 2016). Furthermore, this study also concluded that  $\beta$ -lg prevented anthocyanin degradation during *in vitro* gastric digestion, and its release in the small intestine.

Protein-polysaccharide interactions can also take place to promote the synergy between both structures and thereby improving their functional properties. This kind of interaction can be achieved through chemical (not used in the food industry due to regulatory concerns), physical and enzymatic processes (Ramos et al., 2017; Liu et al., 2017; Damodaran et al., 2007). The Maillard reaction is often used to prepare protein-polysaccharide complexes under monitoring temperature, time, moisture, pH, heating, dry-heating or the use of novel processing technologies, such as pulsed electric field conditions. Briefly, deprotonated amino groups from protein molecules and polysaccharide carbonyl groups can react with each other (i.e., through covalent bonding) to form a N-glycosylamine and one water molecule. The N-glycosylamine can then be rearranged to form the Amadori product (i.e., 1-amino-1-deoxyketose), which is a more stable ketone that can lead to the formation of protein-polysaccharide conjugations. If the reaction takes place under controlled conditions (i.e., temperature, moisture, pH and time), the interaction between proteins and polysaccharides can have enhanced emulsifying, heating stability, antioxidant and antimicrobial properties (Liu et al., 2017; de Oliveira et al., 2016; Semenova, 2017). Mengibar et al. (2017) assessed the emulsifying and antioxidant properties of the Maillard reaction residues from soluble chitosans (with different molecular weights) with  $\beta$ -lg, emphasizing the importance of choosing the appropriate biopolymer (according to its physico-chemical properties), to formulate novel functionalized food products. The authors concluded that the Maillard reaction with high molecular weight chitosans (39 and 56 kDa) resulted in the gelation of the final product. On the other hand, the Maillard reaction with low molecular weight chitosans (1.3 kDa) resulted in the formation of soluble functional residues with significantly higher antioxidant activity.

Micro- and nanoemulsions are often used to protect lipophilic bioactive compounds from environmental conditions, while tensioactive compounds (i.e., surfactants) can be used to stabilize micro- and nanoemulsions, preventing from flocculation and coalescence. Biopolymers can also be used with surfactants in order to increase the repulsive intermolecular interactions between droplets (i.e., influence of repulsive steric forces), resulting in an increased emulsion stability. Proteins, for example, can compete with surfactants in the oil-water interface (i.e., competitive adsorption), as well as interact with surfactants resulting in protein unfolding (i.e., increase in hydrophobicity) or protein precipitation (with charge neutralization leading to an increase in the surface activity of surfactant-protein complex) (Damodaran et al., 2007; Hasenhuttl and Hartel, 2008).

## Application of Bio-Based Micro- and Nanosystems Into Foods

Micro- and nanotechnologies applied in the food industry have been widely studied and reported in the last few decades. Consequently, novel food-grade controlled delivery micro- and nanosystems were developed to be further applied to food products. Therefore, it is very important to understand the interactions of such systems with food matrices and their digestibility (Simões et al., 2017; Wilde et al., 2016). In this section, food products containing micro- and nanosystems that were recently developed are listed, and information regarding to the digestion of such functional food products is provided.

### Novel Functional Foods

Consumer awareness towards the consumption of fortified, healthy food products is gradually increasing in nowadays society. This results in an increased effort from the food industry players to develop novel food formulations with additional functional properties. Consequently, recent developments in food science point to the application of encapsulated bioactive compounds in commercialized food products (Simões et al., 2017; Cerqueira et al., 2014; Gultekin-Ozguven et al., 2016). For instance Gultekin-Ozguven et al. (2016) encapsulated black mulberry extract into anionic liposomes coated with chitosan through layer-by-layer deposition. The resulting mixture was spray-dried to be further applied into chocolate formulations (i.e., natural and alkalized cocoa liquors) at different conching temperatures (i.e., 40, 60 and 80 °C). The authors concluded that it is possible to fortify dark chocolate with encapsulated anthocyanins, although the fortification efficiency depends on the pH and processing temperatures.

Encapsulating delivery systems can also be used to mask off-flavors, as well as the astringency and bitterness which some bioactive compounds can produce. Wilde et al. (2016) used  $\beta$ -lg to encapsulate allicin, the main thiosulphate in garlic, responsible for its taste and smell. The authors incorporated the nanosystem entrapped with allicin into a coffee-based beverage and assessed the sensory profile of this food product. They concluded that the encapsulation of allicin into  $\beta$ -lg masked the characteristic flavor of garlic in the tested food matrix.

Rivero et al. (2013) took a different approach and developed chitosan films loaded with propionic acid to extend the shelf-life of pastry dough. The authors concluded that chitosan films loaded with propionic acid had a significant antimicrobial effect, which makes this system a good sustainable, food-grade alternative to conventional synthetic films and chemical preservatives.

Therefore, food-grade micro- and nanosystems can be used to functionalize food products, through the controlled release of bioactive compounds, to mask off-flavors and unwanted organoleptic characteristics (e.g., smell and taste) that are associated to the application of some bioactive compounds, as well as to extend the shelf-life of food ingredients through the controlled release of antimicrobial and antifungal bioactive compounds.

### In vitro Digestion of Functional Foods

The digestion of food is a complex, yet an important assessment to understand the behavior of controlled delivery nanosystems in the GI tract. Therefore, *in vitro* digestion models can be used to make this assessment since they do not raise any ethical issues and they are an unexpensive alternative to *in vivo* models (Simões et al., 2017; Madalena et al., 2016). Several *in vitro* digestion models were developed in the last few years with different functionalities; some of them are more simple and static (Gultekin-Ozguven et al., 2016), while others are more complex and dynamic (Pinheiro et al., 2016) taking into account the peristaltic movements of the GI tract during digestion. This kind of models (static and dynamic) can be used to study the digestion of functionalized food, fortified with controlled delivery systems, as well as the bioavailability of bioactive compounds incorporated into food matrices. In addition to the development of fortified dark chocolate with black mulberry extract, Gultekin-Ozguven et al. (2016) studied the *in vitro* digestion of this functionalized food product and determined the effect of liposomes encapsulation in anthocyanin activity, under GI conditions. The authors reported that the encapsulation of anthocyanins in liposomes improved their bioavailability by protecting them from the acidic gastric conditions and by allowing their release in the small intestine due to the presence of bile salts (responsible for the disruption of the liposomes). Amine et al. (2014) evaluated the protective effect of alginate microsystems on the survivability of *Bifidobacterium longum* in Cheddar cheese during *in vitro* digestion. The authors concluded that microencapsulated *B. longum* had higher survivability during gastric digestion when compared with that in free form.

Micro- and nanosystems can in fact be applied to food products to promote the protection of bioactive compounds during digestion, thus improving their bioavailability. Despite the increased number of publications regarding the application of micro- and nanosystems in food products, systematic studies on regulation and legislation of the application of these systems are still

lacking, in particular of those at nanoscale, thus, more studies must be conducted to understand the possible interactions or undesired reactions that may occur in food matrices.

## Concluding Remarks

Micro- and nanotechnologies have a significant impact on the food industry through the development of novel food products with additional functional properties, resulting from an increased public awareness towards the consumption of healthy food products. However, at the nanoscale, materials tend to have distinct characteristics (e.g., higher volume/surface area ratio) which results in improved or even novel functionalities. This indicates that nanotechnology is a very promising option and published research efforts corroborate this statement. However, some challenges must be addressed (e.g., potential cytotoxicity) through *in vitro* or *in vivo* studies. Another important bottleneck is the understanding of the possible interactions of nanosystems with food matrices; there is an enormous lack of research in this field. In addition, there is a growing interest in understanding the behavior of such micro- and nanosystems when incorporated into real food products under GI conditions. For this purpose, *in vitro* GI models have been used since they are inexpensive and practical, do not raise any ethical concerns, and, in fact, some simulate closely the GI conditions of the human digestion. Several studies infer that micro- and nanoencapsulation protect the bioactive compounds from the harsh gastrointestinal conditions and consequently increase their bioavailability. But, it is important to point out that more knowledge is required regarding the digestion of functional foods using *in vitro* GI systems mainly due to the complexity of the food matrix and the constraints of existing experimental methodologies. There is still a long way to go, more fundamental and applied research are needed to prove that these functional food products are safe for public consumption and can be commercialized.

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# Oleogelation for Food Structuring Based on Synergistic Interactions Among Food Components

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## Glossary

**Oleogels** Sub-group of organogels where the continuous liquid oil phase is structured into a gel with the help of a dispersed continuous network of either self-assembled structures, crystalline particles, aggregated inorganic particles or polymeric strands.

**Oleocolloids** A broad range of oil continuous soft matter systems comprising colloids at an interface of complex fluids and phase-separated states of matter such as oleogels; biphasic colloids (indirect emulsions and oleofoams); complex colloids such as foamed oleogels and oil-in-water-in-oil (O/W/O) emulsions; and layered structural matrixes embedding a large volume of liquid oil (oleofilms).

**Self-Assembly** A process by which individual molecules form defined molecular assemblies such as aggregates or clusters.

**Self organization** A process by which the self-assembled aggregates or clusters create higher-ordered structures (network).

## Introduction

Oleogels are a sub-group of oleocolloids where a continuous liquid oil phase is structured into a gel through self-assembly and hierarchical organization of oleogelators. These oleogelators or oil structuring agents are low molecular weight molecules that can assemble into a continuous space-filling network which is capable of physically immobilizing liquid oils into 'gel-like' structures. In most cases, a single oleogelator is used to design oleogels (which are often referred to as monocomponent gels). Although this is an interesting strategy, monocomponent gels suffer from certain drawbacks such as requirement of relatively higher concentration of oleogelator and limited tunability of gel properties such as optical clarity, melting range, rheological properties and storage stability. Alternatively, multi component oleogels (mixed gels) which are based on synergistic interactions of two or more materials offer the flexibility of creating oleogels with desired properties at a low total structurant concentration. A low concentration of structurant ensures incorporation of a large proportion of liquid oil in reformulated food products (resulting in the improvement of fatty acid profile) and, the flexibility of altering macrostructural properties (by simply changing the proportion of components) may further broaden their applicability in different product formats.

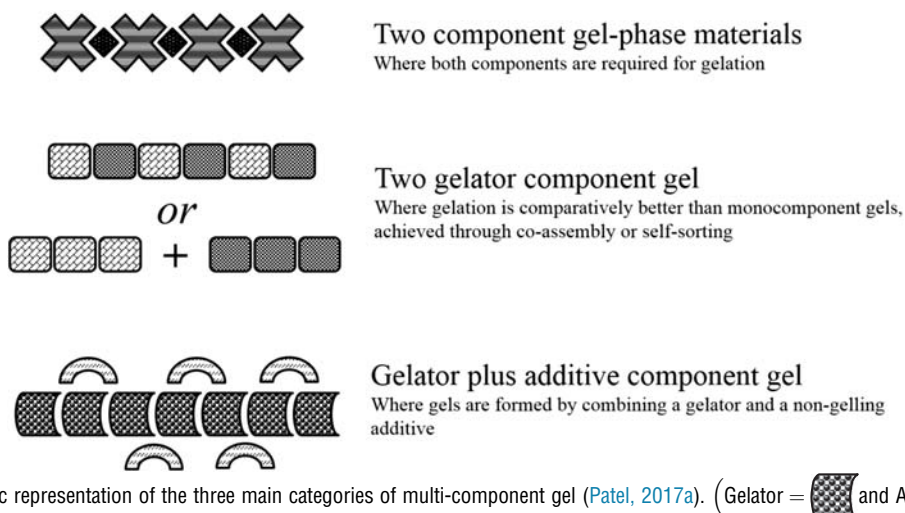
The simplest of these mixed gels are ones in which two components are added to the liquid oil in order to exploit their synergistic interactions to alter the formation of microstructure as well as the structural framework that supports the gel. The three general classes of these kind of gels (Fig. 1) include: (a) two component gel-phase material gels where both components are required for gelation as the individual components cannot form gel on their own (in other words, the two components form gelator *in-situ*, interacting *via* non-covalent forces to form a complex that is the gelator); (b) two gelator component gels where both components are themselves gelators (having properties of forming structured materials) and when used in combination, they are capable of organizing into assembled structures either together (co-assembled) or independent of each other (self-sorting), and (c) gelator plus additive component gels which are formed by a combination of a gelator and a non-gelling additive. The non-gelling additive can impact either the self-assembling properties of gelator or promote an effective spatial distribution of building blocks formed by gelator or strengthen the network linkages among the formed building blocks (Patel, 2017a).

Some selected examples of edible gels prepared from combination of food components are discussed briefly in the following text.

## Two Component Gel-Phase Material Gels

### Phosphatidyl Choline-Tocopherol Oleogels

When phosphatidyl choline (PC) or  $\alpha$ -tocopherol are individually added to vegetable oil, they do not yield to any structural organization, but their mixture (at 1:1 ratio) leads to the formation of solid-like structures (*in-situ* formation of building blocks) (Niki-foridis and Scholten, 2014). Due to its molecular structure (small polar group and large hydrophobic tail), PC likes to pack itself into spherical reverse micelles. However, these spherical structures are incapable of organizing into a spanning network.



**Figure 1** Schematic representation of the three main categories of multi-component gel (Patel, 2017a). (Gelator =  and Additive = .

Incorporation of  $\alpha$ -tocopherol (which has a complementary structure, i.e. large hydrophobic group and a small polar head) leads to the formation of cylindrical micelles that have the ability to grow into a space-filling network which leads to gel formation. Mixed gels with thermoreversible properties can be created at a total solid mass of 25% weight (wt) but these gels display a high sensitivity to shear (Nikiforidis and Scholten, 2014). The requirement of high concentration and shear sensitivity of these gels may limit their practical applications in food structuring.

### Lecithin-Sorbitan Tristearate Oleogels

Individually both lecithin (Lec) and sorbitan tristearate (STS) are incapable of forming oil gels at concentration between 6 and 20 % wt in absence of a polar solvent. However, when mixed in specific ratios between 40:60 to 60:40, Lec:STS can form firm gels at a total concentration as low as 4 %wt (Pernetti et al., 2007). The crystalline units formed in these systems are based on STS, while Lec plays an important role in influencing both the morphology of the crystalline units as well as the network junctions among the formed units. The gel however has limited use as hardstock fat replacer as it starts softening at temperature above 15 °C and undergoes complete collapse at 30 °C (Pernetti et al., 2007).

### Lecithin-Sucrose Esters Oleogels

Lec is also known to show synergistic interactions with sucrose esters (SEs) resulting in the formation of structured units (reverse worm-like micelles) in organic solvent such as *n*-decane (Hashizaki et al., 2009). Formation of these units and the subsequent continuous network results in gel formation. This interaction was reported for hydrophilic SEs (HLB > 10) and the functionality of Lec-SEs combination was found to be influenced both by concentration of Lec and the hydrophobicity of SEs (Hashizaki et al., 2009).

It was recently shown that synergistic interactions of Lec and SEs are also seen in edible vegetable oil (rapeseed oil) when hydrophobic SEs (HLB 1 and 2, also called as sucrose oligoesters) are mixed with Lec at a total structurant concentration of 10 %wt (Bin Sintang et al., 2017c; Patel, 2017b). It was hypothesized that the presence of Lec interrupts the extensive hydrogen bonding among SEs molecules and prevents the aggregation induced contraction seen in mono component gels of SEs. This is in contrast to the reported study done in *n*-decane where Lec micelles were the structuring units and SEs played the role of an additive that modified the morphology of micelles by interacting with the phosphate groups of Lec (Hashizaki et al., 2009).

## Two Gelator Component Gels

### Phytosterols-Oryzanol Oleogels

Mixed component oleogels of  $\beta$ -sitosterol and  $\gamma$ -oryzanol are undeniably one of the most extensively studied oleogel systems. On their own both components form crystalline particles in vegetable oils, but when mixed at certain proportions they co-assemble to form nanoscale tubular structures. Depending on the concentration, these nanoscale tubules aggregate to form a 3-D network that can physically immobilize liquid oil through capillary forces to form a viscoelastic gel. Aside from  $\beta$ -sitosterol, other related phytosterols such as dihydrocholesterol, cholesterol and stigmasterol can also produce gels with  $\gamma$ -oryzanol. Structurally, the presence and position of hydroxyl group was found to be critical for gel formation while the composition of alkyl group did not appear to have

any major impact on the gelling properties of phytosterols (Bot and Agterof, 2006). Both  $\beta$ -sitosterol and  $\gamma$ -oryzanol are reported to have an effect on cholesterol absorption due to the similarity in their chemical structures (Rong et al., 1997; Ikeda et al., 1988). This lipid lowering bioactivity makes these components even more attractive for oil structuring in lipid-based food products.

### Fatty Acid-Fatty Alcohol Oleogels

Fatty acid (stearic acid) and fatty alcohol (stearyl alcohol) mixtures were among the first components used for creating mixed component gels to be potentially used for edible applications (Gandolfo et al., 2006). The synergistic effect in these systems was previously explained based on mixed crystal formation (co-crystallization) which results in finer crystal sizes as well as an altered crystal morphology (needle-like for mixtures versus platelet-like for individual components) (Gandolfo et al., 2006; Schaink et al., 2007). However, a recent study claims that the synergistic effect is not linked to the changes in crystal sizes but rather linked to the increase in the crystalline mass as well as its spatial distribution in the solvent (Blach et al., 2016).

### Phytosterol-Monoglyceride Oleogels

Phytosterols (PS) and monoglycerides or monoacylglycerols (MAGs) are lipidic materials that form well-defined crystalline particles in liquid oils. Crystalline particles formed by MAGs can connect together to form a 3D network that results in oleogel formation. However, these oleogels are usually soft in nature (low firmness) and MAGs are prone to polymorphic transition over time, resulting in the formation of grainy crystals. On the other hand, PS crystallize as large flat-crystals and thus, form weakly structured oleogels that are prone to phase separation caused by aggregation-induced contraction of crystalline phase. Surprisingly, by combining MAGs with PS at certain proportions (and a total gelator concentration of 10 %wt), a synergistic enhancement in the rheological properties of oleogels was observed due to the formation of finer crystalline particles with completely different morphology compared to MAG and PS crystals (Bin Sintang et al., 2017a). Based on the data from several characterization techniques, it was concluded that the synergism was not based on co-crystallization owing to a large difference in the molecular structures of the two components (Bin Sintang et al., 2017b). Instead, a self-sorting behavior was noticed as both components crystallize independent of each other to form characteristic structuring units (fibrous and spherulitic crystals) that contribute to a synergistic enhancement in the rheological property of the gel (Patel, 2017a). In addition, the presence of different structuring units prevents aggregation of crystalline mass (which is an issue in the monocomponent gels) leading to a better spatial distribution of mass in the bulk liquid oil phase.

### Gelator Plus Additive Component Gels

#### Stearic Acid Oleogels

Stearic acid has been reported to form oleogels on its own when used at concentration close to 20%wt. Structuring in these gels comes from network of crystalline particles which are predominantly shaped as plate-like. Uvanesh and co-workers have demonstrated that non-gelling additives such as Span 60 (sorbitan monostearate) and Tween 20 (polyethylene glycol sorbitan monolaurate) can significantly influence the properties of stearic acid oleogels by modulating the crystallization behavior of stearic acid (Uvanesh et al., 2016a,b). Span 60 was shown to promote the crystallization of stearic acid which in turn resulted in significant decrease in the crystal size and microarchitectural changes from plate-like to fibrous mesh-like structures (Uvanesh et al., 2016a). Incorporation of Tween 20 resulted in molecular rearrangement and ordering of stearic acid molecules by influencing the polymorphic transition of stearic acid (Uvanesh et al., 2016b).

### Conclusion

Oleogelation is an emerging approach and has the potential to contribute significantly towards solving some long-standing challenges in the area of food structuring. However, one of the main bottlenecks in the field of oleogelation is finding oleogelators that are effective at low concentrations and have required regulatory approval for use in edible products. By exploring the synergistic interactions of known food-grade materials, a range of mixed gels can be created. The flexibility of tuning the rheological and physical properties of such gels (by changing the proportions of individual components and the total gelator concentration) makes them quite attractive for commercial applications. There is however a lack of information on the influence of formulation matrix and product conditions on synergistic interactions among gelators. Therefore, an in-depth analysis to understand this aspect is needed to fully exploit the potential of this class of oleogels.

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# Protein-Based Nanodelivery Systems for Food Applications

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## Introduction

Over the last two decades, the concept of using functional foods for health promotion has been confirmed by several *in vitro*, animal and human studies. Among the food-derived ingredients, proteins are drawing a lot of attention due to their functionality as essential biopolymers and also their distinct biophysicochemical characteristics such as biocompatibility, amphipathicity and the presence of modifiable moieties. Development of encapsulation system using food-derived proteins has become a major research endeavour to facilitate efficient delivery of functional compounds and probiotic organisms (McClements, 2015). Hence, proteins such as gelatin, collagen, zein, casein and whey proteins represent some of the most investigated groups of ingredients for food and biotechnological applications as delivery systems (Gomez-Guillen et al., 2011; Livney, 2015; McClements, 2015; Raikos and Ranawana, 2017; Rajendran et al., 2016).

The size of a protein-based delivery system in the intestinal environment is critical in ensuring biorecognition, bioavailability, bioadhesion and stability (Santos et al., 2013). Acosta (2009) provided a comprehensive review, using thermodynamic and mass transfer principles, on the essence of nanosize of delivery systems in enhancing their bioavailability and bioadhesion relative to their larger sized counterparts. The decrease in size of delivery systems can increase their surface area-to-volume ratios as well as physicochemical interactions with a corresponding effect on the global characteristics of the delivery system in the human gastrointestinal tract (Cerqueira et al., 2014).

To date, various techniques developed to produce protein-based nanodelivery systems include nanoemulsification, coacervation, antisolvent precipitation, electrospraying and electrospinning (Rajendran et al., 2016). McClements (2015) recently discussed critical parameters that are essential in the development of food-grade nanodelivery systems. There are several considerations required to develop nanoencapsulation systems, including safety, commercial viability, food matrix compatibility, robustness, performance, and labelling requirements (Fig. 1). This chapter discusses the fundamental basis of protein-based nanodelivery systems, together with the key characteristics of the produced nanodroplets/nanoparticles and their drawbacks, for tailored design to suit their final applications in food products.

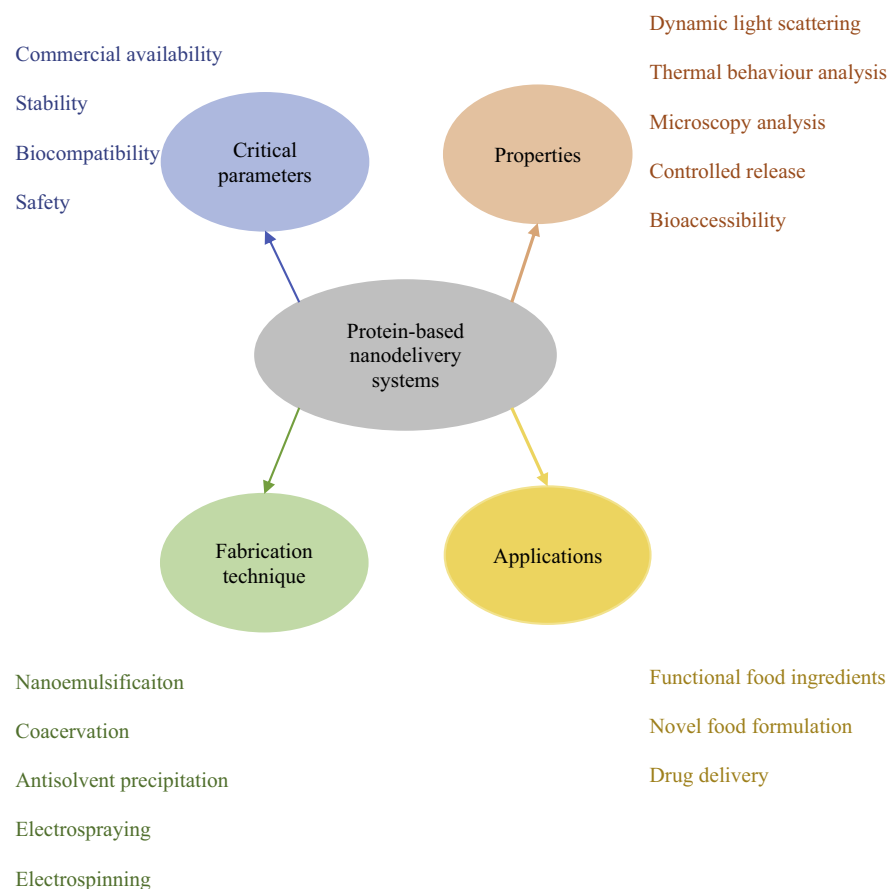
## Preparation and Characterization of Protein-Based Nanodelivery Systems

### Nanoemulsification

Nanoemulsification is a technique that involves dispersion of immiscible liquids and is commonly employed in the production of nanoparticle droplets that are thermodynamically stable, bioavailable, and optically isotropic. Nanoemulsions have a number of potential advantages over conventional microemulsions, including better stability against particle agglomeration and gravitational separation (Tadros et al., 2004), weaker light scattering effects (Mason et al., 2006) and increased bioavailability of some encapsulated lipophilic compounds (Acosta, 2009). Like the conventional oil-in-water (O/W) emulsions, protein-based O/W nanoemulsion can also be prepared using high-energy approaches including high-pressure homogenisation, microfluidisation and ultrasonication (McClements and Rao, 2011). Dynamic light scattering technique is usually used to test the size distribution of the nanoemulsion while the morphology of the nanodroplets/nanoparticles can be revealed by various microscopic techniques including confocal laser fluorescence, scanning electron, atomic force, and transmission electron microscopy.

### Nanoemulsions Prepared by High Pressure Homogenisation

The high pressure method is the most commonly used homogenisation method in the contemporary food industry and for producing fine O/W microemulsions from coarse emulsion prepared with high shear mixers (Schubert and Engel, 2004). As large O/W droplets pass through the narrow valve of the high pressure homogeniser, the O/W droplets are broken down into smaller ones due to the intensively disruptive forces. Generally, the size of droplets decreases with increase in homogenisation pressure and number of passes. By using extremely high homogenisation pressures and multiple passes, sometimes O/W nanoemulsion can also be produced. It should be emphasised that the amount of emulsifier must be sufficient to adsorb onto the O/W interface during homogenisation to prevent re-coalescence of the nanodroplets (Jafari et al., 2008). A study reported the preparation of whey protein-based nanoemulsion using medium-chain triglycerides containing  $\beta$ -carotene with a final emulsion droplet size below 200 nm at the homogenisation pressure of >80 MPa with 3 passes (Mao et al., 2010). Moreover, the delivery of fenofibrate in nanoemulsions of sizes ranging from 100–250 nm was achieved using soy and whey protein isolate as emulsifiers (He et al., 2011). In the study, a more rapid absorption of the nanoencapsulated fenofibrate was observed *in vitro* compared to the unencapsulated compound.



**Figure 1** Protein-based nanodelivery systems: critical considerations for development, physicochemical properties, fabrication technique and their applications.

### Nanoemulsions Prepared by Microfluidisation

Microfluidisation works similarly as homogenisation. The O/W coarse emulsion droplets are also forced to pass through a narrow channel to facilitate the droplets disruption during the process. However, this channel is differently designed, compared to the high pressure homogenisation valve, to produce emulsions with smaller droplets size and narrower size distribution. Using this method, [Dagleish et al. \(1997\)](#) found that protein fractions influence the nanoemulsion droplet size distributions. Moreover, [Klein et al. \(2010\)](#) reported that O/W droplet size in canola O/W nanoemulsion stabilised by whey protein isolate, prepared by microfluidisation, remained unchanged during 30 days of storage. Like high pressure homogenisation, O/W emulsion produced by microfluidisation tends to exhibit a decreased size with increase in pressure, number of passes, emulsifier concentration, and continuous/disperse phase ratio.

### Nanoemulsions Prepared by Ultrasonication

Ultrasonication is a method that uses high intensity ultrasonic waves to disrupt oil and water phase to create small O/W emulsion droplets. This method has been widely used in the nanoemulsification of food-derived products. Like homogenisation and microfluidisation, smaller O/W emulsion droplet size tends to be formed by increasing the energy input (ultrasonic wave intensity), and/or the residence time ([Maa and Hsu, 1999](#)). Using this method, [Mahdi Jafari et al. \(2006\)](#) encapsulated *d*-limonene oil in whey protein concentrate-based O/W nanoemulsion with droplet size below 250 nm. The emulsion size was found to be dependent on process parameters such as emulsifier concentration, *d*-limonene oil-to-water phase ratio, ultrasonic intensity and residence time. Therefore, desired emulsion size can be prepared by adjusting the ultrasonication condition, and this was applied in determining the optimum ultrasonication condition beyond which the emulsion size either increased or remained unchanged with further processing ([Mahdi Jafari et al., 2006](#)).

To date, high pressure homogenisation, microfluidisation and ultrasonication have been widely used for preparing nanoemulsions for food, pharmaceutical and agricultural applications. The fundamental basis of nanoemulsification is simple, but it should be emphasised that the size of nanoemulsion droplets/particles also depends strongly on the characteristics of the oil and protein used. It is usually easier to produce small droplets using the oil phase with low viscosity and/or interfacial tension such as flavour and essential oil, compared to the one with high viscosity and/or interfacial tension, for example, medium and long-chain



triglycerides. This is because, at low viscosity or interfacial tension, the restoring forces holding the droplets can easily be overcome by the homogenizer disruptive forces (McClements and Rao, 2011).

### Coacervation

Coacervation is a method of inducing phase separation by formation of insoluble liquids known as coacervates in a colloidal system. Driven by change in entropy in colloidal systems, this phenomenon can be induced by altering the pH, temperature, dielectric constant and/or ionic strength of the protein mixture (McClements, 2015). Depending on the numbers of polymers used, coacervation can be termed as simple or complex; the former occurs through the surface energy change of the proteins whereas the latter is by means of interactions of oppositely charged colloids at various conditions. The major advantage of coacervation is the ability to regulate the process conditions to produce nanoparticles of desired sizes. Wang et al. (2008) reported the nanoencapsulation of capsaicin into gelatin-based particles with the size of about 100 nm via simple coacervation. In the work of Xing et al. (2005), complex coacervates between gelatin and gum acacia were used to encapsulate tannins into nanoparticles with spherical morphology and mean diameters of 300–600 nm. The mean drug loading content and encapsulation efficiency of the nanocapsules were 20.8% and 81%, respectively. A good dispersion was observed and the particle distribution and morphology were greatly influenced on addition of hydrolysable tannins because of hydrophobic and hydrogen bonding effects. Gelatin is almost the only protein successfully used in nanoparticle production by coacervation, but the increasing vegetarian and/or vegan population limits its food application. Therefore, there is a need to develop non-gelatin based protein delivery systems when using the coacervation technique.

### Antisolvent Precipitation

Antisolvent precipitation occurs via the addition of a non-solvent to a solution to enhance desolvation and production of the active solute of interest (Joye and McClements, 2013). Thorat and Dalvi (2012) described the general steps in the precipitation of nanoparticles by antisolvent precipitation as: 1) mixing of the solution and antisolvent; 2) generation of supersaturation; 3) formation of nucleation and its growth by coagulation and condensation; and 4) particle agglomeration in the uncontrolled system. The properties of formed nanoparticles such as size, morphology and purity are significantly dependent on the flow rate of the antisolvent, magnitude and uniformity of supersaturation (Thorat and Dalvi, 2012). The precipitation methods involve the use of miscible organic antisolvents, heat treatment and application of shear force and/or lyophilisation (Jarzebski and Malinowski, 1995). Although various antisolvent precipitation methods have been successfully developed for pharmaceutical applications, they are fraught with significant bioprocessing concerns such as exposure to oxygen, high shear force, recovery of solvent for reuse, and high temperatures (Thiering et al., 2000).

As a result, gas antisolvent precipitation, where the fluid near its critical or supercritical conditions is used as the antisolvent, has been developed. The process has a competitive advantage over the traditional methods (Yeo et al., 1993). Using antisolvent precipitation, zein was used as a carrier to prepare lysozyme nanoparticles of sizes around 100 nm, by optimising the zein concentration, solvent type and CO<sub>2</sub> flow rate (Zhong et al., 2008). Interestingly, ultrasonication has been used to enhance mass transfer during protein nanoparticle precipitation (Chattopadhyay and Gupta, 2002). This technique can potentially be used in the development of a delivery system with protein as the shell material. However, zein is one of the few protein candidates currently available for this technique due to its solubility in organic solvents miscible with CO<sub>2</sub> and insolubility in water.

### Electrospraying

Electrospraying is an electro-hydrodynamic atomisation method that allows for easy tailored (in terms of architectural form, morphology and composition) nanoformulations from a broad spectrum of precursors such as biopolymers and cells (Jaworek, 2008; Jayaraman et al., 2015). Using an electrical field, it allows for the breakdown of a conductive liquid jet, flowing through a capillary nozzle, into fine droplets with high monodispersity. Hence, the droplet size formed via electrospraying technique only depends on the solution flow rate and charge acquired due to voltage variation (Dasgupta and Ranjan, 2018). In the study by Torres-Giner et al. (2010), docosahexaenoic acid (DHA) was encapsulated in ultrathin zein-based delivery system of 500 nm particle size, and the nonencapsulated DHA exhibited improved oxidative stability at elevated storage temperature and/or relative humidity. Likewise, López-Rubio and Lagaron (2012) produced glycerol in whey protein isolate-based nanocapsule and found that pH affects the surface tension of whey protein solution and consequently the size of the formed nanocapsule. Surface tension of 47.0, 43.3, 42.1, 43.5 and 45.3 mN/m were calculated at pH 4.5, 5.5, 6.4, 7.3 and 8.0, respectively. Surprisingly, smaller protein capsules were not formed in solution with lower surface tension; instead, larger protein capsules were observed. This is an indication that other parameters, such as conductivity of the solution, may play a major role in size determination. Furthermore,  $\beta$ -carotene was electrosprayed into whey protein capsule and this improved its stability against photo-oxidation (López-Rubio and Lagaron, 2012). This demonstrates the direct shielding/protective effect of the protein shell. Recently, Gomez-Estaca et al. (2012) reported the formation of curcumin-rich zein nanoparticles using electrospraying technique. In addition to zein concentration, optimization of the flow rate and applied voltage resulted in nanoparticles in the size range of 80–130 nm, with encapsulation efficiency of over 85%. Despite the prospects, there are some challenges in using electrospraying technique, including low throughput and product yield typically in the range of milligrams/hour (Jayaraman et al., 2015).

## Electrospinning

Electrospinning works on a similar principle as electrospraying but occurs at a higher voltage leading to the formation of polymer nanofibers (Frenot and Chronakis, 2003). In electrospinning, the main processing factors are voltage, solution flow rate, concentration, molecular weight of the polymer, and nozzle-to-ground distance (Dasgupta and Ranjan, 2018). Several nanofibre systems have been developed for the delivery of bioactive compounds including phytochemicals, probiotics, growth factors and hormones (Dong et al., 2009; Zhang et al., 2006). For instance, nanoencapsulation of  $\beta$ -carotene was achieved in electrospun zein fibres with a mean cross-section of 1140 nm and this resulted in improved oxidative stability of the bioactive compound (Fernandez et al., 2009). Similarly, curcumin-loaded zein nanofibers with an average diameter of 310 nm were developed by electrospinning (Brahatheeswaran et al., 2012). In the study, loaded curcumin was well encapsulated within the nanofibre as no aggregates were found on the surface. Also, an increase in fluorescence was reported after encapsulation and curcumin was found to retain its antioxidant property on release. The thermal stability of zein-curcumin nanofibre increased with an increase in curcumin amount, as pure curcumin has higher thermal stability than zein. Initial burst release followed by sustained release was also observed, which is a characteristic of drug release from a polymer-based matrix. The zein-nanofibre exposed a good surface of attachment that can enable the growth of fibroblast making it a promising candidate for drug/nutraceutical delivery and for soft tissue engineering. However, the major bottleneck in using electrospinning is the difficulty of large-scale production of nanofibers with diameters under 50 nm (Wang et al., 2013).

## Binding, Digestion and Bioaccessibility of Protein-Based Nanodelivery Systems

### Binding and Encapsulation

Various types of protein nanoparticles have been engineered from whey, gelatin, collagen, zein, casein (Gomez-Guillen et al., 2011; Livney, 2015; Penalva et al., 2015; Pérez-Masiá et al., 2015; Raikos and Ranawana, 2017; Shen et al., 2018),  $\beta$ -lactoglobulin, lactoferrin (Chapeau et al., 2016), potato protein (David and Livney, 2016), soy protein (Lee et al., 2016), fish protein (Stephansen et al., 2015), and cruciferin (Akbari et al., 2017; Wang et al., 2018). The proteins can be employed in the food, pharmaceutical and medical industry as delivery vehicle for efficient capping, protection and release of bioactive compounds. Protein has inherent structural and physicochemical properties that enhance binding and hence encapsulation of molecules, ligands and ions. For instance, caseins spontaneously bind with calcium and calcium phosphate nanoparticles through their serine-phosphate residues. Alpha-lactalbumin easily binds calcium while lactoferrin is known to bind ferric ion (Fox and McSweeney, 2003).  $\beta$ -Lactoglobulin can bind epigallocatechin (Livney, 2010), vitamin D (Forrest et al., 2005), omega-3 fatty acid (Zimet and Livney, 2009), retinol (Fox and McSweeney, 2003), and resveratrol (Liang et al., 2008). The binding is usually facilitated by hydrophobic interaction, hydrogen bonding or van der Waals attraction. In a recent study, encapsulation and loading efficiency ranges of 68.1–72.0 and 5.4%–6.2%, respectively with particle sizes of 217–227 nm were reported for acylated rapeseed cruciferin protein loaded with curcumin (Wang et al., 2018). Stability study indicates increase in particle size and decrease in encapsulation efficiency with time due to the diffusion of curcumin out of the nanoparticle matrix. Moreover, casein-folic acid nanoparticles stabilized with cationic lysine and arginine, prepared by coacervation, had 95%–97% yield and 40% encapsulation efficiency, with decreases in particle size, polydispersity index and zeta potential when compared with unstabilized casein nanoparticles (Penalva et al., 2015). Furthermore, the nanoparticles stabilized with lysine and arginine were reported to be stable for at least 48 h and 16 h, respectively. Affinity of bioactive compounds to the proteins is important in achieving high encapsulation efficiency, but this can also influence the release of the compounds *in vivo*. Some examples of various types and properties of protein-based nanodelivery systems are presented in Table 1.

### In Vitro Release Studies

Protein has demonstrated efficient binding of bioactive compounds and there is need to consider the release of various encapsulated nutraceutical into the target regions of the human body. Protein nanoparticles digest rapidly and can permeate the mucus layer and epithelium cells of the intestine (McClements, 2018). The small size of the nanoparticle can increase the bioavailability, chemical stability and absorption of bioactive compounds encapsulated within the matrix. The nanoparticle may release any encapsulated agent more rapidly during digestion in the gastrointestinal tract due to their small size and large surface area. Some studies reported efficient release of bioactive compounds encapsulated in protein nanodelivery systems as a result of inherent structural and physicochemical properties of proteins. For instance, caseins can easily undergo proteolytic cleavage due to their open structures, a result of their high proline content and low contents of  $\alpha$ -helix and  $\beta$ -sheet secondary structures. Moreover, acid-soluble calcium-phosphate bridging in casein can facilitate target-activated release mechanism for offloading encapsulated compounds in the stomach (Fox and McSweeney, 2003). Likewise, bovine serum albumin binds small molecules in the blood and offloads them in their target locations (Li and Yao, 2009). Recently, *in vitro* release of curcumin from acylated rapeseed cruciferin protein nanoparticles was demonstrated in simulated gastric fluid (SGF) and intestinal fluid (SIF) within 6 h of exposure (Wang et al., 2018). Initial burst release of about 70% in the first 2 h and sustained release of about 75% after 2 h was observed in the SIF. In the SGF treatment, only about 10% of curcumin was released in the first 1 h followed by slow and up to 25% release after 6 h. Acidic nature (pH 1.2) of the system compared to the isoelectric point of chitosan (pI 6.5) contributed to the slow curcumin release in the SGF. Burst release of curcumin in the SIF in the first 2 h at pH 6.8 was due to deprotonation of the amine group of chitosan giving rise to a weaker polysaccharide-protein interaction. A pH-dependent release of folic acid encapsulated with casein nanoparticles was demonstrated using a similar digestion model (Penalva et al., 2015). No folic acid was released in the SGF (pH 1.2) with pepsin

**Table 1** Types and properties of protein-based nanodelivery systems

<i>Protein-based nanodelivery agent</i>	<i>Preparation technique</i>	<i>Bioactive compound encapsulated</i>	<i>Encapsulation efficiency (%)</i>	<i>Size range (nm)</i>	<i>PDI</i>	<i>Zeta potential (mV)</i>	<i>Loading ability</i>	<i>% Release in SIF</i>	<i>% Release in SGF</i>	<i>Transport across Caco-2 cells</i>	<i>Apparent permeability coefficient (cm/s)</i>	<i>References</i>
Cruciferin	Cold gelation	Coumarin	n.d.	58–334	0.29–0.45	–14.3 to –33.0	n.d.	85	0	2%–50%	$1.4 \times 10^{-6}$ – $3.0 \times 10^{-6}$	Akbari et al., 2017 Wang et al., 2018
	Polyelectrolyte complexation	Curcumin	68.1–79.0	217.7–454.4	0.139–0.204	+15.3 to +18.6	5.4%–6.2%	20–80	25	1.8%	$2.43 \times 10^{-6}$ – $6.00 \times 10^{-6}$	
Casein	Coacervation	Folic acid	40	128–305	0.17–0.45	–9.4 to –17.6	23–31 µg/mg	60–90	0	n.d.	n.d.	Penalva et al., 2015 Shen et al., 2018
Whey protein isolate	Emulsification-Evaporation	Astaxanthin	92.1–93.5	80.4–267	0.21–0.543	–19.3 to –35.0	1%–5%	25–65	0–10	0.25–2.5 µg	$2.0 \times 10^{-6}$ – $3.5 \times 10^{-6}$	Stephansen et al., 2015 Gomez-Estaca et al., 2012 Pérez-Masiá et al., 2015
Fish sarcoplasmic protein	Electrospinning	Insulin	98.6	349–360	n.d.	n.d.	14	75	45	2%–14%	$0.5 \times 10^{-6}$ – $1.5 \times 10^{-6}$	
Zein	Electrohydrodynamic atomization	Curcumin	85–90	175–900	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
Whey protein concentrate	Nanospray drying and electrospraying	Folic acid	80.8–83.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	

n.d., data not available; PDI, polydispersity index; SIF, simulated intestinal fluid; SGF, simulated gastric fluid.

action due to strong binding of the vitamin inside the protein network. Burst release was equally observed at 2 h followed by slow and sustained release in the SIF. This is likely because of the electrostatic repulsion between folic acid and casein at neutral pH.

The interactions can also depend on the protein type and complexity of the matrix. For instance, kinetics of *in vitro* release of curcumin from electrospun zein nanofibre into cell culture media showed initial burst release of about 80% between 5 and 20 h, followed by constant release of about 90% for the nanofibre loaded with 10 wt% curcumin (Brahatheeswaran et al., 2012). The initial burst release is as a result of diffusion of curcumin adsorbed at the outer region of electrospun zein nanofiber, whereas the steady release after 20 h was due to slow diffusion of water molecules into the covered nanofiber, which limited the release of capped curcumin. Larger molecules have also been successfully released from protein nanoparticles. Stephansen et al. (2015) demonstrated the control release of insulin encapsulated with electrospun fish sarcoplasmic protein nanoparticles in simulated intestinal fluid. The encapsulating agent shielded insulin from chymotrypsin-catalysed degradation, which is expected to enhance its bioavailable amount. In contrast, whey protein-stabilized astaxanthin was resistant to pepsin, but trypsin digested the complex leading to the release of astaxanthin (Shen et al., 2018). The rate, extent and trigger mechanism of release of protein-encapsulated components depend on protease activity, temperature, pH, dilution, and ionic strength of the gastrointestinal environment. These factors are critical especially when delivering sparingly water-soluble components with the goal of controlling their release within the mouth, stomach, or small intestine (McClements and Rao, 2011).

### Bioaccessibility and Uptake Studies

Protein-based nanomaterials do not only bind, encapsulate and protect bioactive compounds but also control their release, uptake and transport across cells, thereby reducing the risk of toxicity associated with burst release. For instance, acylated cruciferin-chitosan nanoparticles loaded with curcumin had higher permeability coefficient in cultured Caco-2 intestinal cells, and lower effect on cell viability compared to free curcumin (Wang et al., 2018). The latter may be due to the delayed release of curcumin from the curcumin nanoparticles, among other factors. Properties of the nanoparticles, e.g. surface charge, are critical to the bioaccessibility and uptake of protein-based nanoparticles. Akbari et al. (2017) demonstrated that uptake of negatively charged cruciferin/calcium nanoparticles by Caco-2 cells was far higher than that of positively charged cruciferin/chitosan nanoparticles. Uptake and transport of the nanoparticles was not affected by the mucus secreted by a Caco-2/HT29 cells co-culture, which demonstrate that they are potentially biostable. As demonstrated for cruciferin-curcumin (Wang et al., 2018), an increase in cellular uptake of coumarin was recorded for the digested nanoparticles in simulated gastrointestinal fluids, and this was more with cruciferin/chitosan than cruciferin/calcium nanoparticles (Akbari et al., 2017). Likewise, Stephansen et al. (2015) reported the permeability of insulin encapsulated with electrospun fish sarcoplasmic protein nanoparticles across Caco-2 cells. The protein-based delivery agent was also found to be biocompatible, and insulin uptake in Caco-2 cell was pH-dependent. However, overall insulin transport was not affected by its increased release from the protein nanoparticles, and insulin binding and structural properties were not affected after cell permeation. Lastly, capping astaxanthin with whey protein isolate and polymerized whey protein enhanced its apparent permeability coefficient in Caco-2 cells by 10- and 16 folds, respectively (Shen et al., 2018). If translated *in vivo*, this observation can result in the use of lower amounts of bioactive compounds in food products to achieve beneficial health effects in humans.

### Drawbacks of Protein-Based Nanodelivery Systems

Protein-based nanoparticles have demonstrated efficient binding, encapsulation, protection and controlled release of bioactive compounds. However, there are drawbacks associated with the use of proteins in producing nanomaterials. Allergenicity can occur in some individuals due to the ability of some proteins to elicit immune response. Proteins from egg, soybean, fish, cow's milk, etc. can be allergenic (Hefle et al., 1996), and this would impede their use for producing nanodelivery agents. Protein abundance, resistance to digestive enzymes, and processing may also lead to allergenicity (Bannon, 2004). Furthermore, high specific surface area associated with the small size of nanoparticles could lead to adsorption of digestive or metabolic enzymes, which may consequently interfere with their normal gastrointestinal function (McClements and Xiao, 2017). Also, changes in thermodynamic environment after adsorption of some proteins to the nanoparticle surface can cause denaturation thereby affecting the activity of many digestive enzymes. As a result of the small size associated with nanoparticles, rapid digestion and burst release in the intestinal medium may also lead to adverse effects. Currently, there is no standardized analytical technique for assessing the potential toxicity of protein-based nanoparticles and further studies is required in this area.

### Concluding Remarks

Various techniques have been developed to produce food protein-based nanoencapsulation systems for the delivery of nutraceutical compounds. This chapter was focused on the prospects and drawbacks of nanoemulsification, coacervation, antisolvent precipitation, electrospraying and electrospinning techniques in the production of protein-based nanoparticles, as well as the major characteristics of fabricated nanodroplets/nanoparticles. It is worth noting that due to the uniqueness and complexity of proteins, it is difficult to have predetermined set of standard conditions for different protein nanodelivery systems. Hence, the design and processing of the nanoparticles should be on a case-by-case basis. Furthermore, fabrication conditions for laboratory and industrial

scale applications can also vary significantly. Proteins are easily digested in the gastrointestinal tract making them promising candidates for controlled delivery of drugs/nutraceuticals. Some considerations are required to develop protein-based nanodelivery systems that can be commercialised, such as availability of raw materials, biocompatibility, safety, performance of the entrapped bioactive compounds, efficient gastrointestinal release, and stability of the protein nanoparticles when incorporated into food products.

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# Edible Delivery Systems Based on Favorable Interactions for Encapsulation of Phytochemicals

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## Glossary

**Phytochemicals** Bioactive components produced by plants through primary or secondary metabolism. When orally consumed in forms of fruits, vegetables, nuts, grains, herb extracts, or dietary supplements, these compounds exert health-promoting effects. And the rate and extent to which they are absorbed and become available to the action site determine their health-promoting efficiency or bioefficacy. Major categories of phytochemicals include carotenoids, flavonoids, indoles, isothiocyanates, phenolic compounds, phytosterols, saponins, vitamins, bioactive peptides, etc.

## Introduction

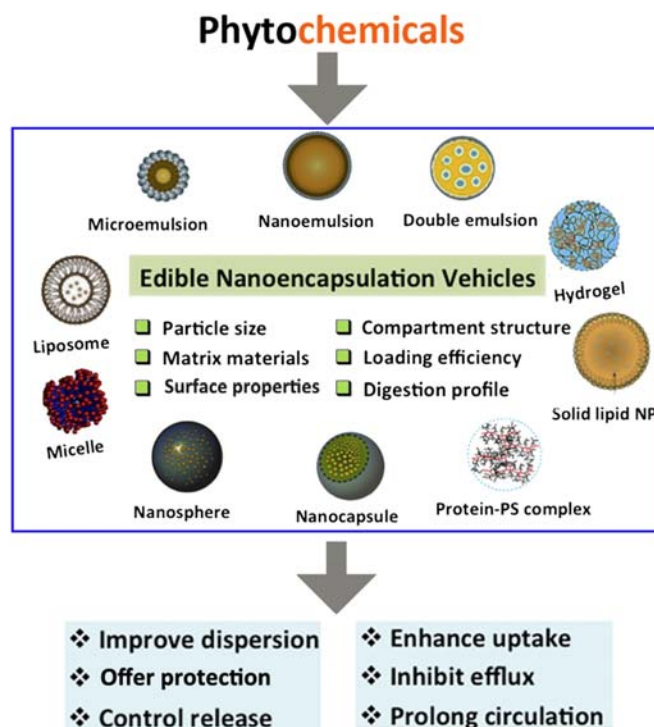
The term “phytochemicals” defined here refers to naturally occurring bioactive compounds found in vegetables, fruits, spices, nuts, grains, and other plants that have health-promoting properties. The major categories of phytochemicals include phenolic acids, carotenoids, flavonoids, isoflavones, indoles, phytosterols, saponins, monoterpenes, bioactive peptides, etc. (Swamy and Sinniah, 2015; Khan et al., 2015; Jiang et al., 2015; Erdem et al., 2015). Although synthesized by plants in low concentrations, a high intake of phytochemicals via frequent consumption of dietary vegetables, fruits, nuts, and herbal extracts usually leads to reduced risk for diseases and enhanced health benefits (Ta and Arnason, 2016; Lu et al., 2016; Chang et al., 2016). The rate and extent to which they are absorbed and become available to the action site determine their health-promoting efficiency or bioefficacy. In reality, the actual quantities of phytochemicals or their metabolites entering the systemic circulation often fall in the micromolar range (which are far less than their initial amounts for oral ingestion), causing their bioefficacy to be limited (Palles et al., 2013). Thus, there is an increasing interest in developing novel encapsulation and delivery strategies for these phytochemicals. Edible delivery vehicles (EDVs), which are constructed using “generally recognized as safe” (GRAS) ingredients (various lipids, polysaccharides, proteins, and synthesized biodegradable polymers featured on the “GRAS” list) with defined shape, size, and compartment structure, are thus proposed as encapsulation and delivery reservoirs for phytochemicals to improve their bioefficacy.

EDVs in forms of biopolymeric nanoparticles, protein–polysaccharides coacervates, emulsions, hydrogels, and inclusion complexes are among the most commonly utilized carriers for phytochemicals. Such EDVs exert effects on (1) the dispersion and gastrointestinal stability; (2) the digestion and release profile; (3) the transportation efficiency across endothelial layer to systemic circulation; (4) the systemic circulation and target distribution; (5) the microflora metabolism process of phytochemicals. Encapsulating phytochemicals within appropriate ENVs would increase their bioefficacy due to one or more of the following effects: (1) protection of sensitive phytochemicals from degradation during storage and gastrointestinal digestion; (2) enhancement in solubility; (3) prolonged contact time with the intestinal wall; (4) enhanced penetrability through mucus and permeability across intestinal walls; (5) improved cellular uptake efficiency; (6) prolonged residence time within the blood; (7) optimized release rate and duration as well as delivery location; (8) optimized interactions with gut microflora.

While the interactions among the building materials of EDVs play key roles in the controlled fabrication of EDVs, the interactions between building blocks and encapsulated phytochemicals determine the loading efficiency, stability, and release profiles of phytochemicals. This article focuses on interactions favoring the establishment of various edible delivery systems and the encapsulation of phytochemicals. Their respective application status as delivery vehicles is also briefly discussed (Fig. 1).

## Biopolymeric Nanoparticles

Biopolymeric nanoparticles are polymer- or lipid-based carriers that have particles sized from 10 to 1000 nm. Compared with inorganic nanoparticles, biopolymeric nanoparticles are biodegradable, less immunogenic, and nontoxic. Building materials include



**Figure 1** Edible encapsulation vehicles for oral delivery of phytochemicals. Copyright © 2017, American Chemical Society. All rights reserved.

naturally occurring proteins, such as albumin, collagen, gelatin, silk sericin or fibroin, zein, and kafirin, or polysaccharides, such as alginate and chitosan. Synthetic biodegradable polymers, including polyalkylcyanoacrylate, poly (lactic-co-glycolic acid), and polyanhydride, are also good candidates for biopolymeric nanoparticles. In addition, lipid-based nanoparticles, referred to as solid lipid nanoparticles, are constructed from physiological lipids and surfactants.

Dominant interactions favoring the biopolymeric nanoparticle formation vary among fabrication methods (Sundar et al., 2010). For instance, alginate nanoparticles can be obtained by producing a water-in-oil reverse microemulsion followed by inducing gelation with calcium ions, where physical cross-linking among polysaccharide chains plays a major role in sustaining the nanoparticle matrix. The use of oppositely charged proteins and polysaccharides to form nanoparticles via a complex coacervation method involves mainly electrostatic interactions. Alcohol-soluble proteins such as zein and kafirin proteins can be fabricated into nanoparticles through an antisolvent precipitation method, where hydrophobic interactions among protein chains dominate the self-assembly process. It is worth to mention that, during the particle formation process, covalently conjugating nanoparticle matrixes with cross-linking reagents (such as glutaraldehyde and formaldehyde) is a commonly utilized technique because it actively manipulates the density, biodegradability, and subsequent release profile of nanoparticles.

Interactions between surface materials of nanoparticles and specific surface coating materials occurring during the surface modification process endow biopolymeric nanoparticles with versatile functionalities. Take the layer-by-layer assembly process for an example, a typical assembly cycle involved the dispersion of nanoparticles into a coating material solution with opposite charge, electrostatic attractive interactions occurring at the interface lead to one deposit layer onto the surface. And subsequent coating is carried on by initiating another cycle of electrostatic attractive interactions between the deposited membrane and the other oppositely charged coating material. Repeating a number of the coating cycles leads to nanoparticles with desired coating thickness and surface chemistry. In this scenario, positively charged chitosan and poly-L-lysine are two of the most commonly utilized external surface coating candidates for nanoparticles because they endow nanoparticles with positive charge that promotes nonspecific binding between nanoparticles and the epithelium, thus enhancing the absorption through the tight junction. Apart from the non-covalent weak interaction, covalent bonding happened between the surface material and address tags such as monoclonal antibodies or peptides, yielding nanoparticles with target delivery feature.

Biopolymeric nanoparticles have long been utilized as efficient delivery vehicles for sustained, controlled, and target release of phytochemicals. Nanoparticles can be structured as nanospheres or nanocapsules (Roger et al., 2011), and phytochemicals are generally loaded through physical interactions within the particle matrix. In the former case, phytochemicals can be entrapped or adsorbed to the particle matrix and distribute evenly within the nanosphere. In the latter case, phytochemicals are physically encapsulated within the aqueous or lipid core. With regard to the release mechanisms, phytochemicals may diffuse out due to

the hydration of nanoparticles, that is, once the nanoparticles are swollen to a point where they stretch, phytochemicals diffuse out from the polymeric matrix. Also, nanoparticles may release phytochemicals via an erosion-dominant diffusion process as the particle matrixes degraded due to the enzymatic degradation during oral digestion process. Usually, nanoparticulate encapsulation is particularly suitable for phytochemicals that suffer to premature degradation or have poor solubility in the human gastrointestinal tract such as curcumin, quercetin, epigallocatechin gallate, and resveratrol, due to their high surface to volume characteristics. By manipulating the particle formation process and/or incorporating target ligands on the surface of the as-prepared biopolymeric nanoparticles, nanoparticles can perform sustained release manner and/or targeted delivery of phytochemicals, all add to the charm of nanoparticulate encapsulation of phytochemicals.

### Protein–Polysaccharide Coacervates

“Complex coacervation” commonly refers to the formation of a dense macroion-rich phase caused mainly by an electrostatically driven liquid–liquid phase separation process. Among the polyanion–polycation interactions that lead to the formation of complex coacervations, electrostatic interactions between oppositely charged residues of proteins and polysaccharides are studied most extensively (Corredig et al., 2011). The size of protein–polysaccharide coacervates may range from 0.1 to 100  $\mu\text{m}$  with their soluble precursors less than 200 nm in diameter. To form the soluble complexes, specific pH values are adjusted so that the electrostatic repulsion forces are minimized and associated interactions between charged patches of molecules are favored. During the complex formation process, hydrogen bonding and hydrophobic interactions also play a role in stabilizing the coacervates. The shift balance of the abovementioned three forces determines the final conformation of protein–polysaccharide coacervates (Turgeon et al., 2007). And the chemical nature of biopolymers (such as molecular weight, charge, distribution of charges), the mole ratio between polymers and their respective concentration, as well as environmental conditions, all exert effects on the force balance.

Because the structure of protein–polysaccharides coacervates is sensitive to environmental changes, especially the pH value and ionic strength, complex coacervates have been utilized as stimuli responsive delivery vehicles for phytochemicals. For application scenarios where sustained release is preferred, stronger coacervates are achieved by initiating cross-linking reactions among coacervate components (Chen et al., 2012). Another application of protein–polysaccharide coacervates lies in their ability in serving as emulsion stabilizer (Li and McClements, 2011). Herein, the emulsion interface can either be stabilized through emulsifying the oil phase with aqueous complex coacervates or via emulsification of the oil phase with the aqueous dispersion of one biopolymer followed by adsorption of the second biopolymer to induce the formation of interfacial complex coacervates (Raikos, 2010). In such cases, complex coacervates do not encapsulate phytochemicals directly (phytochemicals are encapsulated within the dispersed phase instead) but contribute to the smart delivery of phytochemicals by tuning the interfacial stability of emulsions triggered by environmental stimuli.

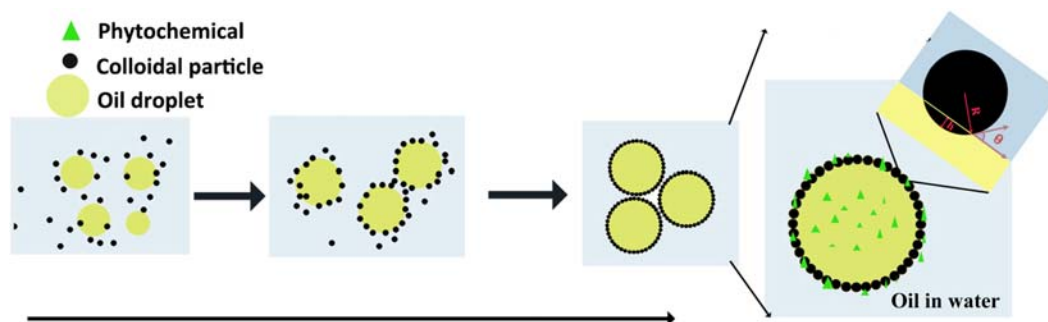
### Emulsions

Emulsion is a mixture of two or more liquids (usually oil and water) that are initially immiscible such as oil-in-water, water-in-oil, and derived multiple emulsions. Under mechanical turbulence and with the aid of surface-active compounds, one liquid (the dispersed phase) is dispersed into the other (the continuous phase). Interactions between emulsifiers and the water–oil interface play critical roles in the formation and stability of emulsions.

Emulsifiers can be classified according to their relative molecular weight into three categories: small molecular weight surfactants, surface-active macromolecules, and colloidal particles. Small molecular weight surfactants, for instance, lecithin, monoglycerides, and diglycerides, stabilize emulsion interface via migrating onto the interface and reducing interfacial tension between two phases. Because small molecular weight surfactants contain both hydrophobic tails and hydrophilic heads, their interactions with the interface are thermodynamically favored: the water-insoluble hydrophobic tails extend into the oil phase, whereas the water-soluble head groups remain in the water phase. On the contrary, interactions among surfactant molecules happen at places other than interface, for example, interactions among excessive surfactants in the bulk aqueous phase, result in formation of micelles and destabilization of emulsion.

Surface-active macromolecules, usually proteins and polysaccharides, stabilize emulsion droplets by creating a viscoelastic film over the dispersed phase, which prevent droplets from coalescence or flocculation via steric hindrance and repulsive electrostatic interactions. Interactions between proteins and the interface is also thermodynamically favorable because the hydrophobic residues of protein backbone orient toward the oil phase, following protein structural rearrangement at the interface (Chen and Dickinson, 1995). Key parameters determining the interaction strength between a protein and the oil–water interface lie in molecular flexibility and surface hydrophobicity (Dickinson, 2010). Again, the interactions between interfacial proteins and components in the aqueous phase can destabilize the emulsion. For instance, increase in ionic strength in aqueous phase exerts masking effect on the charged, adsorbed proteins and thus destabilize emulsion droplets through flocculation (Chen and Dickinson, 1995).

Colloidal particles, usually in the size of 100–2000 nm, can also stabilize emulsions (referred as Pickering emulsions). To stabilize Pickering emulsions, colloidal particles should remain stable in both oil and water phases while having a proper partial



**Figure 2** Emulsion droplets stabilized by colloidal particles via limited coalescence process. Phytochemicals can be loaded either within the colloidal particles or within the internal oil phase.

wettability (Xiao et al., 2016). Due to their large particle size, once migrating onto the emulsion interface, the energy required for detaching colloidal particles from emulsion interface will be several orders of magnitude higher than the kinetic energy for their Brownian motion, thus the attachment of colloidal particles at emulsion interface is regarded as an irreversible process. This is essentially different from the dynamic equilibrium process in cases of small molecular weight surfactant-stabilized and surface active macromolecule-stabilized emulsions. After absorbed onto the interface, colloidal particles stabilize emulsion interface by the physical barrier effect via a “limited coalescence” process (Fig. 2): During emulsification process, a large excess of oil–water interface (compared with the amount that can be covered by the presence of colloidal particles) is produced. As the agitation process is stopped, the partially protected droplets start to coalesce to reduce the surface area of oil–water interface until the interface is sufficiently covered by a rigid layer of colloidal particles. In this way, the colloidal particle layer prevents adjacent emulsion droplets from coalescence.

After the formation of emulsions, their stability and rheological properties are correlated largely to interactions between emulsion droplets, either via repulsive or attractive forces, which in turn depend on the structure and composition of the interfacial layer of emulsion. Emulsions (100 nm–100  $\mu$ m), nanoemulsions (10 nm–100 nm), and microemulsions (2–10 nm), especially in the oil-in-water type of structure, are widely used for the encapsulation and delivery of lipophilic phytochemicals. In case of a Pickering emulsion, phytochemicals can be encapsulated within the internal oil droplets, and the colloidal particle layer also serves as an encapsulation reservoir if phytochemicals are loaded within colloidal particles prior to emulsification. For instance, curcumin was encapsulated within the zein–chitosan particle layer prior to emulsification to achieve an antioxidant Pickering emulsion (Wang et al., 2015). A striking benefit of using emulsions to deliver lipophilic phytochemicals is that when orally digested, lipophilic phytochemicals migrate into micelles formed by free fatty acids after the digestion of the lipid phase, which facilitates the dispersion and subsequent cellular transportation process (Xiao et al., 2015). When fabricated as water-in-oil-in-water emulsions, they can be utilized as a protective carrier of sensitive hydrophilic phytochemicals, dual-delivery vehicle for both oil-soluble and water-soluble phytochemicals (Xiao et al., 2017a; Kaimainen et al., 2015).

## Hydrogels

Hydrogel, a coherent three-dimensional network of at least two components, behaves mechanically like a solid and is capable of retaining a significant amount of water within its structure. Popular building materials for food application-oriented hydrogels include collagen, gelatin, whey protein isolates, starch, alginate, caseinate, pectin, etc. While the water holding property arises from hydrophilic functional groups of the hydrogel matrix, their resistance to dissolution arises from cross-linked polymeric network. The cross-linked junctions can be either permanent or transient depending on their physical or chemical nature. In the former case, hydrogels with tailored properties such as biodegradability desired mechanical strength and response to stimuli can be synthesized through polymerization via interactions between hydrophilic monomers and cross-linkers. In the latter case, cross-linked junctions can arise from polymer chain entanglements; crystallite formation; or chemical and physical interactions such as ionic interactions, hydrogen bonding, or hydrophobic interactions. Particularly, protein-based hydrogels can be formed via a heat- or cold-induced gelation process. Electrostatic interactions, hydrophobic interactions, and disulfide bonding contribute differently in respective aggregation and gelation processes during heat-induced gelation (Alting et al., 2002). Cold-induced gelation takes place via two consecutive steps: protein aggregates are first formed by heating a protein solution and then gelation is induced via the addition of calcium or by lowering the pH.

Hydrogels are usually sensitive to environmental conditions particularly temperature, pH, ionic strength, pressure, and solvent composition. Thus particular advantages of encapsulating phytochemicals within a hydrogel matrix involve the protection effects during storage and the controlled release profiles in response to specific environmental triggers. When encapsulating phytochemicals within a hydrogel matrix, hydrophilic phytochemicals are mixed directly with the polymer solution prior to hydrogel formation and hydrophobic phytochemicals are typically incorporated into emulsified lipid droplets prior to the hydrogel formation.

Hydrogen bonding and steric hindrance effect between phytochemicals and the polymer skeleton of hydrogel contribute to the holding effect. Thus, the release of phytochemicals occurs through mechanisms such as a simple diffusion process, swelling and diffusion process, disintegration and diffusion process, with the chemical nature of phytochemicals (polar or nonpolar), polymer concentration, and gel viscosity affecting the rate of diffusion (Eros et al., 2003). Recent research practices in which beta-carotene and curcumin-enriched lipid droplets were entrapped within hydrogel matrixes have proven the protective effect and controlled release function of the systems (Xiao et al., 2017b; Zhang et al., 2016). In application scenarios where a slow release kinetic is demanded, a chemical cross-linking process following the regular gelation process can be carried out to increase the polymer junction and shrink the pores.

## Inclusion Complexes

Inclusion complexation refers to the entrapment of smaller molecules inside the hollow cavity of a larger molecule. Cyclodextrins (CDs) are the most commonly utilized components for the formation of such cavities. Naturally occurring CDs are a group of cyclic oligosaccharides derived from starch with six ( $\alpha$ -CD), seven ( $\beta$ -CD), eight (commonly  $\gamma$ -CD), ten ( $\epsilon$ -CD), or more glucose residues linked by  $\alpha$ -(1–4) glycosidic bonds in a cylinder-shaped structure (Karoyo and Wilson, 2015). Its central cavity is made hydrophobic by the glycosidic oxygen, while the hydroxyl groups located on the external edges making its outer surfaces hydrophilic. Various methods had been developed for the fabrication of inclusion complexes including kneading, solid dispersion, spray/freezing drying, and coevaporation (Pereva et al., 2016).

A whole range of interactions, including the van der Waals interactions, entropy-driven hydrophobic interactions, hydrogen bonding and electrostatic interactions, have been identified and are vital to sustaining the CDs derived inclusion complexes (Joshi et al., 2016). The same interactions exist between the inclusion complex and guest phytochemicals. The encapsulation efficiency is a combined result of the hydrophobicity of phytochemicals and relative size of the inclusion complex and the guest phytochemicals. After oral digestion, phytochemicals are usually released from the matrix through a Fickian or non-Fickian diffusion process. Benefits of encapsulating phytochemicals within an inclusion complex include improved water solubility, enhanced stability, decreased decomposition rate in GI tract, shortened time lag to reach plasmatic peak, etc. (Singireddy and Subramanian, 2016). Bioavailability enhancement of hydrophobic phytochemicals, such as curcumin, quercetin, myricetin, and capsaicin, has been found when the phytochemicals were encapsulated within an inclusion complex (Marques, 2010; Miskolczy et al., 2016; Mangolim et al., 2014).

## Conclusion

Understanding the dominant interactions among building blocks of EDVs and phytochemicals occurring during the encapsulation and release processes will guide the rational design of EDVs with controlled sizes, surface properties, matrix effects, and compartment structures. EDVs with different structural characteristics, such as polymeric nanoparticles (nanospheres and nanocapsules), protein–polysaccharide complex coacervates, emulsions, CD inclusion complexes, and polymeric hydrogels, can thus be designed to encapsulate various phytochemicals with different loading processes, encapsulation efficiency, release profile after oral ingestion, and application potentials.

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- <http://www.liposomes.org/> – Liposomes.
- <http://nanoparticles.org/> – Nanoparticles.
- <https://phys.org/nanotech-news/> – Nanotech-news.



# Fabrication of Electrospun and Electrosprayed Carriers for the Delivery of Bioactive Food Ingredients<sup>☆</sup>

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## Introduction

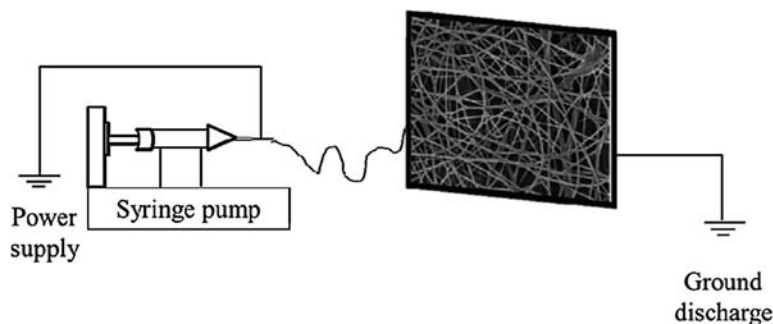
The use of bioactive compounds in foods has been gaining increasing interest over the last years due to the fact that these ingredients can provide health benefits to consumers, beyond nutrition. This category includes vitamins, polyphenols, probiotics, peptides, essential fatty acids, and minerals, among others. However, preserving the health-promoting properties of bioactive compounds along the food supply chain, and also during the transport in the gastrointestinal tract, is still challenging.

Encapsulation technologies constitute an actual path to protect bioactive compounds from adverse environmental conditions (e.g., light, moisture, and oxygen) through the entrapment of core materials within a wall polymer (e.g., polysaccharides and protein-based materials). This strategy allows the improvement of the stability of bioactive compounds, the delivery of compounds at a controlled rate, masking of unwanted odor or taste, and/or improvement of the properties of raw materials (solubility and dispersibility, among others) for easier handling. Different methods have been used to encapsulate bioactive compounds including spray drying, emulsification, coacervation, liposome entrapment, inclusion complexation, nanoprecipitation, and freeze drying, among others (de Vos et al., 2010). However, some of these techniques involve harsh processing conditions (e.g., high temperature and solvents use), which can significantly reduce the functionality of the bioactive compounds (Ghorani and Tucker, 2015). In this context, electrospinning and electrospraying technologies are becoming simple and attractive alternatives to encapsulate sensitive bioactive food ingredients. Nevertheless, the application of electrospinning and electrospraying technologies in the field of food processing engineering remains less explored.

## Principles of Electrospinning and Electrospraying Technologies

Electrospinning and electrospraying are one-step technologies that utilize high electrostatic potentials to draw polymer solutions (or polymer melt) into fibers or particles, respectively. The typical setup for electrospinning and electrospraying is mainly constituted by four components: (1) a high-voltage source (1–50 kV) usually operated in direct current mode (although alternating current mode is also possible), (2) a syringe pump, (3) a spinneret (typically a hypodermic syringe needle), and (4) a grounded collector either a flat plate or a rotating drum (Fig. 1; Bhushani and Anandharamakrishnan, 2014).

In both electrospinning and electrospraying, polymer solutions (or polymer melt) are pumped at a fixed solution flow rate to a capillary spinneret, forming a droplet in the needle tip. When the electrical voltage is applied to the spinneret, the droplet becomes charged and experiences an electrostatic repulsion, forming into a conical geometry known as a 'Taylor cone.' Once the electrical voltage exceeds a critical value at which the repulsion forces overcome the surface tension of the droplet from the tip of the Taylor cone, electrospun fibers are formed if the solution concentration or the molecular chain entanglement in the liquid is high, and the polymer ejects from the Taylor cone and travels toward the collector as a stable jet. On the other hand, if the solution concentration is low, aerosol droplets are produced due to the liquid jet breakup. These highly charged droplets are deposited on the collector as



**Figure 1** Schematic representation of the electrospinning process.

<sup>☆</sup> *Change History:* This is an update of Alex López-Córdoba, Jose Maria Lagarón and Silvia Goyanes, Fabrication of Electrospun and Electrosprayed Carriers for the Delivery of Bioactive Food Ingredients, In Reference Module in Food Science, Elsevier, 2016, ISBN 9780081005965. March 2018 Alex López-Córdoba, Jose Maria Lagarón and Silvia Goyanes updated the text, figures and references.

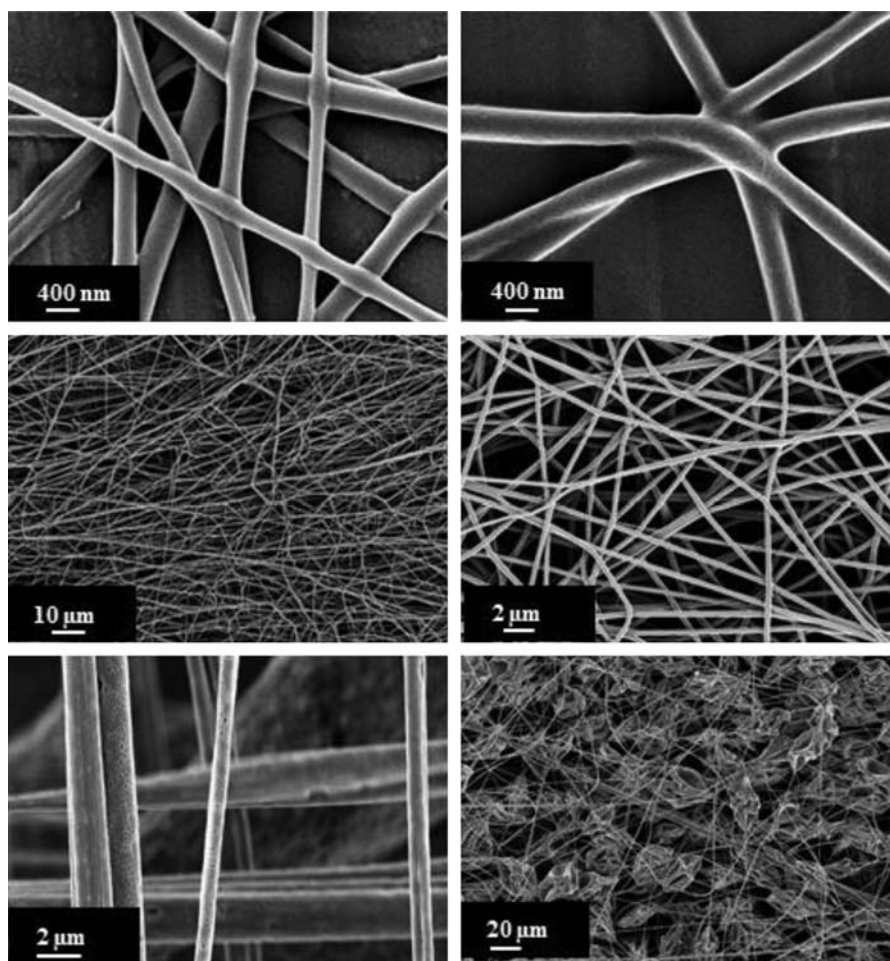
micro- or nanoparticles. One important advantage of electrospinning and electrospraying is that the evaporation of the solvent occurs during the process and no heating is needed, which makes these technologies suitable for dealing with thermally sensitive materials (e.g., probiotic bacteria).

The most relevant factors that affect the fabrication of electrospun fibers or electrosprayed particles are the solution properties (e.g., conductivity, viscosity, and surface tension), the instrumental parameters (e.g., applied electrical field, solution flow rate, and the distance between the tip of the needle and the collector), and the ambient conditions (e.g., temperature and humidity). By changing these parameters, the size and the morphology of the encapsulation structures obtained can be tailored for specific applications (Fig. 2).

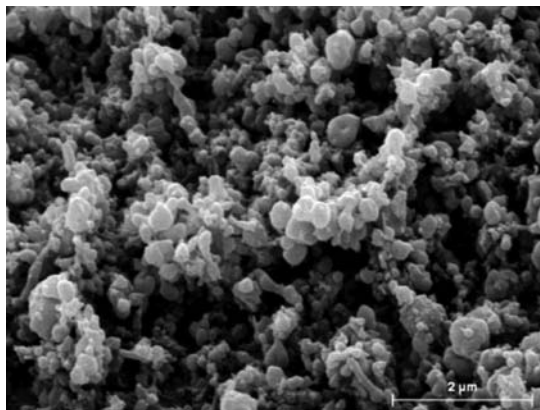
### Food-Grade Materials for Electrospinning and Electrospraying

Polymers from natural sources are the preferred materials for the encapsulation of bioactive food ingredients by electrospinning and electrospraying because these have the advantages of being nontoxic, edible, and biocompatible. Moreover, many of them can be dissolved using GRAS (generally regarded as safe) substances (e.g., water or ethanol), avoiding the use of toxic solvents. Proteins (e.g., whey protein isolate, whey protein concentrate, soy protein isolate, egg albumen, collagen, gelatin, zein, and casein) and polysaccharides (e.g., chitosan, alginates, cellulose and its derivatives, and dextran) have been the wall materials most frequently used for encapsulation of bioactive ingredients by electrospinning and electrospraying.

Pérez-Masiá et al. (2014) studied the ability to form fibers or particles from aqueous solutions of selected hydrocolloids such as dextran (molecular weight (Mw)  $\sim 70\,000$ ), maltodextrin (Mw  $\sim 1300$ ), resistant starch (Mw  $\sim 1700$ – $2700$ ), pullulan (Mw  $\sim 100\,000$ ), fructooligosaccharides (Mw  $\sim 330$ – $8100$ ), whey protein concentrate from milk (Mw  $\sim 20\,000$ – $70\,000$ ), and a soy protein isolate (Mw  $\sim 30\,000$ – $350\,000$ ). Only dextran, pullulan, and whey protein were able to form capsules (sizes about  $1\,\mu\text{m}$ ). This fact was attributed to their higher molecular weight, which led to a significant viscosity increase when increasing the hydrocolloid concentration in the dispersion. As an exception, the soy protein, despite its high molecular weight (Mw  $\sim 30$



**Figure 2** Examples of different morphologies obtained by electrospinning/electrospraying.



**Figure 3** SEM image of capsules obtained from 10% (w/v) soy protein solutions after denaturation. From Pérez-Masiá, R., Lagaron, J., López-Rubio, A., 2014. Development and optimization of novel encapsulation structures of interest in functional foods through electrospraying. *Food Bioprocess Technol.* 7(11), 3236–3245, reprinted with permission.

000–350 000), did not lead to the capsule formation, probably due to the presence of strong inter- and intramolecular forces, which impeded chain entanglements in its globular structure. In this case, the electrosprayability of the solutions was improved by the denaturation of the protein through a thermal treatment (Fig. 3). It was stated that denaturation leads to protein unfolding and exposure of the functional groups, which could improve intermolecular interactions, both between the different protein chains and with the solvent, resulting in increased viscosity (Pérez-Masiá et al., 2014). On the other hand, the effect of the addition of gums (guar gum or xanthan gum) and/or surfactant (Span 20) was also analyzed as a strategy to improve the electrosprayability of the hydrocolloids (Pérez-Masiá et al., 2014). This approach led to important changes in the solution properties (conductivity, viscosity, and surface tension) allowing to obtain capsules from all wall materials.

In several studies, the use of synthetic polymers, such as polyvinyl alcohol (PVOH) and polyethylene oxide, as spinning aids has been also reported (Sousa et al., 2015; Wongsasulak et al., 2014).

Compatibility between active compounds and polymer carriers is a key factor to consider when choosing materials for encapsulation by electrospinning or electrospraying. Active compound loading capacity is determined mainly by several driving forces such as hydrophobic interaction, electrostatic interaction, hydrogen bonding,  $\pi$ - $\pi$  stacking and van der Waals force (Li and Yang, 2015). Other factors affecting active compound loading capacity are loading space, preparation methods, crystallinity and glass transition temperature (T<sub>g</sub>) of carrier materials (Li and Yang, 2015).

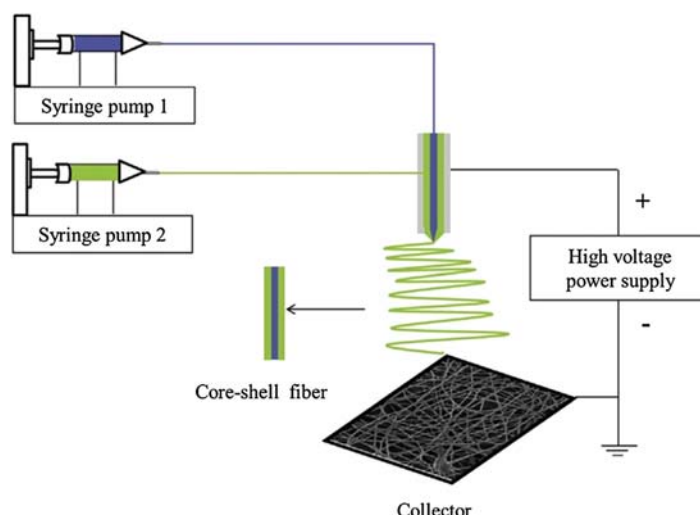
Variations in composition or chemical modifications such as cross-linking or the addition of functional groups into loading sites have been used as strategies in order to generate specific attractive or repulsive active compound-carrier interactions, thus controlling the retention and release of bioactive compounds (Deng et al., 2017; McClements, 2018). If there is a strong attraction between the bioactive compounds and polymers, then the bioactive will tend to be retained inside the carrier, but if there is no attraction or a repulsion then the bioactive compounds will tend to be released (McClements, 2018). Also, the sign or magnitude of the interactions between bioactive ingredient and carrier materials can be altered by changing environmental conditions (such as pH, ionic strength or temperature), which can be used to develop triggered release mechanisms (McClements, 2018).

## Encapsulation of Functional Food Ingredients by Electrospinning and Electrospraying Technologies

Different strategies are available for encapsulation purposes using electrospinning and electrospraying. The direct dissolution of the active components in the polymeric carrier is the most common encapsulation approach. Using this path, the bioactive component is randomly distributed throughout the fibers or the particles. Another strategy is the fabrication of core-sheath structures using a couple of capillary tubes where a smaller one (inner) is inserted concentrically inside the bigger (outer) capillary (Fig. 4). The active component is fed through the inner capillary spinneret, while the polymer solution is extruded through the outer capillary spinneret simultaneously in order to acquire core-sheath structures (Yao et al., 2016). This approach is highly versatile because the core fluid does not need to be electrospinnable.

Core-sheath structures can also be fabricated by emulsion electrospinning using a single feeding capillary. In this approach, the immiscible solutions are mixed into an emulsion and then electrospun or electrosprayed. In order to avoid phase separation, vigorous mixing of the polymer and core materials and the incorporation of an emulsifying agent are almost always necessary. This technique shows high encapsulation efficiency of immiscible drugs as well as their sustained release. Some examples of recent studies using electrospinning or electrospraying to encapsulate bioactive compounds of interest in the food area are out-lined below.

Antioxidants are considered attractive ingredients to encapsulate because these compounds are frequently used in the food industry in order to prevent the spoilage of oxidation-sensitive foods (e.g., baked goods, biscuits, mayonnaise, meat products,

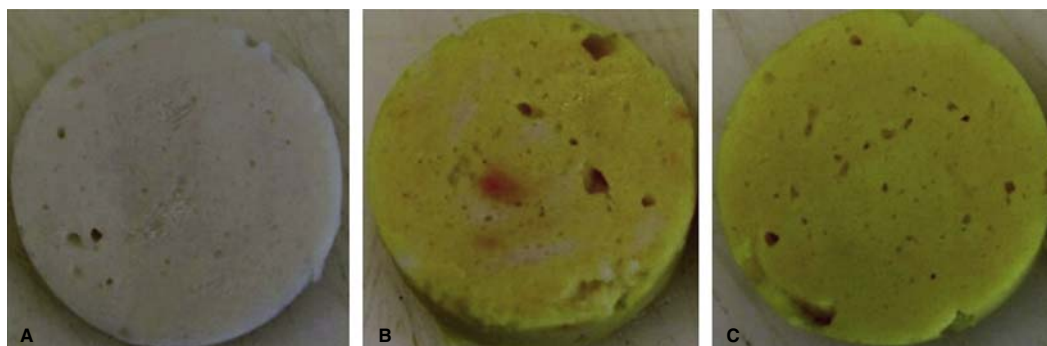


**Figure 4** Schematic representation of coaxial electrospinning device setup.

nuts, oils, and fats). The use of antioxidants from natural sources is preferred due to the association of synthetic additives with the generation of adverse health effects. Several studies have been carried out dealing with the fabrication of electrospun fibers and electrospayed particles containing natural antioxidants. For instance, the entrapment of gallic acid within zein electrospun fibers was first reported by [Neo et al. \(2013\)](#). In this work, electrospinning proved to be an efficient tool for one-step preparation of gallic acid-loaded zein electrospun fibers, yielding a loading efficiency of almost 100%. The active fibers were examined using Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy method, finding interactions between gallic acid and zein at the molecular level which promoted the compatibility between the active compound and the polymer carrier. Despite such interaction, gallic acid has retained its antioxidant activity.

Curcumin, besides being a commonly used natural food dye, exhibits numerous biological activities, including antioxidant, anti-inflammatory, antiviral, antibacterial, and antifungal properties. However, the use of curcumin in foods is limited due to its low water solubility and photostability. Recently, [Gómez-Estaca et al. \(2015\)](#) reported the fabrication of electrospayed gelatin microspheres containing curcumin. The encapsulation process allows increasing the antioxidant activity, the water solubility, and the bioavailability of curcumin. Moreover, encapsulated curcumin was better dispersed into a jellified fish product than commercial curcumin (i.e., a nonencapsulated ingredient) ([Fig. 5](#)). Recently, [Deng et al.](#) evaluated the effect of different surfactants (non-ionic Tween 80, anionic sodium dodecyl sulfonate (SDS) and cationic cetyltrimethyl ammonium bromide (CTAB)) on the release behavior of curcumin from gelatin electrospun nanofibers ([Deng et al., 2017](#)). Tween 80 and CTAB greatly improve the release of curcumin into polar solvents, resulting in a higher radical scavenging activity and a stronger antimicrobial activity, while the addition of SDS provoked chemical interactions which do not favor the release of curcumin. Fourier transform infrared spectroscopy and differential scanning calorimetry analysis suggested that gelatin and SDS intimately interacted via electrostatic and hydrophobic interactions.

$\alpha$ -tocopherol is a form of vitamin E that is well known for its ability to act as a hydroperoxyl radical scavenger, therefore playing an important role in protecting the organism against oxidative damage. [Wongsasulak et al. \(2014\)](#) reported the fabrication of



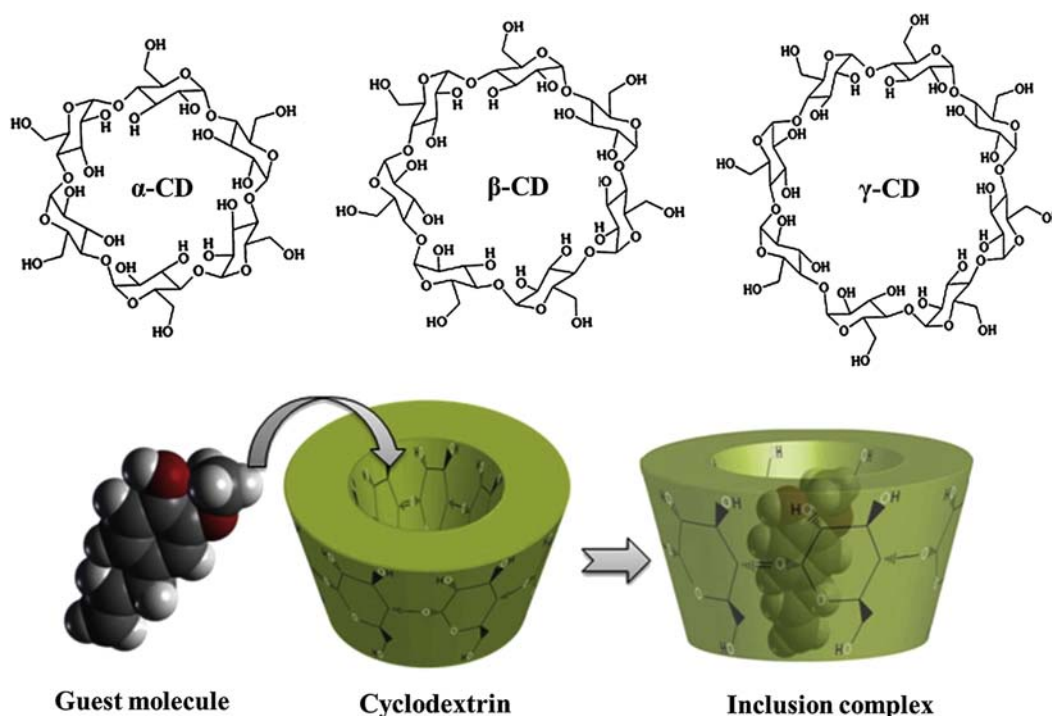
**Figure 5** Photographic images of control fish gel (A), fish gel supplemented with commercial curcumin (B), and fish gel supplemented with 10% curcumin-loaded gelatin microparticles obtained by electrospaying (C). Curcumin content for B and C gels was 0.05 g/100 g of fish mince. From [Gómez-Estaca, J., Gavara, R., Hernández-Muñoz, P., 2015. Encapsulation of curcumin in electrospayed gelatin microspheres enhances its bio-accessibility and widens its uses in food applications. \*Innov. Food Sci. Emerg. Technol.\* 29, 302–307, reprinted with permission.](#)



composite electrospun fibers based on zein/poly(- ethylene oxide)/chitosan blends embedding  $\alpha$ -tocopherol (a hydrophobic bioactive compound). A non-polar surfactant, Tween 40, was added to facilitate manipulation of the composite and the gastro-mucoadhesivity was tested using porcine gastric mucosa. The presence of  $\alpha$ -tocopherol significantly enhanced the mucoadhesivity of the nanofibers, thereby aiding in improved bioaccessibility and bioavailability. Mucoadhesion is considered a highly effective delivery method to conserve the functional properties of a bioactive compound delivered to the gastrointestinal tract. In this process, the attachment first arises from molecular chain diffusion/interpenetration between the polymeric molecules of the mucoadhesive material and mucin glycoprotein (solid main component of the mucosa), followed by molecular bonding (e.g., ionic interaction, covalent bonds, hydrogen bonding, dipole-dipole interactions, as well as hydrophobic interaction) between the mucoadhesive material and the mucin glycoprotein (Wongsasulak et al., 2014).

Lycopene, a naturally occurring red carotenoid pigment found in tomatoes and other fruits, is another antioxidant which is well-known because of its potential to reduce the risk of chronic diseases such as cancer and coronary heart disease. However, lycopene presents several unsaturated bonds in its molecular structure, which makes it very susceptible to oxidants, light and heat. Moreover, it has a very low water solubility, which limits its industrial applicability in aqueous-based systems. Pérez-Masiá et al., 2015 addressed these limitations through the encapsulation of lycopene by electrospaying assaying dextran, a whey protein concentrate (WPC), and chitosan as wall materials (Pérez-Masiá et al., 2015). Since lycopene is a poorly water-soluble compound and WPC and dextran are water dispersible polymers, emulsion and coaxial electrospaying were used in these cases. For chitosan, an acetic acid solution was used, where lycopene could be easily dissolved and, thus, uniaxial electrospaying was carried out. The antioxidant-containing electrospayed capsules were compared with those obtained through the traditional spray drying process. In overall, electrospaying process allow to obtain higher encapsulation efficiencies than the spray drying method, mainly when emulsion electrospaying was carried out and WPC was used as wall material (encapsulation efficiency  $\sim 75\%$ ). This behavior was attributed to the high temperature involved in the spray drying process which led to a partial degradation of the antioxidant. When chitosan was used as a matrix to encapsulate lycopene through both techniques (electrospaying and spray drying), the acid conditions needed in this case in order to dissolve the polymer affected lycopene stability and very poor encapsulation efficiencies were found ( $\sim 2\%$ ).

Acetuno-Medina et al. (2015b) studied the encapsulation of two bioactive compounds, quercetin and ferulic acid, within amaranth protein isolate/pullulan electrospun fibers. In general, quercetin was released in a most sustained manner during in vitro digestion than ferulic acid. At the end of the in-vitro intestinal digestion stage, the amount of quercetin and ferulic acid released corresponded to 82% and 99%, respectively. This behavior was attributed to the composition of the polymeric matrix (i.e., the ratio between amaranth protein isolate and pullulan) and to the interactions of the bioactives themselves with the encapsulating matrices.



**Figure 6** Chemical structures of cyclodextrins ( $\alpha$ -CD,  $\beta$ -CD and  $\gamma$ -CD) and schematic representation of formation of inclusion complexes (B). Adapted from Kayaci, F., Ertas, Y., Uyar, T., 2013. Enhanced Thermal Stability of Eugenol by Cyclodextrin Inclusion Complex Encapsulated in Electrospun Polymeric Nanofibers. J. Agric. Food Chem. 61(34), 8156–8165, reprinted with permission.

Folic acid is a water-soluble vitamin that is vital for a variety of physiological functions in humans. However, this bioactive compound undergoes degradation reactions when it is exposed to light, temperature, moisture, acid or alkaline medium, and oxygen atmosphere. In order to overcome these disadvantages, the entrapment of folic acid into electrospun nanofibers and electro-sprayed particles has been suggested as viable alternative. Aceituno-Medina et al. (2015a) reported the fabrication of folic acid-loaded protein isolate/pullulan electrospun fibers with high encapsulation efficiency (>95%). The encapsulation strategy led to enhanced thermal stability and photostability of folic acid. In another study, the encapsulation of folic acid in both a whey protein concentrate matrix and a commercial resistant starch was also carried out by electro-spraying, finding that the folic acid stability was also improved (Perez-Masia et al., 2015).

Probiotics are widely used bioactive food ingredients due to their health-related benefits. However, the survival of probiotic bacteria into food formulations can be affected by several factors (e.g., pH and temperature). López-Rubio et al. (2012) studied the feasibility of using the electro-spraying technique to encapsulate bifidobacterium strains in whey protein concentrate and pullulan.

Whey protein concentrate provided higher protection for the probiotic bacteria than pullulan, extending the survival of the cells even at high relative humidity.

The formation of inclusion complexes of cyclodextrins with bioactive compounds has been proposed as a strategy to enable the encapsulation of bioactive compounds within electrospun or electro-sprayed nanostructures. Cyclodextrins ( $\alpha$ -CD,  $\beta$ -CD, and  $\gamma$ -CD) possess hydrophobic cavities of different sizes, which render them capable of forming inclusion complexes with a range of hydrophobic compounds (Fig. 6). This means that cyclodextrins can be used as formulation aids to increase the aqueous solubility and enhance the stability of guest molecules. For example, Kayaci et al. (2013) reported the fabrication of PVA nanofibers containing inclusion complexes of cyclodextrins ( $\alpha$ -CD,  $\beta$ -CD, and  $\gamma$ -CD) with geraniol (a natural component of plant essential oils that presents several bioactive properties such as antioxidant, anti-inflammatory, and anticancer properties). Higher complexation efficiency was obtained when  $\gamma$ -CD was used, and, therefore, these complexes were chosen to be incorporated within PVOH nanofibers by electrospinning. This strategy allows obtaining a higher thermal stability and a prolonged durability of geraniol.

## Conclusions

Electrospinning and electro-spraying technologies have proven to be an excellent method for the encapsulation of bioactive food ingredients. These processes have the advantages of being simple and easily adaptable. Moreover, through the control of the solution properties and the optimization of the processing variables, a huge variety of micro- or nanocarriers with unique properties may be engineered. The large-scale production and successful commercialization of electrospun fibers or electro-spraying particles are still in their infancy, despite that industrial equipment of electrospinning and electro-spraying is available. Therefore, close linkages between the scientific community and the industrial sector are highly necessary in order to identify and commercialize more application for these technologies in micro- and nanoencapsulation of bioactive food ingredients.

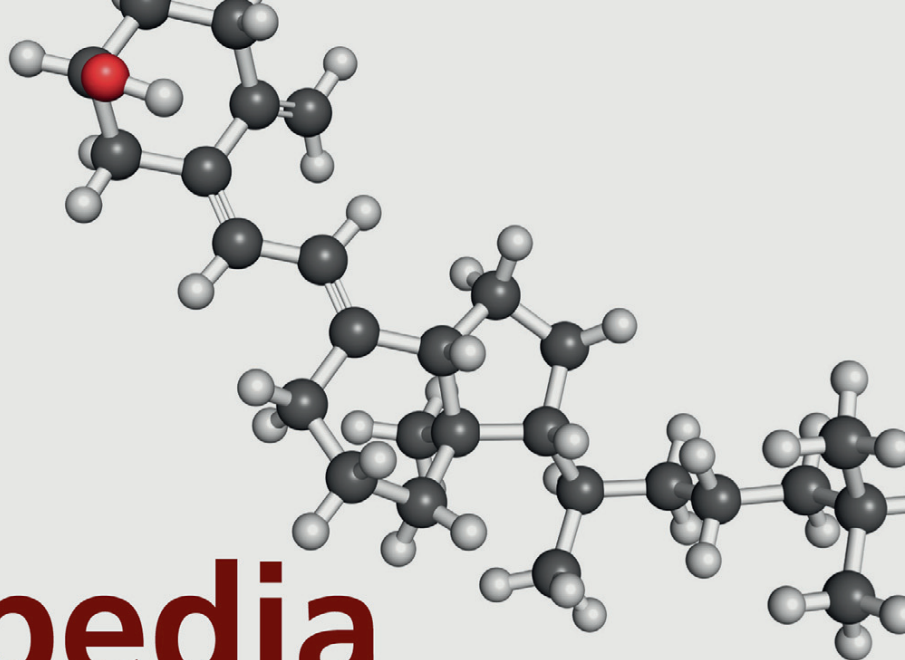
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Volume 3

# Encyclopedia of Food Chemistry

Editors

Peter Varelis, Laurence Melton and Fereidoon Shahidi



# ENCYCLOPEDIA OF FOOD CHEMISTRY

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# ENCYCLOPEDIA OF FOOD CHEMISTRY

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VOLUME 3





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## PREFACE

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This is an exciting time for all who wish to understand the nature of food and how it changes during storage, processing, cooking and digestion. This is because science has turned its focus onto soft matter and food is very largely soft matter. Earlier scientific effort was preoccupied with simple molecules in solution and solids (preferably pure crystalline proteins). When you think about it, knowing the total protein, carbohydrate, lipid and vitamin contents of a food doesn't tell you much about the nature of the food and how it will provide nutrients for us. Now we are seeing a huge effort to understand soft matter, which is directly applicable to food. Meaning we are increasing our knowledge of how food components (both macromolecules such as proteins and polysaccharides as well as smaller molecules) interact chemically with each other; leading ultimately to understanding the complex chemical structure of foods (everything from apples and ice cream to vegetable protein meat-substitutes and food for a visit to Mars).

A golden age for food research has begun, with so many new techniques available such as CRISPR, isothermal calorimetry (ITC), analytical ultracentrifugation (AUC), small angle neutron and X-ray scattering (SANS, SAXS), surface plasmon resonance, electronic tweezers, and computer modelling (for example molecular dynamics simulations for understanding the interactions of proteins and polysaccharides, enzymes and substrates, bioactives and encapsulating agents). This is in addition to the tried and tested techniques of nuclear magnetic resonance (NMR), mass spectroscopy, electron and confocal microscopy, high performance liquid chromatography (HPLC), and gas chromatography. Biosensors publications are huge and nanotechnology is a hot topic for research.

The *Encyclopedia of Food Chemistry* is for people who have a basic knowledge of food chemistry and wish to expand their understanding of a topic based on information from a reliable source. No fake news here! We welcome you to enjoy the excitement captured here by experts from across the planet.

Laurence D. Melton

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Figure 4. Formation of Selected Heterocyclic Flavor Chemicals in Beverages.

Figure 1. Interactions between polyphenols and macromolecules: effect of tannin structure.

Figure 2. Interactions between polyphenols and macromolecules: effect of tannin structure.

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Table 1. Next-generation sequencing.

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# Fruit and Vegetable Texture: Role of Their Cell Walls

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## Overview

Appearance, flavour, texture and nutritional value are the four main quality factors in foods. The first three are referred as 'sensory acceptability factors' because they are perceived directly by the senses. In fruit and vegetables, the latter defined as any edible part of plants consumed for food that is not a mature fruit or seed (e.g. leaves, roots, stems, bulbs, tubers ...) and mature fruit that are consumed as part of a main meal rather than dessert (e.g. tomato, pepper) (Radovich, 2011), texture has a significant contribution to the overall quality, and it is one of the main attribute for the acceptance in the market from the consumer's point of view (Contador et al., 2015). Texture also determines the acceptability of some part of fruit or vegetable and the way they are usually eaten, e.g. the skin of some fruit or vegetables is eaten with the fleshy portion when it is thin, as in the strawberry, but it is not eaten when it is thick, hard, hairy or fibrous, as in mango or peach. The definition of 'texture' is difficult since it includes a wide range of attributes which determine the feel of food within the mouth and the way these attributes can be measured. For this reason, it is better to talk about 'textural properties', a group of related properties, rather than 'texture', a single parameter. Bourne (2002) defined the textural properties of a food as '*that group of physical characteristics that arise from the structural elements of the food, are sensed primarily by the feeling of touch, are related to the deformation, disintegration, and flow of the food under a force, and are measured objectively by function of mass, time, and distance*'. Among the different textural properties that can be perceived or measured in a food, firmness and juiciness are the most important parameters in the case of fruit or vegetables (Toivonen and Brummell, 2008). Firmness and juiciness are complex traits depending upon physical anatomy of the tissue (cell shape and size), cell wall thickness and strength, the way in which cells bind to form the flesh tissue (extension and strength of adhesion areas between adjacent cells) and cell turgor. Fruit and vegetables are mainly comprised by parenchyma cells, thin-walled cells with metabolic activities such as photosynthesis of storage sites of starch or other compounds. The presence of non-parenchyma cells (epidermal cells, vascular elements, fibers and sclereids) influences texture since most of these cells contain thickened walls (Harker et al., 1997a; Toivonen and Brummell, 2008; Mercado et al., 2011). Vegetable tissues generally have a greater proportion of cells with thickened and lignified cell walls, being usually harder than ripe fruit. Also, fleshy fruit and vegetables with thick highly impermeable cuticle, reduced water loss and whole turgor could contribute significantly to fruit firmness (Gómez-Galindo et al., 2004; Saladié et al., 2007).

Fleshy fruit undergoes a ripening process that results in the modification of textural properties, acquiring firstly the desired texture for eating but later an undesirable texture that leads to economically important postharvest losses. Physiologically, fruit are classified as climacteric or non-climacteric, depending on the role of ethylene in the promotion of fruit ripening. From a textural point of view, fruit can be classified in those that soften greatly as they ripen (e.g. peach, apricot, melon, plum, tomato, strawberry), and those that soften moderately as they ripen (e.g. apple, quince, nashi pear, cranberry, watermelon) (Bourne, 1979). Fruit from the first category tend to have a short postharvest life, acquiring a melting texture as they ripen, and show a poor correlation between firmness at harvest and after storage. By contrast, fruit belonging to the second category generally have a long storage life, a crisp, fracturable texture, and show a close relationship between firmness at harvest and after storage. The climacteric or non-climacteric condition does not correlate with the textural properties, e.g. some climacteric fruit are soft and melting (peach) while others are crisp (apple); similarly, some non-climacteric fruit are soft (strawberry) while others display a crisp texture (watermelon). During ripening, cell walls are extensively modified, changing their mechanical properties, and inter cellular adhesion is reduced due to the dissolution of the middle lamella (Brummell, 2006; Mercado et al., 2011). These modifications in conjunction with the reduction of turgor pressure lead to fruit softening. Juiciness is determined by cellular turgor and the pattern of tissue breakage during biting and chewing. Juicy texture is associated with large cells which break open releasing intracellular content; fruit with a dry texture tend to have small cells that separate along the middle lamella with little cell rupture (Harker et al., 1997b). Ultimately, firmness and juiciness depend on cell wall strength and inter cellular adhesion. Thus, when intercellular adhesion areas are stronger than cell walls, cells break and liberate their content making crunchy and juicy textures, while when the cell walls are stronger than middle lamella, the weak intercellular adhesion produces soft-melting textures, attributing to the main mode of tissue failure, either cell breakage or cell separation, a central role on the perception of fruit and vegetable texture (Christiaens et al., 2016). The postharvest physiology of vegetables varies widely because of the broad range of tissue types. Vegetables not derived from fruits do not suffer a ripening process; the loss of textural quality in these vegetables is associated to cell wall modifications, that generally occur to a lesser extent than in fruit, senescence, specially in leafy vegetables, and reduced turgor due to transpirational water loss and leakage of osmotic solutes (Brummell and Toivonen, 2018; Gómez-Galindo et al., 2004; Goulao et al., 2010; Ranganathan et al., 2016).

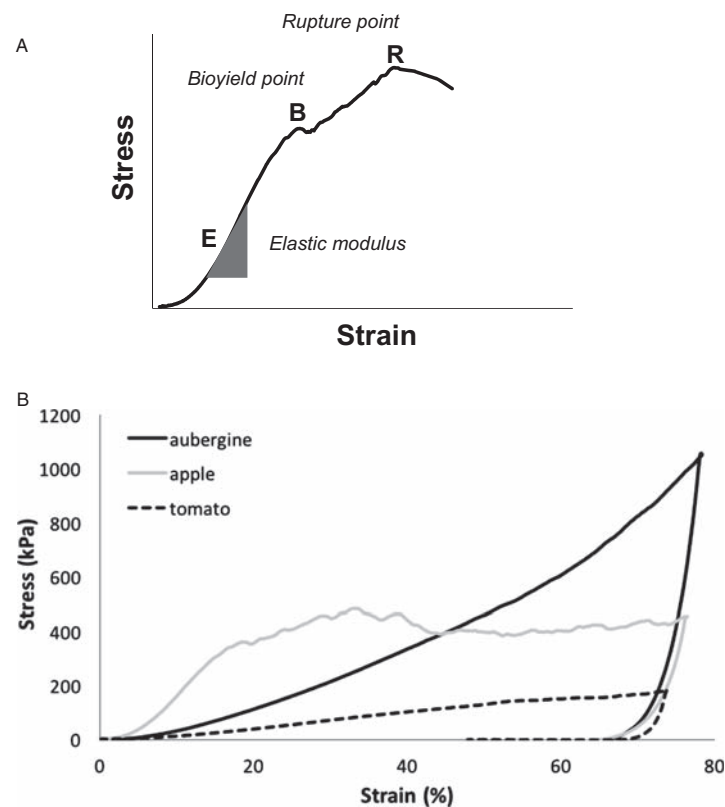
## Mechanical Assessment of Fruit and Vegetable Texture

Firmness has been mostly assessed by both, puncture testing machines or penetrometers, widely used in food industry, and universal testing machines (UTM) like Instron or Texture Analyser, preferred in food science (Abbott, 2004). Penetrometers offers a quick and handy assessment of firmness based on a unique output parameter defined by the maximum force to disrupt the tissue

(herein named as hardness). In contrast, UTM are time-consuming but provides an output of precise measurements of stress, strain, distance and time. Here, stress ( $\sigma$ ) is defined as force per unit area, and strain ( $\epsilon$ ) is dimensionless and defined by dividing the difference between the final and initial deformations by the initial deformation (Niklas, 1992). Thus, in the simplest and widely used uniaxial compression test (compared to a whole hand squeeze) a typical stress–strain curve (Fig. 1A) includes relevant texture parameters: elastic zone or linear limit at the initial phase, where the sample recovers its original shape once stress ceased; elastic modulus (E) defined by the slope of the curve in the elastic zone; bioyield point (B), shortly after the end of the elastic zone sometimes there is a first noticeable decrease in slope which indicates the start of cell disruption at local level; and rupture point (R), as the first prominent positive peak with a substantial decrease in force as deformation increases, that marks a major tissue failure beyond which the force can behave either increasing again, level off or decreasing (Abbott, 2004; Contador et al., 2015). Fig. 1B depicts several possible scenarios of different agronomic samples. Overall, the hardness obtained by hand penetrometers is a good enough approach for agronomical purposes in the field, but UTM are advisable when a deeper texture analysis is required or when several types of fruit textures are compared (Cornuault et al., 2018). Juiciness is often measured according to the amount of juice released from a cut tissue or during tissue homogenization (Harker et al., 1997b). Apart from the disruptive mechanical assessments described here, other texture analysis includes the sensory analysis by panelists as well as non-destructive approaches (eg. acoustic-based, NMR, spectroscopy-based) (Chen and Opara, 2013), which are considered emerging techniques for its potential in high throughput analysis of crop texture at industrial level.

### Cell Wall Composition and Structure

Primary cell walls are composed of polysaccharides, a small proportion of glycoproteins and, in some specialized cells, noncarbohydrate substances such as lignin, suberin or cutin. Primary cell wall comprises a load-bearing framework of cellulose together with other polysaccharides and glycoproteins embedded in a more soluble matrix of pectic polysaccharides (Carpita and Gibeault, 1993). Type I walls, those present in most plants contain about 30% each of cellulose, hemicelluloses and pectins, and 1%–10% of structural glycoproteins. The amount of pectins can be much higher in fleshy fruit, accounting up to 60% of cell wall mass (Redgwell et al., 1997a; Prasanna et al., 2007). Type II walls, characteristics of some monocots contain lower amount of pectins and structural proteins. Additionally, middle lamella is the first layer deposited after cell division, so it is located at the interface where the walls of



**Figure 1** (A) Typical stress–strain diagram from a uniaxial compression test. (B) Stress–strain curves from texture analysis by compression test at maximum strain of 70% in three fruit with contrasting texture. E: elastic modulus defined as the slope in the elastic zone; B: bioyield point; R: rupture point.



neighbouring cells come into contact. This thin layer, enriched in pectins, functions as a flexible adhesive layer between cells (Willats et al., 2001).

Cellulose is the main scaffolding of plant cell walls. It is composed by linear chains of  $\beta$ -(1–4) linked D-glucose (Glc) that associate together by hydrogen bonds to form microfibrils. Hemicelluloses are formed by (1–4)- $\beta$ -linked backbones of Glc, xylose (Xyl) or mannose (Man), decorated with neutral or slightly acid sugar side chains. Xyloglucan (XG), linear chains of (1–4)- $\beta$ -D-glucose with numerous Xyl units linked at regular sites to the O-6 position of Glc units, is the most abundant hemicellulosic component in Type I walls. Xyloglucans adhere to cellulose microfibrils by hydrogen bonds and are long enough to span the 20–40 nm distance between adjacent microfibrils, linking them together forming a network (Park and Cosgrove, 2012). Glucuronoarabinoxylans (GAXs) is the main cross-linking glycan in Type II walls.

Pectins are complex polymers largely composed of GalA (Yapo, 2011a). These carbohydrates are classified in three main domains: homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII). HG, the most abundant pectin domain, is formed by a linear backbone of  $\alpha$ -(1–4) GalA units. Xylogalacturonan (XGA) is a HG substituted with xylose residues which is also abundant in storage tissues of reproductive organs. RGI are highly ramified pectins formed by a backbone of the disaccharide (1–2)- $\alpha$ -L-rhamnose-(1–4)- $\alpha$ -D-galacturonic acid, with 20%–80% of rhamnose residues substituted with arabinan, galactan or arabinogalactan side chains (Yapo, 2011b). Finally, RGII is a branched polymer composed of a 9 GalA residues backbone substituted with four heteropolymeric side chains containing more than 20 uncommon sugar residues. The glycosyl sequence of RGII is highly conserved in all vascular plants (O'Neill et al., 2004). RGII in the cell wall are usually cross-linked by a borate diester (O'Neill et al., 2004). How the different pectin domains are interconnected is poorly understood. The classical model describes pectin as a HG linear backbone (smooth regions), interspersed with rhamnosyl residues and alternated with branched RGI regions (hairy regions) (Voragen et al., 2009). The analysis of pectins at nanoscale level by atomic force microscopy has revealed novel characteristics such as HG branches attached to HG backbone that challenge current model of pectin structural organization (Posé et al., 2018; Wang et al., 2018). HG chains can be cross-linked by calcium (Carpita and Gibeau, 1993). Pectins, XG and cellulose microfibrils can also be covalently linked (Zykwinska et al., 2008; Wang et al., 2015). As regard as the localization of these pectin domains, demethylated HG is concentrated in the tricellular junction zones between cells and in the middle lamella; RGI would be located in the cell wall, where they might contribute to interlink pectin and cellulose microfibrils; RGII seems to be widespread in the wall except in the middle lamella (Vincken et al., 2003; Posé et al., 2018; Wang et al., 2018).

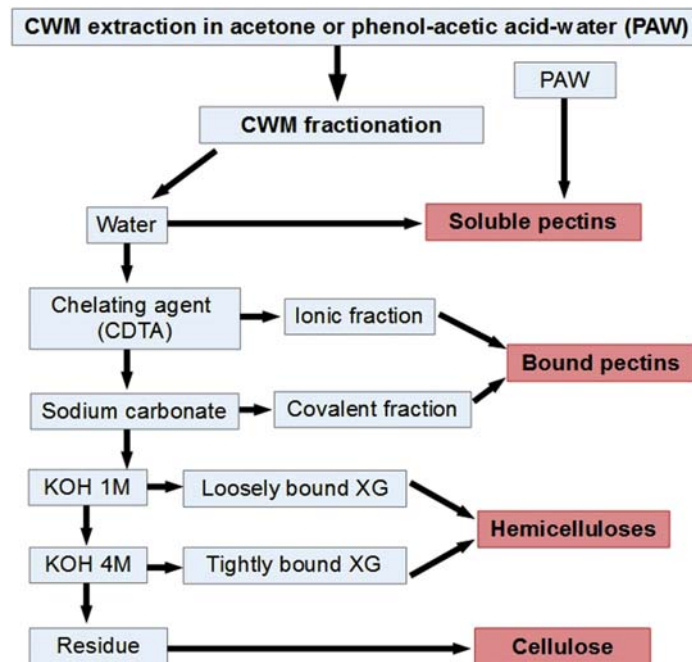
The most accepted hypothesis about the structure of primary walls shows it as a fundamental framework of cellulose-hemicellulose embedded in a pectin matrix (Doblin et al., 2010). XG might facilitate a close contact between cellulose microfibrils in certain regions, called “hot spots”. It has been suggested that pectins also play a key role in wall biomechanics (Park and Cosgrove, 2012). Supporting this role, functional analyses of genes encoding pectin degrading enzymes in fruit with contrasting texture, such as strawberry, tomato or apple, have found that the suppression of their expression increases fruit firmness and extends shelf life, as it will be discussed later.

## Cell Wall Analysis

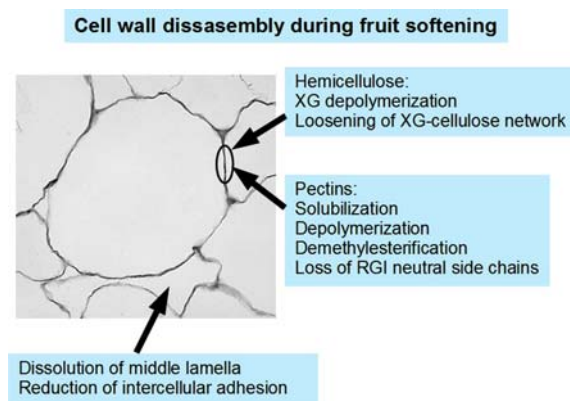
A brief description of general procedures for cell wall extraction and analysis is necessary to understand the role of its different components in fruit texture. *In situ* studies of the cell wall can be performed by different analytical techniques, e.g. immunolocalization of cell wall components, solid state NMR and AFM (Vicente et al., 2007). However, most studies use a destructive procedure based in the isolation and purification of the different cell wall components by solubilisation using a series of extractants (Fig. 2). This approach involves the irreversible breakage of bonds among the different wall components and therefore important information about their *in situ* localization is lost. This could be important in ripening fruit since this process is not uniform over the whole fruit tissues. For cell wall extraction, different solvents, such as phenol based solutions, are used to inactivate cell wall hydrolases from plant homogenates (Huber, 1991; Fry, 2000). Then, the residue enriched in cell wall material (CWM) is treated with dimethylsulphoside or digested with amylase to remove starch. CWM is sequentially extracted by solutions to produce fractions enriched in a specific wall component (Fig. 2). Despite the limitations of this chemical extraction above commented, the cell wall fractions obtained reflect to some extent particular polymer moieties within the cell wall. First fractions (phenol and water) extract polysaccharides that are readily soluble or poorly bound to the matrix; chelating agents extract pectins rich in HG from middle lamella; sodium carbonate extracts pectins loosely bound to the wall and enriched in neutral sugars; finally, KOH solutions solubilise xyloglucans loosely and tightly bound to the wall. The insoluble residue mainly contains cellulose.

## Fruit and Vegetable Softening and Cell Wall Modifications

Textural changes in fruit during ripening and postharvest storage are mainly associated with the dissolution of the middle lamella and the disassembly of the primary cell wall (Brummell, 2006; Goulao and Oliveira, 2008; Mercado et al., 2011) (Fig. 3). Although the temporal sequence of cell wall disassembly events and the degree of these changes is fruit-specific and even cultivar-specific, some modifications are common to most species, such as the depolymerization of hemicelluloses, the solubilization and depolymerization of pectins and the loss of neutral sugars from pectin side chains (Brummell, 2006). These changes in cell wall are mediated by the action of a broad variety of cell wall degrading enzymes or proteins secreted from the symplast into the cell wall space,



**Figure 2** Schematic diagram of the general procedure for cell wall extraction and fractionation into the different cell wall components. CWM: cell wall material; PAW: phenol-acetic acid-water; XG: xyloglucans.



**Figure 3** Main processes taking place in the cell wall during fruit softening.

generally encoded by ripening-related genes (Goulao and Oliveira, 2008; Goulao et al., 2010; Mercado et al., 2011). Additionally, some evidence indicates that pectins can be degraded spontaneously by hydroxyl radicals generated as the fruit ripens (Airianah et al., 2016). In general, cellulose crystallinity is not affected during fruit softening (Newman and Redgwell, 2002).

XG depolymerization during ripening is common to many fruit (e.g. tomato, avocado, melon, kiwifruit, peach); however, others fruit soften without apparent changes in XG molecular weight (e.g. apple, raspberry) or show a cultivar dependent response (e.g. strawberry) (Brummell, 2006). Besides the reduction of hemicellulose size, the loss of the structural integrity of the cellulose-XG network has also been suggested as a primary cause of softening. Two different proteins participate in this process, expansins and xyloglucan endotransglycosylase/hydrolase (XTH). Expansins disrupt non-covalent interactions between hemicelluloses and cellulose microfibrils promoting cell wall loosening and extension (Cosgrove, 2000; Choi et al., 2006). The XTH genes encode proteins that can potentially have XG endo-transglycosidase activity, non-hydrolytic cleavage and ligation of XG chains, and endo-hydrolase activity, yielding irreversible chain degradation (Eklöf and Brumer, 2010). Expansins and XTH are encoded by large gene families including specific members involved either in the maintenance of the cell wall during fruit growth or in the cell wall disassembly during fruit ripening.

Pectins are the cell wall polysaccharides subjected to most dramatic changes during fruit ripening. These modifications involve an increase in their solubilization, depolymerization and degree of demethylesterification, and a loss of neutral sugars (Paniagua et al., 2014). The disentanglement of the pectin network may also facilitate the swelling of the cell wall due to the penetration of

water into enlarged intermicrofibrillar spaces (Redgwell et al., 1997a). This process would allow increased mobility of wall enzymes within cell wall matrix and increased accessibility of hydrolytic enzymes to their substrates. Pectin solubilization refers to an increase in the content of pectins loosely bound to the cell wall, those easily extracted with water, and reflects a modification of their extractability due to changes in their structure and/or chemical composition. Usually, the higher amount of soluble pectins is paralleled to lower contents of ionically and/or covalently bound pectins, suggesting that these bound fractions are the source of soluble pectins (Brummell, 2006). Pectin solubilization is directly related with the texture of ripe fruit, i.e. fruit with a soft and melting texture (e.g. avocado, kiwifruit, blackberry, tomato, persimmon, melon, peach or strawberry) show a high to moderate solubilization, while those with a crisp texture (e.g. apple, watermelon or nashi pear) display a low or undetectable solubilization (Mercado et al., 2011). In apple, it has been found that sensory evaluation of juiciness is positively correlated with the GalA content in water soluble pectins while sensory crunchiness and firmness correlate negatively with this component (Billy et al., 2008).

Several processes can contribute to the solubilization of pectins (Paniagua et al., 2014). A marked loss of neutral sugars, mainly Ara and Gal, from RGI side chains is an early event in the ripening of some fruit (Redgwell et al., 1997b). Side chains from RGI might anchor pectins to the wall by physical entanglement with other wall polymers or by binding to the cellulose-hemicellulose network (Zykwinska et al., 2008; Wang et al., 2015), and, consequently, their loss could weaken the pectin network. However, some fruit show extensive solubilization without significant loss of arabinose or galactose (e.g. plum), while others (apple and nashi pear) display an extensive loss of galactose but slight pectin solubilization (Redgwell et al., 1997b; Ng et al., 2015). A progressive demethylesterification of HG mediated by pectin methylesterase (PME) is common to the ripening of many fruit (Mercado et al., 2011). Demethylesterification facilitates the hydrolysis of HG by endo-polygalacturonase (PG) or pectate lyase (PEL) since these enzymes needs at least 4 demethylated GalA residues of the HG backbone to cleavage the glycosidic bonds (Chen and Mort, 1996). Additionally, demethylesterification might create large regions of negatively charged HG which would contribute to the loosening of pectins by electrostatic repulsion (Brummell, 2006). The methylesterification status of pectins seems to be more related to the middle lamella and tissue integrity rather than to fruit firmness. Transgenic suppression of PME activity in tomato reduced pectin methylesterification but did not affect fruit firmness; however, fruit shelf life was notably shortened due to the loss of tissue integrity as result of a reduced amount of bound  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the apoplast (Tieman and Handa, 1994).

Pectin depolymerization is not as general as pectin solubilization and in many fruit it takes place late in ripening (Mercado et al., 2011). Some species show a significant loss of high molecular weight pectins (e.g. avocado, tomato, peach, kiwifruit), while others display a low to moderate depolymerization (e.g. strawberry, blueberry, melon, apple). This process can be also cultivar-specific. Despite this diversity, functional analyses of genes encoding pectinases in fruit with contrasting textural properties have demonstrated that pectin depolymerization is a fundamental process in fruit softening. In strawberry, a soft fruit, transgenic suppression of PG or PEL genes increased fruit firmness and extended postharvest shelf life (Jiménez-Bermúdez et al., 2002; Quesada et al., 2009). These improvements were related to a lower depolymerization of bound pectins, a lower degree of pectin solubilization and a higher tissue integrity (Santiago-Doménech et al., 2008; Posé et al., 2013). The visualization of pectins from these transgenic fruit with an atomic force microscope revealed a positive correlation between the preservation of the nano-structural complexity of pectins (pectin length, branching and number and size of pectin aggregates) and fruit firmness (Posé et al., 2015). In tomato, transgenic suppression of PG did not affect fruit firmness but improved shelf life due to a higher middle lamella integrity (Smith et al., 1990; Kramer et al., 1992). Recently, it has been found that suppression of a PEL gene also reduces softening in this fruit (Uluisik et al., 2016). Down-regulation of an endo-PG in apple also results in firmer and juicier fruit (Atkinson et al., 2012).

Our knowledge about cell wall modifications in fresh vegetables is limited when compare with fruit mainly due to the less dramatic textural changes that suffer these products. By contrast, there are ample literature about the effect of processing on textural properties of vegetables (Van Buggenhout et al., 2009; Ranganathan et al., 2016). As previously described for fruit, pectin size, branching and solubility has been related to firmness in some root and storage organs such as potato or carrot (Gómez-Galindo et al., 2004; Ranganathan et al., 2016). The reduction of arabinan and galactan side chains in RGI makes potato tubers more brittle when subjected to compression (Scheller and Ulvskov, 2010), indicating a key role of the pectin matrix in textural properties. In stem and inflorescence vegetables like asparagus, broccoli and cauliflower, hardening may occur after harvest due to tissue lignification (Goulao et al., 2010). Textural changes in leafy vegetables are mainly associated with the loss of cell turgor. However, modifying cell wall properties can have a direct impact on texture and shelf life. The transgenic silencing of XTH genes in lettuce produces smaller and thicker leaves with an extended shelf life (Wagstaff et al., 2010).

## Conclusions

Firmness and juiciness are the most important textural properties in fruit and vegetables. Both parameters depend on the anatomy of the plant tissue, the strength of cell walls, the cohesiveness of the middle lamella and the cell turgor. Fruit undergo a ripening process that substantially modify their texture, leading to fruit softening. The rate of this process determines the postharvest shelf life of the product. Loss of cell turgor can be important in some fruit; however, cell wall disassembly during fruit ripening is considered the main factor causing softening. Among the different wall components, both hemicellulose and pectins are modified during fruit ripening, although the mechanisms involved in these processes are largely unknown. Genetic transformation studies with genes encoding pectin degrading enzymes, such as polygalacturonase or pectate lyase, suggest that the metabolism of pectins is a major event regulating fruit firmness and shelf life. During ripening, pectins are subjected to solubilization, depolymerization and elimination of neutral side chains, being the pectin

solubilization process the change that best correlate with softening. Vegetables generally are firmer and less susceptible to softening than fruit due to the presence of thicker cell walls. The physiological basis underlying textural changes in these vegetables are less known due to the broad range of tissues and organs that comprise this group of products. Some of them suffer cell wall alterations in the pectin matrix that correlate with textural properties. In others, textural changes are more related to senescence and loss of cell turgor.

## Supplementary data

Supplementary data related to this article can be found online at <https://doi.org/10.1016/B978-0-12-814026-0.21679-X>

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## Relevant Websites

- CAZy: Carbohydrate active enzymes database. A database that describes the families of structurally-related catalytic and carbohydrate-binding modules of enzymes that degrade, modify, or create glycosidic bonds. <http://www.cazy.org/>.
- Glycans at NCBI: A website dedicated to all glycoscience content at NCBI. Useful glycome-related content including publications, chemical records databases, and resources for better integrating glycomic studies (eg: symbol nomenclature for glycans; software tools; Glycan Informatics advisory group). Also includes a list of external resources (Expasy Glycomics, GlyTouCan, CAZy, MonosaccharideDB, Carbohydrate Structure DB), <https://www.ncbi.nlm.nih.gov/glycans/>.



# Atomic Force Microscopy (AFM) and X-Ray Micro-Computed Tomography (Micro-CT): Applications in Cell Wall Imaging of Softening Fruit

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## Glossary

**Artefact** something observed in a scientific investigation or experiment that is not naturally present but occurs as a result of the preparative or investigative procedure

**Fixation** procedure to preserve cell structures in their current state. Inactivates enzymes that might disrupt cell morphology and toughens cell structures to ensure they do not move or change during staining and observation. Common chemical fixatives include glutaraldehyde, formaldehyde and ethanol.

## Overview

Texture is critical for consumer acceptability of fresh fruits and vegetables. Apples for example should be crisp and firm, however when stored inappropriately, they can become mealy and soft. These and many other textural properties relevant to postharvest and processing behaviour are related to the microstructure of tissue. Tissue microstructure has conventionally been visualized using light microscopy coupled with chemical staining or immunolabelling, or by scanning electron microscopy (SEM) (for examples, see [Ng et al., 2013](#)). These techniques are informative, but often require extensive sample preparation, making them prone to artefacts. This review explores two microscopy imaging techniques and their applications in providing better understanding of fruit microstructure: atomic force microscopy (AFM), revealing the arrangement and changes of cell wall polymers during softening, and X-ray micro-computed tomography (micro-CT), allowing visualization of tissue structure largely in their native state.

## Atomic Force Microscopy (AFM)

### Principle

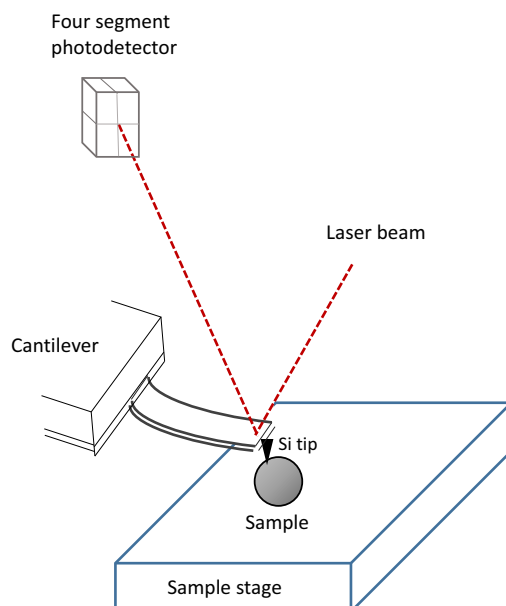
AFM forms images of surfaces using a physical probe that scans the specimen surface, most commonly either close enough for forces associated with atoms to be detected (non-contact; AC mode) or touching the surface continuously (contact; DC mode) or intermittently (tapping mode) ([Fig. 1](#)). A detailed description of the mechanics of an AFM can be found in [Torre et al. \(2011\)](#). AFM can operate both in air and in liquids. A detailed description of the various operating modes and principles are described in [Kirby et al. \(1996\)](#), [Kirby \(2011\)](#) and [Morris et al. \(2010\)](#). For a guide on AFM experimental design, refer to [Gavara \(2017\)](#).

### Applications

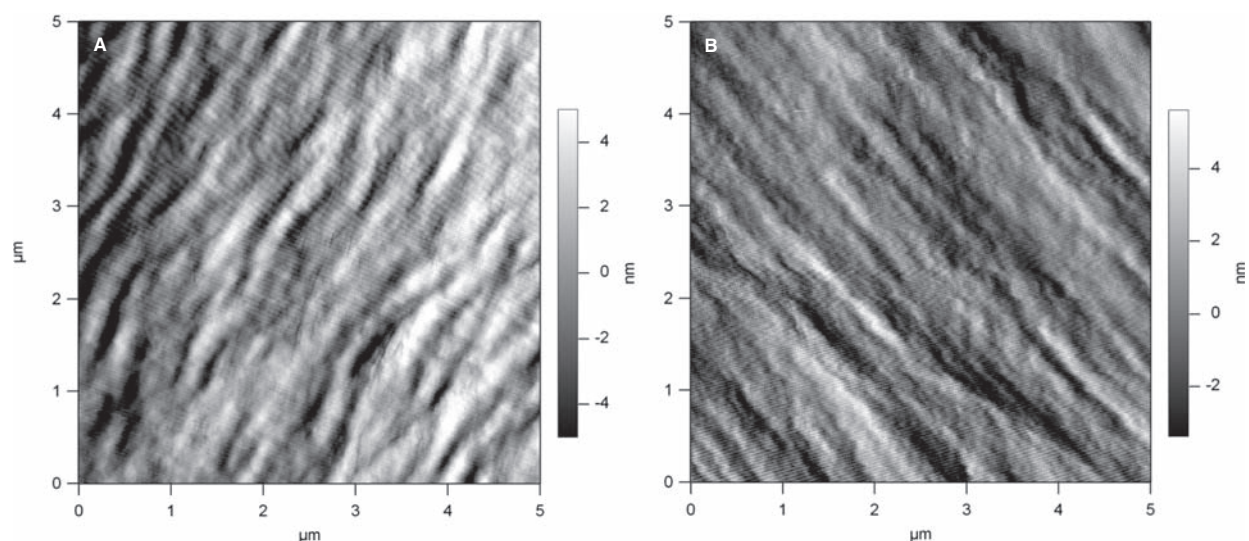
AFM has been used to investigate isolated cell walls, sub-fractions enriched for pectin, or cellulose. During fruit softening, biochemical analyses of cell walls suggested a decrease in frequency of pectin branching and chain length ([Ng et al., 2013](#)). Using AFM, these changes were confirmed on the atomic scale; pectin chains in ripe fruit were shorter and the molecular width decreased ([Paniagua et al., 2014](#)). However, molecular width assessments need to be interpreted carefully, as AFM can be susceptible to artefacts such as from 'probe broadening' effects ([Morris et al., 2010](#)). Other artefacts can be introduced from the way that the sample is prepared; e.g. to expose the cellulose microfibrils for imaging, pectin and hemicelluloses are removed by sequential extraction, resulting in induced aggregation and increased diameter of the observed underlying microfibrils ([Thimm et al., 2009](#)). Example AFM amplitude images (non-contact mode) of apple cell wall microfibrils revealed after pectin removal are shown in [Fig. 2](#). For example deflection images (contact mode), refer to [Thimm et al. \(2009\)](#). For technical issues, practical solutions and a description of common image artefacts, consult [Kirby \(2011\)](#). For in-depth reviews on the application of AFM in plant cell wall polysaccharide studies, refer to [Paniagua et al. \(2014\)](#) and [Wang and Nie \(2018\)](#).

**Table 1** summarizes observations of nanostructural changes in cell wall polysaccharide extracts during softening. **Table 2** summarizes studies using various preparation methods to image cellulose microfibrils in their closest to native form.





**Figure 1** Block diagram of an atomic force microscope. A tip is moved across the sample surface and changes in magnitude of the force between tip and sample are measured and used to produce a three-dimensional profile of the sample's surface. The tip is attached to a flexible cantilever allowing vertical movement. A laser beam is focused onto the back of the cantilever, which reflects onto a position-sensitive photodetector consisting of photodiodes. The difference in electrical potential between the photodiode signals indicates the position of the laser spot, the angular deflection of the cantilever and the respective displacement of the tip, generating a topographical image of the sample.



**Figure 2** AFM amplitude images of 'Scifresh' (also known as 'Jazz™') (ripe, firm) (A) and 'Royal Gala' (ripe, soft) (B) apple cell walls. Native cortex tissue was excised and rinsed with 50 mM CDTA buffer pH 6.5 to remove pectic matrix to reveal the underlying cell wall structures, hemicellulose and cellulose. Apples were harvested at optimum maturity and stored at 0.5 °C for 20 weeks prior analysis. Images were captured using an MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA, USA) operating on non-contact mode using silicon tip with nominal spring constant of 2.5 N m<sup>-1</sup>, NSG II (ND-MDT CSG II Moscow, Russia). The drive amplitude was set between 2.0 and 4.0 V, with drive frequency between 50 and 100 Hz. The scan size was 5 μm<sup>2</sup>, scan rate 1–5 Hz, and scan angle 0–45°. Images were captured and processed using MFP-3D software running parallel with IGOR Pro v4. The firmer apple 'Scifresh' showed higher frequency of larger diameter fibrils, with mean fibril width of 269 nm, while the softer apple 'Royal Gala' had mean fibril width of 248 nm. For further supporting biochemical analysis, the reader is referred to [Ng et al. \(2013\)](#).

## X-Ray Micro-Computed Tomography (Micro-CT)

### Principle

In Micro-CT, a series of X-ray images is captured as the sample rotates (180° or 360°) and these images are used to visualize the sample in 3D through back projection ([Fig. 3](#)). Micro-CT occurs without destructing samples, as they are scanned under normal

**Table 1** AFM nanostructural observations of cell wall extracts isolated from cell walls of softening fruit

<i>Fruit</i>	<i>Cell wall extract</i>	<i>Observation during postharvest softening</i>	<i>References</i>
Chinese cherry	SSP	SSP became narrower, shorter and less entangled during softening. Crisp cultivar had wider and shorter chains than soft cultivar.	Zhang et al. (2008)
	WSP	Molecular width of WSP and CSP chains decreased.	Lai et al. (2013)
	CSP		
	HC	Higher percentage of thicker HC chains in ripe crisp fruit than soft fruit. HC fibrils regular in ripe crisp fruit but irregular in ripe soft fruit.	Chen et al. (2009)
Tomato	CSP	CSP became narrower, shorter and more linear during softening.	Xin et al. (2010)
Peach	WSP	WSP and CSP chain lengths similar between crisp and soft fruit of two cultivars. Only crisp fruit showed CSP and SSP branching. SSP chains of crisp fruit were significantly longer than those of the soft fruit.	Yang et al. (2009)
	CSP		
	SSP	Controlled-atmosphere conditions inhibited the degradation of WSP and CSP as molecules had a greater width than fruit stored in air. Decrease in amount of branched SSP and reduction in size and number of aggregates observed over storage.	Yang et al. (2005); Yang, Lai, An & Li (2006a); Yang, Feng, An & Li (2006b)
		In unripe fruit, SSP occurred as large aggregates, whereas CSP occurred as single linear chains with less aggregates. SSP chain width decreased with cold-storage time and increasing storage temperatures.	Zhang et al. (2010, 2012)
Chinese jujube	WSP	CSP and SSP molecular width and length decreased with softening.	Wang et al. (2012)
	CSP		
	SSP		
Pear	WSP	Compared to ripe soft fruit, crisp fruit had larger WSP chains, thicker and more branched CSP molecules, a denser network of SSP molecules, and thinner but more homogeneous distribution of hemicellulose molecules that tended to tangle. Cellulose microfibrils were similar.	Zdunek et al. (2014)
	CSP		
	SSP		
	HC		
Carrot	WSP	During cold-storage, CSP and SSP chains decreased in length and width, WSP chains were short overall, and dense SSP network structure disappeared.	Cybulska et al. (2015)
	CSP		
	SSP		
Strawberry	WSP	Widths and lengths of pectin chains reduced during cold-storage. CaCl <sub>2</sub> treatment decreased the degradation of CSP and SSP.	Chen et al. (2011)
	CSP		
	SSP	CSP chains shortened and pectin branching decreased during softening.	Posé et al. (2012)
Apricot	CSP	CSP chain branching and length and HC chain width decreased during cold-storage. CaCl <sub>2</sub> reduced pectin degradation.	Liu et al. (2009); Liu et al. (2017)
	HC		
Chinese quince	HC	Entangled and branched HC chains in ripe fruit. Treating fruit hydrothermally degraded large chain polysaccharides to shorter ones and exposed branched chains.	Liu et al. (2018)

Abbreviations: SSP, ester-bound pectin; WSP, water-soluble pectin; CSP, calcium-bound pectin; HC, hemicelluloses.

ambient conditions without much preparation (Hertog et al., 2012). Coupled with synchrotron radiation, micro-CT is capable of visualizing cell walls (Karahara et al., 2015). Using Micro-CT, tissue porosity, void sizes, void count, shape factor and the degree of cell connectivity can be quantified rapidly and non-destructively, requiring no sample fixation, hence minimizing creation of artefacts. Prolonged scanning times, however, can cause drying out of samples, especially when small. To prevent this, the sample can be coated in a layer of wax, parafilm, or cling-film. For further information on underlying physics, technical background and manipulation and analysis of the data, refer to Landis and Keane (2010) and Karahara et al. (2015).

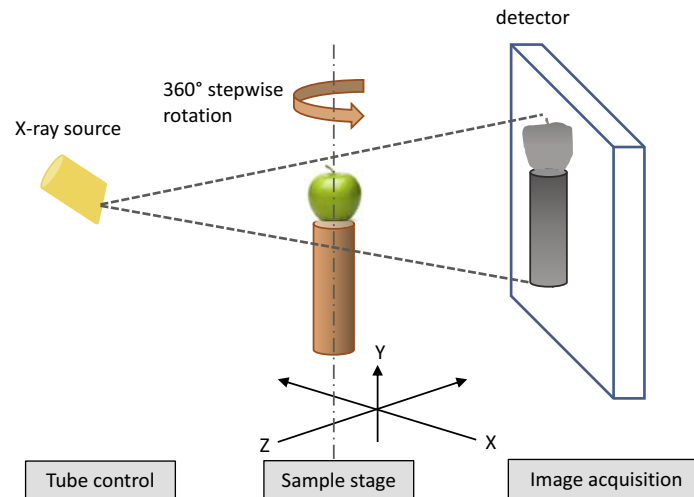
## Applications

Micro-CT is a powerful tool for non-destructively assessing fruit internal quality and to evaluate internal development of structural changes in a fruit and their relationship with texture. Micro-CT has for example been used to investigate physiological internal

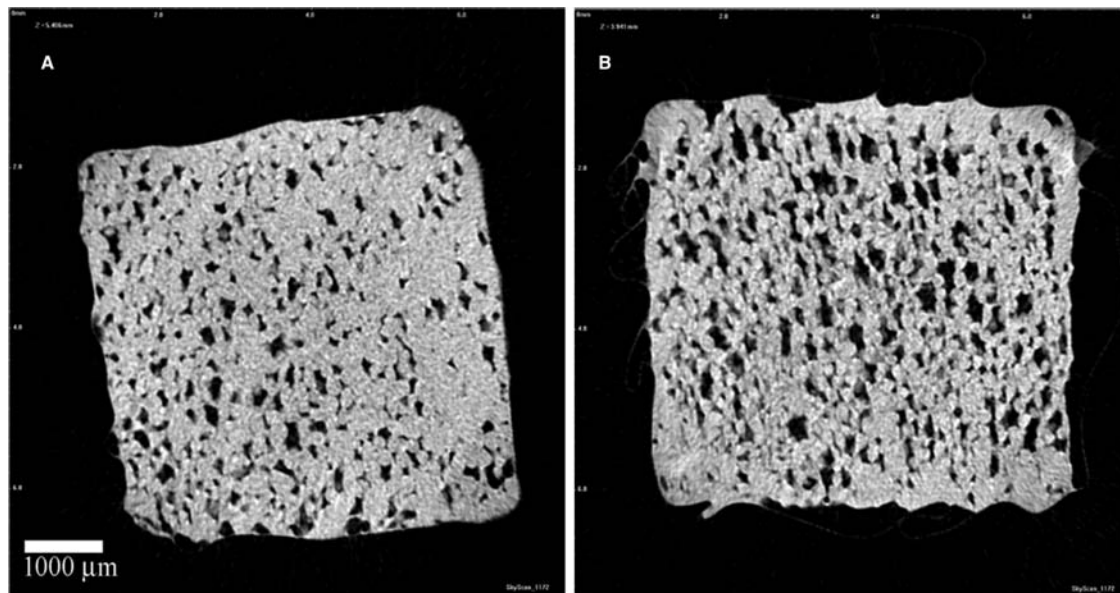
**Table 2** AFM sample preparation techniques and observations of cellulose microfibrils in plant cell walls

<i>Plant material</i>	<i>Sample preparation method</i>	<i>AFM operating mode</i>	<i>Observation</i>	<i>References</i>
Apple Water chestnut Potato Carrot Celery	Tissue homogenized, filtered, re-suspended in sodium dodecyl sulfate and Na <sub>2</sub> SO <sub>5</sub> , ball-milled and frozen. Frozen suspension deposited on mica  (a) Freshly-cut epidermal peel imaged directly (b) Sample freeze-dried and mounted to a metal-disk  CWM isolated using HEPES-buffered phenol and ethanol. Pectin and hemicellulose removed by sequential extraction with chelator and alkali	Imaged in air; DC mode  Imaged in air, water or 50% ethanol; DC mode  Imaged in water; DC mode	No structural changes during freeze-thawing. Microfibrils appear as uniform structures in the order of 25 nm diameter.  Imaged under water, never-dried cellulose microfibrils had diameter of 6–25 nm. With decreasing water content, microfibril diameter increased. Freeze-dried material showed larger microfibril diameter than air-dried material.  Microfibril diameter increased after pectin and HC removal, with tendency to self-associate and form aggregates. Treatment with chelator increased the gaps between microfibrils, and severely disrupted structure and orientation of microfibrils. Treatment with strong alkali restored parallel microfibril orientation.	<a href="#">Kirby et al. (1996)</a>  <a href="#">Thimm et al. (2000)</a>  <a href="#">Thimm et al. (2009)</a>
Cucumber hypocotyls	Samples bisected longitudinally, fixed in paraformaldehyde. Dehydrated in 100% ethanol, critical point dried, and coated with platinum	Imaged in water, PBS buffer, air; DC mode	Cucumber hypocotyls could only be imaged partially-hydrated, with microfibrils around 50 nm in diameter.  Cell walls in the coated samples were similar to fresh, fixed, or partly dehydrated samples. Images showed undulated and meandered surfaces. Dehydration had negligible effect on microfibril organization.	<a href="#">Marga et al. (2005)</a>
Strawberry Peach Rambutan Apple	Tissue blended in acetone, then NaClO <sub>2</sub> + NaOH + acetic acid, freeze-dried. Sonication in water, spread onto freshly-cleaved mica and air-dried  CWM was prepared, and pectin and HC removed by sequential extraction with chelator and alkali. Samples were suspended in 50% ethanol and vacuum-dried onto glass slides	Imaged in air; DC mode  Imaged in air; tapping mode	Cellulose microfibrils were ultrathin and 1–2 nm in width, composed of approximately 10 glucan chains.  After removal of pectin and HC, a fine network of cellulose microfibrils was observed (random orientation for some cultivars, others more aligned). Thicker microfibrils correlated with crisper, harder and juicier texture; softer cultivars had thinner microfibrils.	<a href="#">Niimura et al. (2010)</a>  <a href="#">Cybulska et al. (2013)</a>

Abbreviations: CWM, cell wall material; DC, direct contact; HC, hemicellulose; PBS, phosphate-buffered-saline.



**Figure 3** Diagram of the principle of X-ray micro-computed tomography (micro-CT). Two-dimensional projection images of an object are obtained by using X-ray beams from multiple angles, due to rotation of the object. X-rays passing through the material are detected by a charge-coupled detector. The 2D projections are used to construct a 3D dataset using a set of algorithms, which effectively simulate the back projection of the whole image set to reconstruct the structure that would have given rise to it. Examples of virtual slice images of two different apple cultivars are shown in **Fig. 4**. For example image stacks and 3D reconstruction of ripe apple cortex tissue, the reader is referred to the **Online Supplementary Multimedia Material Movie 1 and 2**, respectively.



**Figure 4** Micro-CT single virtual slice image of 'Scifresh' (ripe, firm) (A) and 'Royal Gala' (ripe, soft) (B) apple cortex tissue. Apples were harvested at optimum maturity and stored at 0.5 °C for 20 weeks prior analysis. Sample preparation and micro-CT imaging as described in [Hall et al. \(2016\)](#). Micro-CT revealed that the firmer apple cultivar 'Scifresh' had greater cell-to-cell connectivity (grey) and lower frequency of intercellular air spaces (black), while the softer apple cultivar 'Royal Gala' showed presence of more intercellular air spaces, that had larger variation of sizes, and that were distributed more uniformly throughout the tissue scanned.

defects or storage-associated disorders in fruit, e.g. translucency in pineapples ([Haff et al., 2006](#)), core breakdown in pears ([Lamertyn et al., 2003](#)), watercore disorder of apples ([Herremans et al., 2014](#)), chilling injury in pomegranates ([Magwaza and Opara, 2014](#)) and bruise volume in apples ([Diels et al., 2017](#)). **Table 3** summarizes the use of micro-CT for investigating microstructural characteristics associated with fruit texture. For an in-depth review on the fundamentals and application of micro-CT in other food structure investigations, refer to [Schoeman et al. \(2016\)](#).

**Table 3** X-ray micro-CT applications on various fruits

Fruit	Sample preparation	Observations during postharvest storage	References
Apple	Excised cylindrical samples placed in plastic tube and wrapped with cling-film	Ripe 'Jazz' apples that were firmer were less porous with smaller intercellular air spaces than softer 'Golden Delicious'.	Ting et al. (2013)
	Excised cylindrical samples in plastic tube	Air spaces at random patterns and with complex distributions in apples after controlled-atmosphere storage. Multifractal Generalized Dimensional Analysis was used to characterise the heterogeneity of pore space structure.	Mendoza et al. (2010)
	Excised cylindrical samples	Development of statistical correlation functions to precisely quantify spatial distribution of cell versus void spaces in apple tissue is presented. Descriptors such as degree of cell connectivity, cell phase and clusters can be used to accurately quantify structure changes over cold-storage.	Derossi et al. (2017)
Apple Pear	As above	Describes an automated analysis of 3D structure to provide numerical microstructural parameters such as cell shape, size, volume, void elongation, void volume, which were used to correlate with effective diffusivity.	Herremans et al. (2015)
Pear	Excised cylindrical samples wrapped in parafilm	Mealy pear fruit were more porous with larger pores compared with non-mealy fruit. Mealy fruit had larger and more oval-shaped cells, while non-mealy fruit cells were more spherical.	Muziri et al. (2016)
Kiwifruit	As above	Fruit with different firmness, weight, soluble solids and dry matter content showed a high variability in porosity and pore size without apparent correlations.	Cantre, East, Verboven et al. (2014a)
Cucumber	As above	Percentage of long and continuous gas-filled intercellular spaces decreased with storage, as water blocked them due to senescing cells.	Kuroki et al. (2004)
Mango	As above + packed in polystyrene foam	Softening was associated with a decrease in pore size and an increase in pore fragmentation and pore specific surface area. Ripe and unripe fruit could be classified correctly using micro-CT, allowing for determining ripeness of fruit non-destructively.	Cantre, Herremans, Verboven, Ampofo-Asiama & Nicolai (2014b)

## Appendix A Supplementary Data

Supplementary data related to this article can be found online at <https://doi.org/10.1016/B978-0-12-814026-0.21680-6>.

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## Legume Microstructure

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### Glossary

**Cytoplasm** A clear substance enclosed within the cell membrane, excluding the nucleus.

**Embryo** A rudimentary plant contained within a seed.

**Exalbuminous** Lacking of endosperm at the maturity stage. Most of the endosperm is absorbed by the developing embryo and nutrients are stored in the cotyledons.

**Gelatinisation** An irreversible process of disrupting the crystalline structure of starch granules and solvating the exposed hydroxyl groups of amylose and amylopectin by water molecules in the presence of heat and water.

**Glycemic index (GI)** A classification system for carbohydrate-containing foods based on the postprandial blood glucose response after ingestion of such foods.

**Integument** A protective cell layer surrounding the ovule that develops into the seed coat after fertilisation.

**Organelle** A small, specialised structure within a cell that performs a specific function.

**Ovule** A part of the female reproductive organ of seed plants that develops into the seed after fertilisation.

**Parenchyma** A ground tissue with thin primary cell walls, mainly found in the softer parts of the plant.

**Vascular bundle** A strand of vascular tissue that transports fluids and dissolved nutrients throughout the plant.

### Introduction

Legumes have been cultivated as foods for human consumption since ancient times. They are the edible seeds of leguminous plants in the *Leguminosae* family. Food legumes are classified into two groups of pulses and oilseeds. Pulses are the dried seeds of cultivated legumes, whereas oilseeds are mainly used for their oil content. Food legumes provide an important source of nutrients (i.e. carbohydrates, proteins, lipids, fiber, vitamins and minerals) for people in many parts of the world (Aykroyd and Doughty, 1982). They generally have a low glycemic index (GI) and only produce a moderate postprandial glycemic response following ingestion. Therefore, it is recommended to incorporate legumes as part of the diets for the glycemic management of diabetes (Rizkalla et al., 2002). The low GI characteristic is largely attributed to the cotyledon cell structure, which plays an important role in modulating digestion of starch in legumes (Berg et al., 2012). This article reviews the legume microstructure and its relationship to starch digestion.

### Seed Structure

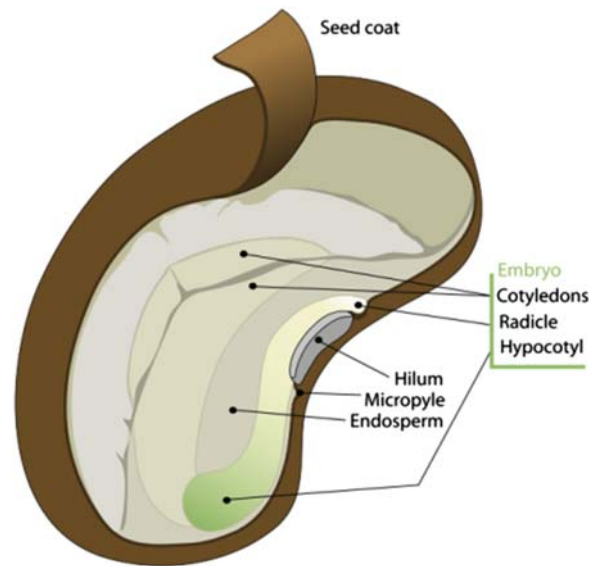
The structure of a typical leguminous seed is shown in Fig. 1. Embryo constitutes the largest proportion of the mature seed. Seed coat (testa) forms a protective outer shell encapsulating the embryo. The principal structural features of the embryo include cotyledons, a hypocotyl (embryonic axis), and a radicle (embryonic root). Legumes are classified as dicotyledonous or dicots, since the embryo consists of two cotyledons that become a pair of embryonic leaves upon germination. Leguminous seeds are exalbuminous or nonendospermic in nature. Endosperms are present at the rudimentary stage of seed development, however they become vestigial upon maturity. The cotyledons contain the storage reserves of nutrients required for the early growth of the seedling. The external topography of the seed notably possesses two specialised markers residing on the seed coat: a hilum that is a funicular scar left where the seed is formerly attached to the maternal plant during development, and a micropyle that is a minute opening underneath one end of the hilum. Both are involved in controlling water entry to the seed (Stanley and Aguilara, 1985; Yousif et al., 2007).

### Seed Coat

According to Souza and Marcos-Filho (2001), the seed coat derives from the formation of inner and outer integuments surrounding the growing ovule. During seed development, the inner integument disappears while the outer one progresses into several distinct cell layers, forming the seed coat (testa) structure. Despite great variations in composition and microstructure among different legume species, the seed coat fundamentally comprises of four layers (Fig. 2):

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\* corresponding author



**Figure 1** Structure of a leguminous seed. Reproduced with permission from: [https://commons.wikimedia.org/wiki/File:Dycotyledon\\_seed\\_diagram-en.svg](https://commons.wikimedia.org/wiki/File:Dycotyledon_seed_diagram-en.svg).

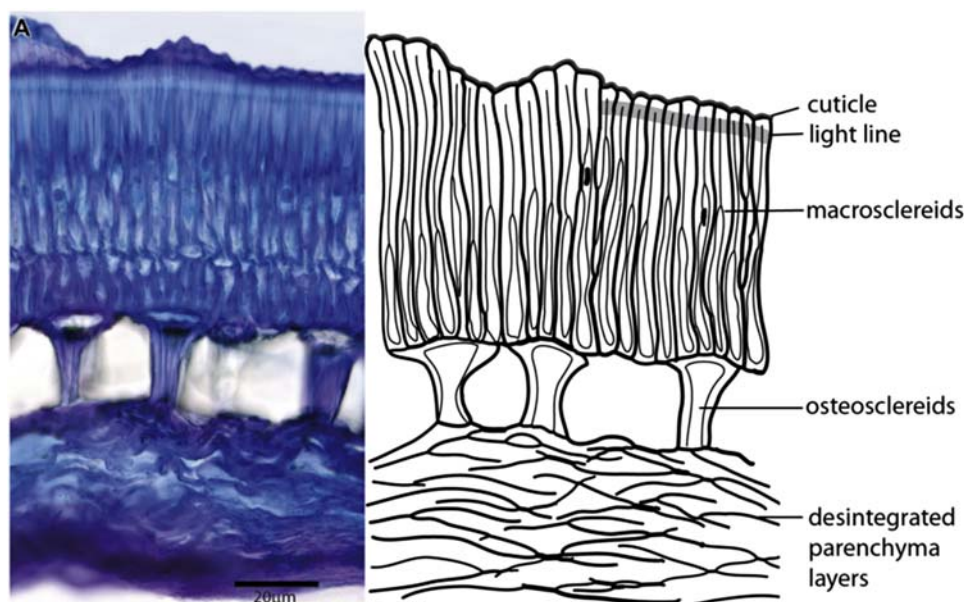
- *Cuticle*: an outermost layer of non-living, non-cellular membrane containing waxy depositions. The cuticle covers the seed, excluding the hilum, and is considered as the primary barrier to seed imbibition.
- *Epidermis*: a single layer of thick-walled and radially elongated macrosclereids or palisade cells. The appearance of a lucent region extending across the macrosclereid layer, also called light-line or *linea lucida*, might be observed due to the local variation in refractive indices of this region in some species.
- *Hypodermis*: a single layer of osteosclereids, also called pillar cells or hourglass cells depending on the pattern of cell thickness and shape. These cells are spaced widely apart and separated by air-filled intercellular spaces.
- *Interior parenchyma*: an innermost part of the seed coat, consisting of 5–12 layers of thin-walled and tangentially elongated parenchyma cells. Upon maturation of the seed coat, these cells lose the protoplast and the innermost layers are crushed, leaving behind disintegrated parenchyma fragments.

## Cotyledons

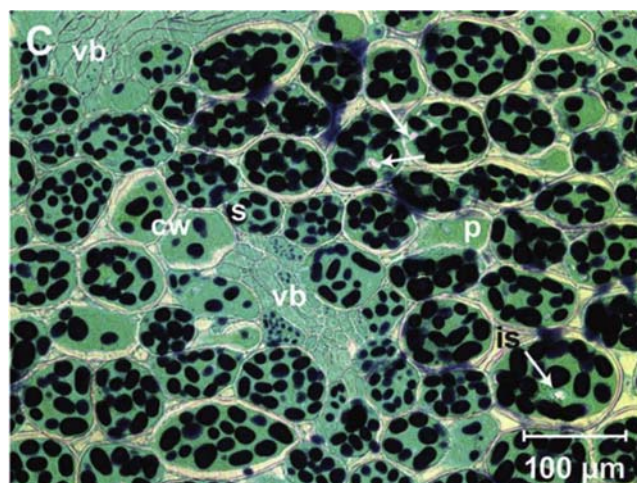
The cotyledons constitute a significant proportion of the seed weight and volume. These are highly organised structures with major components distributed within discrete cellular compartments (Fig. 3). The periphery of the cotyledons is overlaid with a single layer of epidermis cells. These thick-walled cells are filled with protein and devoid of starch granules. Vascular bundles, containing protein-filled, starch-free cells, are occasionally present in the cotyledons. The cotyledon tissue, for the most part, is made up of large parenchyma cells. The cell walls of two adjacent cells are joined to each other by the pectin-rich middle lamella. A cytoplasmic network fills up the space enclosed within the cell membrane of each parenchyma cell, and surround subcellular organelles including starch granules, protein bodies and oil bodies (Young and Schadel, 1990; Wood et al., 2011). The cotyledon cell microstructure of starch-rich legumes (e.g. beans, peas, chickpeas and lentils) differs greatly from that of oil-rich legumes (e.g. soybeans and peanuts).

## Starch-Rich Legumes

The inner parenchyma cell contains tightly-packed starch granules and small protein bodies that are embedded in a proteinaceous matrix, all encapsulated within a thick, mechanically robust cell wall (Fig. 4). The cell shape and size are highly variable. The cells are spherical, angular, or ellipsoid in shape, and normally range from 50 and 100 µm in size (Berg et al., 2012).



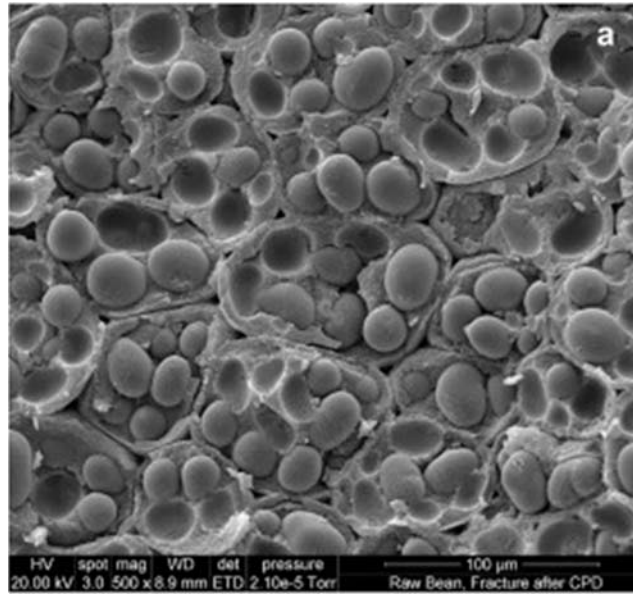
**Figure 2** Transversal section of seed coat structure (left) with a schematic drawing (right) showing four distinctive layers: the cuticle is an outermost layer covering the seed. Underneath the cuticle is the epidermis, which differentiates into macrosclereids (palisade cells). The centre of the seed coat is hypodermis, which differentiates into osteosclereids (hourglass cells). The innermost layer is the interior parenchyma, composed of disintegrated parenchyma layers. Reproduced with permission from Smýkal, P., Vernoud, V., Blair, M.W., Soukup, A., Thompson, R.D., 2014. The role of the testa during development and in establishment of dormancy of the legume seed. *Front. Plant Sci.* 5, 1–19.



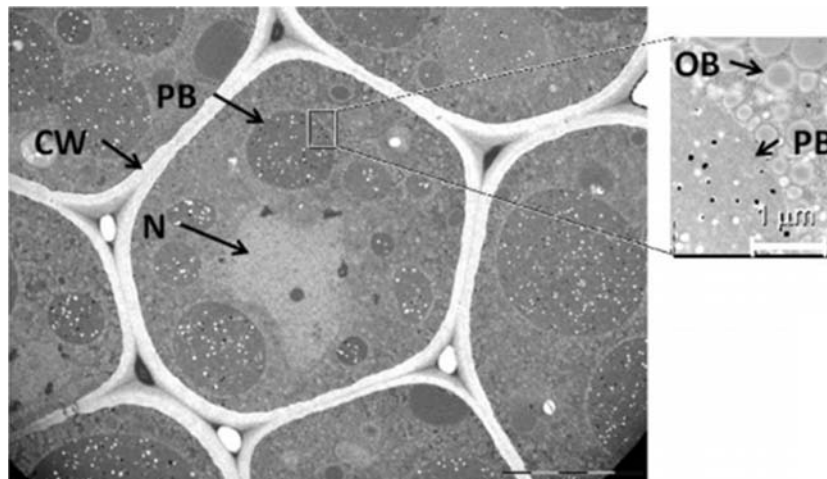
**Figure 3** Cross sections of the cotyledons showing structures of various cellular components: cw, cell wall; vb, vascular bundle; p, protein; s, starch granules; is, intercellular space. Arrow points to organelles. Bar = 100 µm. Reproduced with permission from Wood, J.A., Knights, E.J., Choct, M., 2011. Morphology of chickpea seeds (*Cicer arietinum* L.): comparison of desi and kabuli types. *Int. J. Plant Sci.* 172, 632–643.

### Oil-Rich Legumes

The cotyledons of oil-rich legumes such as soybeans possess distinctive structures (Fig. 5). At the subcellular level, two major constituents of proteins and lipids are assembled in specialised organelles, commonly called protein bodies (PBs) and oil bodies (OBs) respectively. The PBs are surrounded by numerous OBs, and together they are tightly packed and embedded in a cytoplasmic protein network of the cell (Campbell and Glatz, 2009). Soybean seeds generally contain 43%–48% protein and 18%–21% oil, with the protein content significantly higher than that of other plant-based foods. Starch granules accumulate in the parenchyma cells of the cotyledons during seed development, but rapidly decline to trace quantities with maturation (Stevenson et al., 2007).



**Figure 4** Scanning electron micrograph showing cross section of cotyledon cells of raw navy beans. Bar = 100  $\mu\text{m}$ . Reproduced with permission from Berg, T., Singh, J., Hardacre, A., Boland, M.J., 2012. The role of cotyledon cell structure during *in vitro* digestion of starch in navy beans. Carbohydr. Polym. 87, 1678–1688.



**Figure 5** Transmission electron micrograph showing cross section of soybean cotyledon cells. CW, cell wall; PB, protein body; OB, oil body; N, cell nucleus. Reproduced with permission from Campbell, K.A., Glatz, C.E., 2009. Mechanisms of aqueous extraction of soybean oil. J. Agric. Food Chem. 57, 10904–10912.

### Starch Granules

Starch granules are the primary carbohydrate storage reserve, making up between 22% and 45% of the starch-rich leguminous seeds. The size, shape and surface morphology of native starch granules vary according to legume species and stages of seed maturity. The granule size ranges from 4 to 85  $\mu\text{m}$ . The granule shape is chiefly oval, though in some cases can be spherical, elliptic or irregular. The granule generally has a smooth surface with the absence of fissures (Hoover and Sosulski, 1991). However, the presence of deep fissures can sometimes be observed due to the compact packing of starch granules within the protein matrix (Hsieh et al., 1999). The molecular structure of legume starches is characterised by a mixture of linear amylose and highly branched amylopectin, with a high amylose content (24%–65%). Starch granules possess a semi-crystalline structure that contains both crystalline and amorphous regions. Legume starches typically display a C-type X-ray diffraction pattern (crystallinity), which is a mixture of A-type (cereal starches) and B-type (tuber starches) (Hoover and Sosulski, 1991).



## Protein Bodies

Protein bodies or aleurone grains are typically small, spherical organelles in the size range from 0.1 to 25  $\mu\text{m}$  in diameter. The PBs store up to 80% of the total protein in leguminous seeds, and supply a source of nutrients (i.e. carbon, nitrogen and sulfur) for the future needs of plant growth and development. The PB contains an amorphous protein matrix enclosed by a single lipoprotein membrane. The PB of some legume species contains structural inclusions of globoid crystals embedded in the proteinaceous matrix. The globoid is a phytic acid and cation storage compartment. The two major storage proteins, primarily located within the PBs, are vicilin-type (7S) and legumin-type (11S) globulins (Lott and Buttrese, 1978).

## Oil Bodies

Oil bodies or oleosomes are discreet, specialised organelles. They are usually spherical in shape and smaller than the PBs in size, ranging from 0.5 to 2.5  $\mu\text{m}$  in diameter. Triacylglycerol (TAG) is the predominant form of storage lipids. The structure of an OB consists of a central core of hydrophobic TAG molecules that are encapsulated and stabilised by a phospholipid (PL) monolayer embedded with integral proteins (mainly oleosins). The specific conformations of surface proteins and PL impart remarkable stability to the suspended OBs against destabilisation (coalescence and aggregation) in the aqueous environment of the cytoplasm (cytosol) (Tzen and Huang, 1992). The storage lipids in leguminous seeds are rich in fatty acids, including oleic, linoleic, linolenic acids and palmitic acids. Linoleic and linolenic acids are essential fatty acids that cannot be synthesised by the human body and must be obtained from dietary sources (Stanley and Aguilera, 1985).

## Cell Walls

According to Yousif et al. (2007), the cotyledon cell is enveloped by a thick, rigid, outer cell wall and an inner cell membrane. The cell membrane is a thin, semi-permeable, lipid bi-layer with embedded proteins. Its role is to protect the cellular interior from the outside environment by controlling the movement of ions and molecules in and out of the cell. The cell walls are divided into two classes based upon their mechanical properties and chemical composition: primary and secondary walls. In general, the cell wall comprises of three distinctive layers:

- *Primary wall*: a complex three-dimensional network composed of interacting structural components. The highly ordered, crystalline cellulose microfibrils (organised phase) are dispersed in a continuous noncellulosic matrix (amorphous phase). The matrix phase consists of pectins, hemicelluloses (mainly xyloglucan), and some minor structural proteins. The cellulose – xyloglucan framework acts as a major load-bearing structure, while the matrix of pectic polysaccharides functions as a plasticiser and controls wall porosity.
- *Secondary wall*: a significantly thicker, stiffer and stronger layer due to lignification process as compared to the non-lignified primary wall.
- *Middle lamella*: an intercellular layer, which is rich in pectic substances, cements the walls of adjacent cells together.

The composition of cell walls varies depending on legume species, cell type and developmental stage. In general, the primary wall consists of 9%–25% cellulose, 25%–50% hemicellulose, 10%–35% pectin, and ~10% protein, whereas the secondary wall consists of 41%–45% cellulose, 30% hemicellulose, and 22%–28% lignin (Yousif et al., 2007). The cotyledon cell wall of legumes is characterised to be predominantly composed of a mixture of polymers including arabinose-rich pectins, beta-glucans, galacturonans and xyloglucans (Shiga and Lajolo, 2006).

## Effects of Processing on Legume Microstructure

Food processing induces varying degrees of physical and chemical changes to the legume cotyledon structure, such as gradual plasticisation and solubilisation of pectic components in the middle lamella, leading to separation of intact cells (Tan et al., 2011). Other changes include rapid protein denaturation, partial starch gelatinisation, oil coalescence, disruption of cell wall integrity and release of intracellular substances (Campbell and Glatz, 2009; Berg et al., 2012). Traditional cooking of legumes by soaking, boiling or steaming is required for softening and textural tenderisation of the seeds prior to consumption. The common techniques employed in food legume processing and their effects on the cotyledon structure are summarised in Table 1.

## Effects of Legume Microstructure on Starch Digestion

Starch-rich legumes contain a significant proportion of a slowly digestible starch fraction, which can reduce the rate of starch digestion and lower the postprandial blood glucose response. A small fraction of indigestible starch in legumes, also referred to as physically inaccessible starch or resistant starch (RS), escapes the small intestine and arrives at the large intestine for fermentation by the colonic bacteria, generating beneficial physiological consequences (Tovar, 1996). The slow digestion properties of carbohydrates achieved upon the cooking and consumption of legumes can be attributed to various factors, including intrinsic properties of starch

**Table 1** Common processing techniques and their effects on legume cotyledon structure

Processing	Effects on legume cotyledon structure
Milling to flour	<ul style="list-style-type: none"> <li>• Disruption of cotyledon cells</li> <li>• Rupture of cell walls</li> <li>• Release of intracellular materials</li> <li>• Multi-cell flour particles containing disrupted cells close to the surface and intact cells in the interior</li> </ul>
Cooking in high-moisture environment (e.g. boiling and steaming, autoclaving)	<ul style="list-style-type: none"> <li>• Solubilisation of the pectin-rich middle lamella leading to separation of intact, swollen cotyledon cells</li> <li>• No to little disruption of cell walls</li> <li>• Leaching of soluble materials (e.g. amylose and soluble sugars) out of cells</li> <li>• Rapid denaturation of proteins</li> <li>• Partial gelatinisation of starch granules inside cells and distortion of the granule shape</li> </ul>
Oil extraction	<ul style="list-style-type: none"> <li>• Crushing and degradation of cotyledon cells and cell walls</li> <li>• Distortion of protein bodies and starch granules</li> <li>• Coalescence of oil droplets into larger drops of free oil</li> </ul>

Adapted from Berg, T., Singh, J., Hardacre, A., Boland, M.J., 2012. The role of cotyledon cell structure during *in vitro* digestion of starch in navy beans. *Carbohydr. Polym.* 87, 1678–1688; Campbell, K.A., Glatz, C.E., Johnson, L.A., et al., 2011. Advances in aqueous extraction processing of soybeans. *J. Am. Oil Chem. Soc.* 88, 449–465; and Young, C.T., Schadel, W.E., 1990. Microstructure of peanut seed: a review. *Food Struc.* 9, 317–328.

granules (e.g. amylose content and retrograded amylose), interactions between starch and fibers, and presence of amylase-inhibitors (Thorne et al., 1983). Yet, recent evidence has emerged indicating that rigidity and intactness of cotyledon cell structure is the predominant contributing factor to the and slow glycemic features of legumes (Berg et al., 2012; Brummer et al., 2015).

Previous studies have shown that, during cooking of cotyledon tissues (e.g. boiling and steaming), the cell walls and the protein matrix form physical barriers that impose restrictions on water availability, heat transfer and space, required for the swelling and gelatinisation of starch granules inside cells. Consequently, the progression in granular expansion and deformation is suppressed, and the starches in cooked tissues retain some of their ordered structures evidenced by the presence of residual birefringence under polarised light (Edwards et al., 2015; Hahn et al., 1977). In addition, cooking promotes solubilisation of pectic polymers involved in cell–cell adhesion, followed by separation of intact cotyledon cells rather than cell rupture. Since the strong cell walls cannot be disrupted by cooking and exhibit a high level of resistance towards enzymatic hydrolysis, they remain mostly intact throughout subsequent *in vitro* digestion. Moreover, the partially-gelatinised starch is of too high molecular mass to escape via the cell wall pores and remains encapsulated by the cell walls, its exposure to amylases is therefore reduced to a large extent during digestion. The combined effects of the restricted starch gelatinisation and the enzyme barrier properties of the cell walls cause a retardation in the *in vitro* rate and extent of starch digestion in legumes (Berg et al., 2012). Gelatinisation and digestion behaviours of starch inside individual cells isolated from cotyledon tissues have also been investigated. A single intact cell has been shown to have sufficient capacity to limit *in vitro* starch digestibility by providing an effective cell wall barrier against swelling and gelatinisation of intracellular starch granules as well as starch–amylase interactions (Dhital et al., 2016).

An *in vivo* study on the starch digestion in healthy humans after ingestion of cooked white beans provided further robust evidence. A significant proportion of RS was recovered in samples of ileal digesta (the effluent collected at the end of the small intestine). The ileal content, which was collected 3 hours after meals, contained part of the RS in a physically inaccessible form (trapped within intact cells). The formation of the RS was observed to be largely due to the partially degraded starch molecules that escaped small intestinal digestion due to the effect of plant cell wall encapsulation of starch (Noah et al., 1998).

Drastic food processing can be destructive to the cotyledon cells. Indeed, milling or high pressure treatment can disrupt the cell wall integrity and release the starch granules from the intracellular matrix. This makes the starch more accessible to amylolytic attack, resulting in a significant increase in the starch hydrolysis rate (Berg et al., 2012). Therefore, minimal processing for preservation of the intact cell structure is a crucial strategy for maintenance of the low and slow glycemic features of legumes and targeted delivery of RS to the large intestine.

## Conclusion

In short, food legumes are consumed as an important source of energy for humans. The majority of nutrients are assembled in the parenchyma cells of the cotyledons, and encapsulated within the thick, resilient cell walls. The cell walls serve as a physical barrier



that restricts the swelling and gelatinisation of intracellular starch granules, and impedes the access of amylolytic enzymes to the starch. This, in turn, results in the reduced rate and extent of starch digestion following the ingestion of cooked legumes. The study of legume microstructure can further our understanding of the important role it plays in regulating nutrient digestion, and can lead to better utilisation of food legumes.

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# Meat Structure During Processing

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## Glossary

**Comminuted meat products** Meat products that are manufactured from meat ingredients and that are reduced in size by means of grinding, chopping, flaking, or mincing. Commminuted meat products include products that have undergone a restructuring or reformulation.

**Differential scanning calorimetry (DSC)** DSC is a thermoanalytical technique, and the fundamental principle for this technique is that when a sample undergoes a physical transformation such as denaturation or phase transition, more or less energy (heat) is needed. For the measurements the difference in the amount of heat required to increase the temperature of a sample and a reference is monitored as a function of temperature. Whether less or more heat must flow to the sample depends on whether the process is exothermic or endothermic.

**Fourier transform infrared (FT-IR) spectroscopy** FT-IR is a technique used to obtain signals in the infrared spectral range. Infrared radiation is emitted or absorbed by molecules when they change their rotational–vibrational movements and thereby contain information about the structure of the molecule. The term Fourier transform infrared spectroscopy originates from the fact that a Fourier transform, which is a mathematical process, is carried out to convert the raw data into an actual spectrum.

**Meat curing** Curing is a processing method where salt or the combination of different salts (e.g., nitrite, nitrate, phosphate salts) is added to meat to reduce water activity and thereby obtain a preservation effect.

**Myofibrillar proteins** Constitute the structural protein skeleton of the muscle cell. Myofibrillar proteins consist mainly of myosin and actin, but other proteins such as titin are also present. The myofibrillar proteins are organized into thick and thin filaments that give rise to a striated appearance of the muscle fiber in a microscope.

**Proton NMR relaxometry** Spectroscopic technique where nuclei such as protons located in an external magnetic field are excited using a specific radiofrequency. The created signal is influenced by the local environment that the nuclei experience, and thereby information about biophysical/biochemical properties of the sample is provided.

## Nomenclature

AFM Atomic force microscopy

CMC Carboxymethyl cellulose

CLSM Confocal laser scanning microscopy

DSC Differential scanning calorimetry

MCC Microcrystalline cellulose

NMR Nuclear magnetic resonance

SEM Scanning electron microscopy

T<sub>2</sub> relaxation Transverse or spin–spin relaxation

## Curing-Induced Structural Changes

Curing technologies is commonly applied in meat industry to either preserve or enhance meat products. During the process the curing agents (salts) will interact with constituents of the meat and thereby induce structural alterations at the molecular, microscopic, and macroscopic level. At the microscopic level [Offer and Trinick \(1983\)](#) visualized in pioneering work how the myofibrillar structures are modified by curing, which was ascribed to the binding of Cl<sup>−</sup> ions to the myofibrillar proteins with a resultant increased electrostatic repulsion. In their microscopic examinations, [Offer and Trinick \(1983\)](#) also noticed that curing-induced microstructural changes furthermore involve the solubilization of structural constraints in the organization of the myofibrils. This has been corroborated in nuclear magnetic resonance (NMR) T<sub>2</sub> relaxation studies of meat curing, which have confirmed a swelling of myofibrillar spaces caused by a salt-induced solubilization of the myofibrillar proteins ([Andersen](#)

et al., 2007). The analysis of intrinsic water populations in meat by NMR relaxation as a function of curing has also suggested that the separation of myofibrillar and extra-myofibrillar water that exist in fresh meat is disrupted during curing, resulting in the formation of a more “souplike” swollen protein matrix (Andersen et al., 2007).

At the molecular level, probing of protein secondary structures by Fourier transform infrared (FT-IR) microspectroscopy has also revealed that meat curing is associated with the formation of aggregated  $\beta$ -sheet structures in the myofibrillar proteins at the expense of  $\alpha$ -helical structures (Wu et al., 2006; Böcker et al., 2006).

In recent years, attempts have been made to relate curing-induced structural changes at the molecular, microscale, and macroscale level, respectively. Bertram et al. (2008) combined proton NMR relaxometry with confocal laser scanning microscopy (CLSM) and atomic force microscopy (AFM) to identify the associations between detailed structural changes and changes in water–protein interactions and water mobility and distribution. Different curing agents including  $\text{NaHCO}_3$ ,  $\text{Na}_4\text{O}_7\text{P}_2$ , and  $\text{NaCl}$  were examined, and from the CLSM examinations, it was evident that  $\text{NaHCO}_3$  resulted in a much more extensive swelling of the myofibrillar structures (Fig. 1). AFM analyses suggested that the different actions of  $\text{NaCl}$  and  $\text{NaHCO}_3$  could be attributed to less solubilization and more severe protein denaturation after  $\text{NaCl}$  curing. NMR relaxation analyses confirmed that the different curing agents influenced water–protein interactions differently, probably reflecting the differences in structure at the molecular level caused by variations in the effect of curing agent on protein conformations and protein denaturation (Bertram et al., 2008).

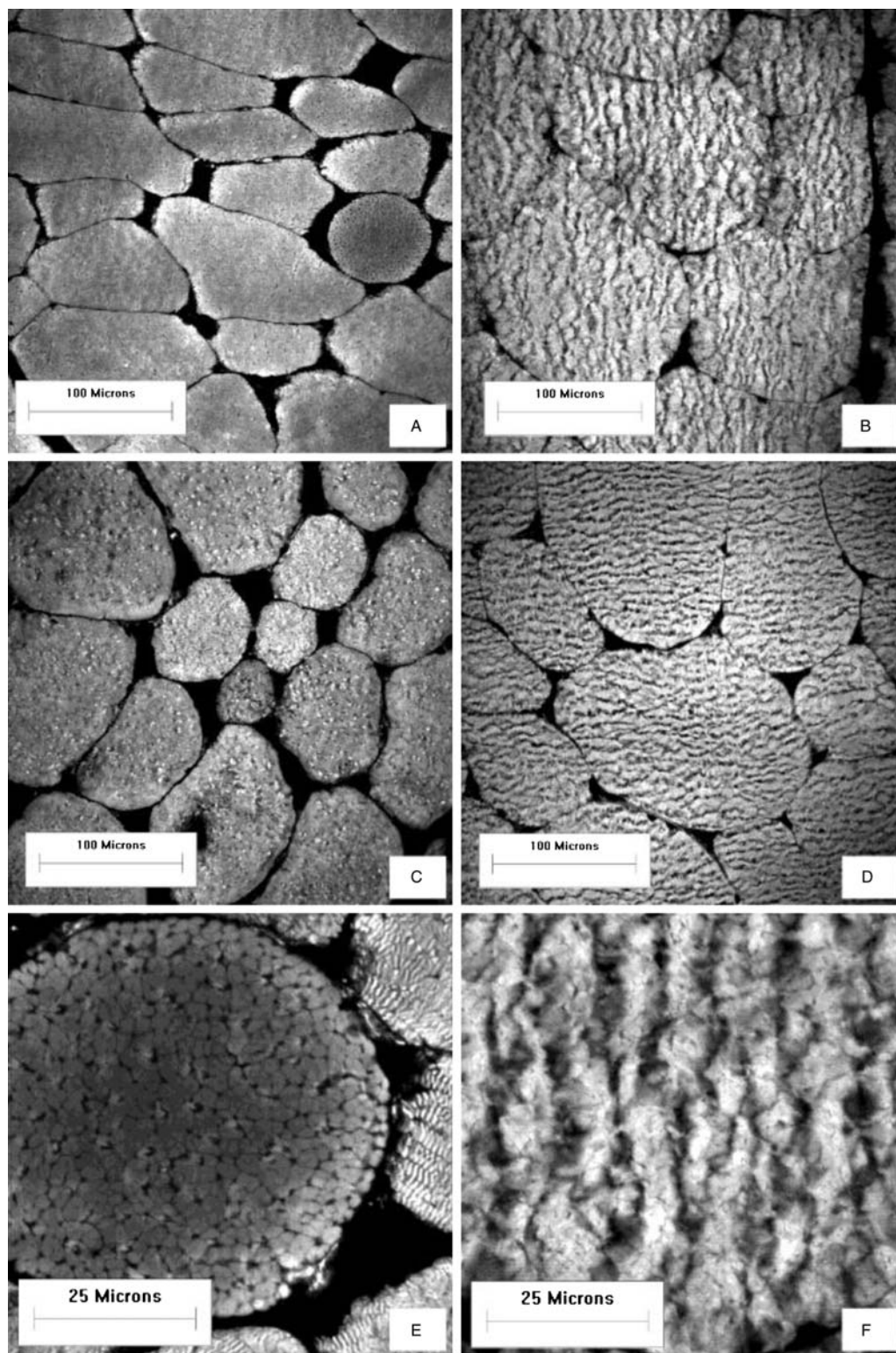
### Heat-Induced Structural Changes

Meat commonly undergoes heat-treatment as part of manufacturing or during cooking before consumption. Heat-treatment of meat is a multifaceted process involving several biophysical and chemical changes giving rise to structural changes. At the macroscopic level, an overall shrinkage of structures is seen during heat-treatment of whole meats. Bendall and Restall (1983) studied the physical changes during heat-treatment of myofiber bundles and individual muscle fibers up to 90 °C and found that up to about 63 °C the main physical change is a transverse shrinkage while above this temperature shortening of the muscle fibers also takes place. Later studies have reported similar results (Tornberg, 2005).

At the molecular level, heat will typically cause proteins to lose their native conformation (denature). Thus, the application of a heat-treatment (cooking, boiling, frying) to meat will cause a denaturation of the meat proteins, which is constituted of myofibrillar proteins (myosin, actin), connective tissue proteins (collagen), and sarcoplasmatic proteins. Differential scanning-calorimetry (DSC) has been used to examine heat-induced denaturation of meat proteins (Stabursvik et al., 1984; Bertola et al., 1994). DSC determines the endothermal or exothermal energy generated from a phase transition and can thereby provide information about the denaturation state of a protein. In the analysis of meat, up to four peaks corresponding to four different thermal transitions have been detected in the DSC thermogram (Zielbauer et al., 2016). Several studies have attempted to assign the detected DSC peaks to specific meat protein fractions. However, such an assignment is complicated because intermolecular interactions between the different meat proteins and the molecular environment (pH, ionic strength) will influence the transition temperature of the individual proteins. However, comparison of DSC measurements on isolated protein fractions and intact meat is to some extent plausible, and it is generally assumed that an endothermic peak in the temperature range 45–55 °C is reflecting the denaturation of myosin heads and that peaks in the temperature range 55–75 °C is reflecting the combined denaturation of myosin tails and sarcoplasmatic and collagen proteins, whereas actin denaturation is reflected by an endothermic peak around 72–75 °C (Macfarlane et al., 1981; Stabursvik et al., 1984; Pospiech et al., 2002; Zielbauer et al., 2016).

As the temperature increases, an unfolding of the meat proteins sets in with a resultant loss of their native conformation. When tertiary and secondary structures are severely disrupted, the proteins may aggregate. This phenomenon and other changes in protein secondary structures can be studied by using FT-IR microspectroscopy, which probes the molecular structure based on IR energy absorption of chemical bonds at specific frequencies related to molecular vibrations. FT-IR studies have revealed that heating of meat is accompanied by an increase in aggregated  $\beta$ -sheet structures at the expense of a decrease in  $\alpha$ -helical structures (Kirschner et al., 2004; Wu et al., 2007).

Meat is comprised by approximately 75% water, and the heat-induced structural changes in the meat proteins will concomitantly influence intrinsic protein–water interactions. Several studies have probed these heat-induced changes in protein–water interactions in meat by low-field proton NMR relaxometry (Fjellkner-Modig and Tornberg, 1986; Borisova and Oreshkin, 1992; Bertram et al., 2004; Christensen et al., 2011). Intriguingly, dynamic NMR relaxation measurements conducted during heating of meat have revealed that the water–protein interactions undergo substantial changes at 43 °C and 56 °C (Micklander et al., 2002; Bertram et al., 2005). These major shifts in water–protein interactions have been attributed to the onset of myosin denaturation and collagen denaturation, respectively, where the latter causes structural constraints and shrinkage (Bertram et al., 2005). A study combining DSC measurements and proton NMR relaxometry during meat cooking has confirmed a close relationship between meat protein denaturation and changes in protein–water interactions and intrinsic water mobility and distribution in the meat (Bertram et al., 2006).



**Figure 1** Confocal laser scanning microscopy (CLSM) images of unheated samples: (A) control sample, 40 $\times$  magnification; (B) sodium bicarbonate-treated sample, 40 $\times$  magnification; (C) sodium chloride-treated sample, 40 $\times$  magnification; (D) sodium pyrophosphate-treated sample, 40 $\times$  magnification; (E) control sample, 60 $\times$  magnification; (F) sodium bicarbonate-treated sample, 60 $\times$  magnification. Reprinted with permission from Bertram, et al., 2008. Water Distribution and Microstructure in Enhanced Pork. *J. Agric. Food Chem.* 56 (16), 7201–7207. Copyright (2008) American Chemical Society.



## Structural Elucidation of Comminuted Meat Products

Comminuted meat products represent a major proportion of processed meat sold in retail. Typical product types include sausages manufactured by the addition of various ingredients to enhance and optimize functional attributes, texture, and taste. These comminuted meat products are traditionally based on raw materials with a relatively high fat content and/or addition of lard and typically contains 20%–30% fat and high water content as well. In the manufacturing of comminuted meat products, the addition of salt to the beef patties will solubilize the meat proteins, which will then act as a “glue” to stabilize the matrix. During a subsequent heat-treatment, the solubilized meat proteins will assist to form a continuous network with the fat particles and other coarse ingredients incorporated. The exact role of the proteins in the formation of a comminuted meat product has been subject for investigations, and different models have been proposed. Thus, it has been proposed that the structure of comminuted meat products can be considered and explained as a matrix exhibiting behavior as an emulsion with fat in a protein solution where the solubilized meat proteins act to stabilize the fat–water interface. The structure of comminuted meat products has also been explained based on a model of physical entrapment of fat particles and other coarse ingredients within a protein gel matrix formed during heat-treatment where an interfacial film made of meat proteins may surround the fat particles to stabilize these and hinder their coalescence (Gordon et al., 1992). A combination of CLSM and scanning electron microscopy (SEM) for examination of the structure of comminuted meat products coupled with detailed analyses of fat and water retention (determined gravimetrically) has revealed that although an interfacial film of protein forms at the surface of fat droplets, it is the intrinsic structure in terms of pore characteristics (pore size and distribution in the network) that is decisive for fat and water retention in a comminuted meat product (Liu et al., 2016).

Over the last decade, an increasing awareness of a strong link between dietary patterns and human health has emerged, and concern in relation to how intake of especially processed meat products affects our health has also arisen. Consequently, even though it is still a matter of dispute what role animal fat plays in relation to lifestyle diseases (Astrup et al., 2011; Lawrence, 2013), an interest in fat replacement or enrichment of comminuted meat products with health-promoting ingredients such as dietary fiber has emerged. Components that have been investigated as fat replacers in comminuted meat products include a variety of hydrocolloids, modified starches, and dietary fibers. A major challenge with fat reduction of meat products is to maintain the same textural attributes (Claus, 1991; Weiss et al., 2010). In this context, it is essential to understand how the intrinsic meat components as well as added ingredients influence matrix structure to be able to obtain a product that meets consumer satisfaction. Gibis et al. (2015) investigated how the use of carboxymethyl cellulose (CMC) and microcrystalline cellulose (MCC) as fat replacers influences the microstructure of fried beef patties. Using CLSM, Gibis et al. (2015) found that CMC and MCC exhibited very different effects on the microstructure of the beef patties. Even at a concentration of 1% (w/w) CMC, severe changes in the structure of the protein network were observed during frying. And at higher concentrations of CMC, a highly porous and uneven network was observed, revealing a low incorporation of CMC fibers into the protein-formed network. In contrast, MCC could be added in concentrations up to 3% (w/w) without negatively influencing the microstructure of the fried beef patties, and MCC displayed a much better capability for incorporation in the network formed by the meat proteins (Gibis et al., 2015). However, studies on other comminuted meat products have shown that the addition of CMC is very efficient in reducing moisture loss during heat-treatment (Barbut and Mittal, 1996; Han and Bertram, 2017). Dynamic NMR relaxation measurements during heat-treatment of a model meat system added a range of different fibers revealed that in the temperature range between 60–70 °C, the expulsion of water located in myofibrillar spaces was counteracted better by the addition of CMC than any other of the tested fibers (Han and Bertram, 2017). NMR relaxation measurements have also been used to indirectly probe the structural features at the molecular level in the study of addition of other components to comminuted meat products. A recent study investigated the addition of cassava starch to pork sausages, and proton NMR relaxometry could reveal that cassava starch addition increased the proportion of water located within the myofibrillar protein matrix (Zhang et al., 2014).

In addition to fat replacement with carbohydrate-based components, fat in comminuted meat products may also be substituted with alternative, healthier fat types. Thus, substitution of fat with olive oil (Ruiz-Capillas et al., 2012) and canola oil (Barbut et al., 2016) incorporated in a gel matrix has been investigated in Frankfurter-like sausage types. Structural features and interactions between protein and lipid constituents were investigated using Raman spectroscopy (Herrero et al., 2014). Raman spectroscopy showed that fat replacement with the olive oil bulking agent was associated with an increase in the amount of  $\beta$ -sheet structures (band at 1620–1720  $\text{cm}^{-1}$ ) in the proteins. This finding is likely reflecting the formation of aggregated intermolecular  $\beta$ -sheet structures, which has been found to be associated with network formation accompanied by a firm texture (Herrero et al., 2014). Specific Raman bands originating from lipid molecules also revealed differences in the structure of the lipids associated with fat replacement with the olive oil bulking agent that could indicate enhanced lipid–protein interactions in the reformulated product (Herrero et al., 2014).

## Concluding Remarks

The introduction of advanced spectroscopic and microscopic techniques in meat research has enhanced our understanding of meat structural characteristics at both the molecular and microscopic level. It is anticipated that these techniques will be advantageous in future where meat industry likely will be facing increasing demands for new, healthier product solutions. Such a demand will entail

product developments that require a comprehensive understanding of associations between raw material and ingredient properties, structural and functional attributes, and product texture and taste.

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# The Structure and Properties of Eggs

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## Structure of an Egg

The predominant function of an egg is a vessel for a developing embryo. At the centre of an egg is the yolk, surrounded by the egg white (albumen) and membranes within a hard calciferous shell. The yolk forms and develops in the ovary of the avian female and is released into the oviduct following ovulation, where it develops further, with first the albumen and then the shell membranes and shell deposited (Chambers et al., 2017). In a chicken egg, the albumen accounts for most of the mass at approximately 60%, followed by the yolk, 30% and the shell at 10% (Chambers et al., 2017).

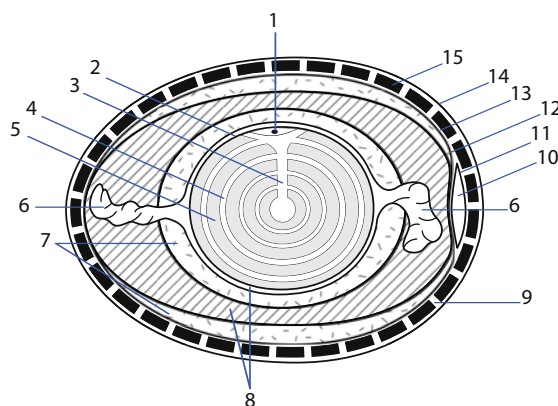
The components inside the egg are organised by membranes. Two closely adhering membranes; the inner and outer shell membranes are composed of a network of various protein polysaccharide fibres (Belitz et al., 2009). These act as a barrier against bacteria, separate the albumen and shell, provide the scaffolding for shell formation in the oviduct and form a pocket of air between themselves, known as the air cell. The air cell is formed at the blunt end of the egg by the cooling and contracting of egg contents in the oviduct (Chambers et al., 2017). It is initially 5 mm in diameter, but this increases upon storage and provides a useful marker for estimating the age of an egg (Belitz et al., 2009).

The egg yolk is held in the centre of the egg by opaque twisted albumen 'ropes', which twist clockwise at the blunt end of the egg and counterclockwise at the tip. When an egg is cracked open, the chalazae separate from the albumen and remain with the yolk. On one side of the yolk, there is a latebra on which the blastoderm or germinal disc is found. The yolk is composed of plasma and granules (Guha et al., 2018) and is prevented from leaking into the albumen by the transparent vitelline membrane (Chambers et al., 2017) (Fig. 1).

## EggShell

The eggshell is essential for avian breeding and has three main functions; to protect the egg from the outside environment, to control water and gas flow during chick development and provide calcium for chick development after the yolk stores have been exhausted (Nys et al., 2004). It is a porous semi-permeable structure, which is, comprised mainly of calcium carbonate (94%) (Chambers et al., 2017). The shell can be divided into 4 parts; cuticle or bloom, spongy layer, mammillary layer and pores. The cuticle is the thinnest and outermost layer, approximately 10 µm and is composed of mucilaginous protein (Belitz et al., 2009). The spongy layer makes up the bulk of the shell thickness and is cemented to one side of the mammillary layer, with the other side of the mammillary layer adhered to the shell membrane (Belitz et al., 2009). Pores allow for the limited flow of gases in and out of the shell while the cuticle serves as a barrier against microorganisms, dust and debris (Chambers et al., 2017). The composition of the shell provides a protective environment for the developing embryo.

Eggshells vary in colour, generally due to the need for environmental camouflage dependent on the birds habitat. Domestic chicken shells are generally white or brown but some genotypes of hen produce blue and green eggs (Chambers et al., 2017). Duck eggs vary from white through blue and green, whereas many wild birds and game birds; quail, pheasant, guinea fowl for example lay spotted eggs. It has been observed that Japanese quail seek out substrates to lay their eggs based upon those that provide



**Figure 1** Schematic cross-sectional diagram of a chicken egg modified from (Belitz, Grosch, & Schieberle, 2009). 1 germinal disc (blastoderm), 2 yolk membrane, 3 latebra, 4 light colored yolk layer, 5 dark colored yolk layer, 6 chalaza, 7 egg white (albumen) thin gel, 8 albumen thick gel, 9 pore, 10 air cell, 11 shell membrane, 12 inner egg membrane, 13 shell surface cemented to the mammillary layer, 14 cuticle, 15 spongy calcareous layer.

the most camouflage. A study by Lovell et al. (2013) showed that the selection of laying substrate by the females was influenced by the degree of egg maculation (or spots on the eggshell), with females choosing laying substrate that matched the degree of maculation of their own eggs. This suggested that these quail inherently “understood” their own eggshell patterning and were able to choose substrate for optimal camouflage based upon their own phenotype. In the commercial sector, the colour of chicken eggs does drive regional consumer demand although there are no differences in the quality and sensory properties of the egg such as taste, based on its colour (Bell, 2002).

### Egg White (Albumen)

Accounting for the bulk of the volume of an egg, the albumen is an aqueous solution of various globular proteins, transparent with a yellow tint in colour. Albumen is 88% water, 11% protein, 1% carbohydrates and trace amounts of lipids (<1%) (Kovacs-Nolan et al., 2005). Both thin and thick albumen are present in the egg, with the latter differing in viscosity due to an increased presence of ovomucin protein. Albumen from a freshly laid egg has a pH of 7.6–7.9 and during storage increases to 9.7 because of solubilized CO<sub>2</sub> diffusing through the shell (Belitz et al., 2009). There are several important proteins present in albumen, these include the major proteins; ovalbumin, ovotransferrin, ovomucoid, lysozyme and ovomucin and the minor proteins; avidin, cystatin, ovomacroglobulin, ovoflavoprotein, ovoglycoprotein and ovomucoid. Of these proteins, the most abundant is ovalbumin, a phosphorylated glycoprotein, which accounts for 54% of the total protein in egg white (Kovacs-Nolan et al., 2005). Ovalbumin is denatured quite easily during whipping or vigorous shaking of its aqueous form (Belitz et al., 2009), a property of great use in the food industry when making whipped desserts, souffles, baking etc. The proteins in albumen exhibit biological activity, many of which are related to protecting the egg from spoilage microorganisms. They act as enzymes, for example, lysozyme, as enzyme inhibitors in the case of ovomucoid and ovomucoid, as well as complex-forming agents for coenzymes, for example, flavoprotein and avidin (Belitz et al., 2009).

### Egg Yolk

The egg yolk is a complex system containing a number of insoluble suspended particles, approximately 0.3–2 µm in size (granules) in a clear-yellow fluid (plasma) (Guilmineau and Kulozik, 2006). It is an oil-in-water emulsion composed of lipids (65%), proteins (31%), carbohydrates, vitamins and minerals (4%) by dry weight (Belitz et al., 2009). Yolk plasma accounts for 80% of the yolk fraction (Freschi et al., 2011) and is comprised of 85% low-density lipoproteins (LDLs) and 15% globular glycoproteins, predominantly α-, β- and γ-livetins (Laca et al., 2015). The granules on the other hand consist of High-density lipoproteins (HDLs) (70%), phosphatidylcholine (16%) and LDLs (12%) (Anton, 2013; Belitz et al., 2009).

## Functional Properties of Eggs

### Nutrition

Eggs from many different birds are a staple of the human diet and are consumed worldwide. The Food and Agriculture Organization of the United States (FAO), estimates that to 8.9 kg of eggs are consumed per capita, per year worldwide, amounting to approximately 167 eggs per person, per year. This corresponds to an increase of 41% since 1990 and global egg production is now in excess of  $6.5 \times 10^7$  ton per year (FAO, 2016).

Reasons behind the popularity of eggs is that they are often called a ‘complete food’ as they contain many essential nutrients (Table 1) and are versatile with numerous uses as a meal on their own or an ingredient in sweet and savory foods (Song and Kerver, 2000). They are inexpensive, accessible to most people and are an important source of high-quality protein (Chambers et al., 2017). The average chicken egg contains over 6 g protein and is a source of the 9 essential amino acids for human health (Lunven et al., 2007).

Eggs contain the fat soluble vitamins A, E, K and D as well as B vitamins and are a good source of B2 and B12 (Chambers et al., 2017). They contain many essential minerals and also antioxidants. Carotenoids, specifically xanthophylls, lutein and zeaxanthin to name a few, are absorbed from the hens feed and are responsible for the colour of the yolk, the feed can be manipulated in terms of the carotenoid type and content to intensify the yolk colour (Belitz et al., 2009).

The lipid profile of chicken eggs contains the essential long-chain polyunsaturated fatty acids (LCPUFA), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and also arachidonic acid. EPA and DHA have many proven health benefits, in areas such as cardiovascular, brain and eye health (Fraeye et al., 2012). The amount and type of LCPUFA in eggs can also be modulated through the feed.

Eggs do contain a high amount of cholesterol, approximately 200 mg and thus they have been vilified in the past and the subject of many health warnings (Brown and Schrader, 1990), however, cholesterol is essential for normal function of the human body and nowadays guidelines indicate that in moderation eggs are a good food choice as part of a balanced diet (HHS and USDA, 2015).

**Table 1** Nutrient composition of 1 whole fresh egg  
(1 large egg = 50 g)

<i>Nutrient</i>	<i>Concentration</i>
Water	38.08 g
Energy	72 kcal
Energy	300 kJ
Protein	6.28 g
Total lipid (fat)	4.75 g
Ash	0.53 g
Carbohydrate, by difference	0.36 g
Fibre, total dietary	0 g
Sugars, total	0.18 g
<b>Minerals</b>	
Ca	28 mg
Fe	0.88 mg
Mg	6 mg
P	99 mg
K	69 mg
Na	71 mg
Zn	0.65 mg
Cu	0.036 mg
Mn	0.014 mg
Se	15.3 µg
F	0.6 µg
<b>Vitamins</b>	
Vitamin C, total ascorbic acid	0 mg
Thiamin	0.02 mg
Riboflavin	0.229 mg
Niacin	0.037 mg
Pantothenic acid	0.766 mg
Vitamin B-6	0.085 mg
Folate, total	24 µg
Folate, DFE	24 µg
Choline, total	146.9 mg
Betaine	0.1 mg
Vitamin B-12	0.45 µg
Vitamin B-12, added	0 µg
Vitamin A, RAE	80 µg
Retinol	80 µg
Cryptoxanthin, beta	4 µg
Vitamin A, IU	270 IU
Lutein + zeaxanthin	252 µg
Vitamin E (alpha-tocopherol)	0.53 mg
Tocopherol, beta	0.01 mg
Tocopherol, gamma	0.25 mg
Tocopherol, delta	0.03 mg
Vitamin D (D2 + D3)	1 µg
Vitamin D3 (cholecalciferol)	1 µg
Vitamin D	41 IU
Vitamin K (phylloquinone)	0.1 µg
<b>Lipids</b>	
Saturated FA	1.563 g
Monounsaturated FA	1.829 g
Polyunsaturated FA	0.956 g
22:5 n-3 (DPA)	0.004 g
22:6 n-3 (DHA)	0.029 g
Trans FA	0.019 g
Cholesterol	186 mg
<b>Amino Acids</b>	
Tryptophan	0.083 g
Threonine	0.278 g
Isoleucine	0.336 g

(Continued)

**Table 1** Nutrient composition of 1 whole fresh egg  
(1 large egg = 50 g)—cont'd

<i>Nutrient</i>	<i>Concentration</i>
Leucine	0.543 g
Lysine	0.456 g
Methionine	0.19 g
Cystine	0.136 g
Phenylalanine	0.34 g
Tyrosine	0.249 g
Valine	0.429 g
Arginine	0.41 g
Histidine	0.154 g
Alanine	0.367 g
Aspartic acid	0.664 g
Glutamic acid	0.837 g
Glycine	0.216 g
Proline	0.256 g
Serine	0.485 g

RAE, retinol activity equivalents; USDA, United States Department of Agriculture.

Modified from USDA Nutrient Database [USDA, 2018](#).

Overall, the nutrient composition of the egg remains relatively consistent among its own species but differences in diet, age of the chicken and environment can alter the composition somewhat ([Rizzi and Marangon, 2012](#)).

### Physicochemical Properties

Eggs exhibit a range of properties that are of use in the food industry. Eggs can be used to impart colour in foods such as fresh egg pasta or to add a distinct aroma or flavor such as in egg liquors. However, their most important functions are; emulsifying, foaming (whipability) and heat or pH induced coagulation, which will be briefly discussed here.

### Emulsification

An emulsion of two immiscible liquids such as oil in water where oil droplets are dispersed throughout a water phase often requires an emulsifier for stability. Egg is used as an emulsifier in a range of products, most commonly in mayonnaise and salad dressings. Both the egg yolk and whole egg have good emulsifying properties, due to the high proportion of LDLs and proteins that are present ([Anton et al., 2001](#)) and are used in the food and pharmaceutical industries. For industrial applications the egg yolk in its entirety can be used as an emulsifying agent or separated into granules (the precipitate) and plasma (the supernatant) by way of a fractionation method of dilution and centrifugation. Plasma shows the better emulsification properties compared to granules ([Anton, 2013](#)), however, granules are thermally stable, with greater heat-resistance than plasma. Seeing as protein solubility is also related to emulsifying properties, the greater heat sensitivity of plasma causes protein solubility to decrease when the temperature reaches 69 °C and a concurrent increase in viscosity is observed ([Le Denmat, Anton and Gandemer, 1999](#)). This makes granules a good option as an emulsifier in products that undergo pasteurization ([Anton, 2013](#)). From a nutrition standpoint using separated granules instead of the whole yolk decreases the total cholesterol content ([Laca et al., 2010](#)).

### Coagulation or Gelation

The coagulation properties of eggs make them effective binding agents and have applications in a range of food products ([Belitz et al., 2009](#)). Egg protein coagulation is a heat and pH dependent process, which is key to a range of physical and sensory properties in a variety of products. The proteins in egg white start to coagulate first at approximately 62 °C and the egg yolk at 65 °C and if pH is modified the egg white can also form a gel at room temperature at approximately pH 11.9 ([Belitz et al., 2009](#)).

When heat is applied to egg, the proteins undergo a conformational change and become partially denatured followed by aggregation of the denatured proteins. This causes an exponential increase in viscosity and the development of a continuous gel network, which needs to occur slowly to ensure a well-organized network, not a random network, which is prone to syneresis ([Phillips et al., 1994](#)). Formation of the network is due to the creation of covalent linkages, modifications to the thiol group-disulfide linkages and hydrophobic interactions ([Phillips et al., 1994](#)).

## Foaming

A foam is a 2-phase liquid-air system in which the mechanical action of whipping entraps air in a dispersed phase, increasing surface area and denaturing proteins. These denatured proteins form a thin layer, the surface phase (Li et al., 2018). The foaming properties of whipped egg white makes it an excellent leavening agent and thus a key ingredient in various foods (Belitz et al., 2009) such as soufflés, meringues, and cakes. Foaming agents must have the ability to rapidly adsorb at the air–water interface, experience conformational change at the interface and through intermolecular interactions form a cohesive viscoelastic film (Du et al., 2002). The physiochemical properties of the foam and its resulting stability are dependent on a number of factors including; microenvironment, temperature and composition of the solution (Ahmed et al., 2003; Bovskova and Míková, 2011).

The foaming characteristics are different between the different albumin proteins and in terms of importance in foam formation and stability can be ranked as follows: ovalbumin, ovotransferrin, lysozyme, ovomucoid, and ovomucin (Du et al., 2002). The superior foaming properties of Ovalbumin in particular, are enhanced by mild heat treatments, where its native rigid conformational state changes to an intermediary molten globule (partially unfolded stable) state, which due to its greater surface hydrophobicity and flexibility exhibits greater foam stability (Relkin et al., 1999). However, if heating increases, the intermediary becomes unstable and reaches the point of coagulation, causing a decrease in foaming ability (Relkin et al., 1999). The whipability of egg white is measured by the volume of foam and its stability over time, if the eggs are not separated cleanly, significant reduction in foam formation occurs even with as little as 0.1% yolk present (Belitz et al., 2009).

However, the yolk itself also has the ability to form foams, albeit with different properties to egg white foams. A stable foam of 6-fold volume increase is possible in egg yolk at elevated temperatures, 72°C producing the best results (Belitz et al., 2009). Heat stable sauces frequently use egg yolk as a foaming agent. Protein coagulation (and thus foam volume reduction), which occurs if temperatures increase further can be inhibited by the addition of a pH lowering ingredient, often acetic acid (Belitz et al., 2009).

## The Egg Industry

### Eggshell Waste

The eggshell and membranes account for approximately 10.2% of the whole egg and is a major waste product of the food industry (King'ori, 2011). Concern is growing on how to curb eggshell waste as costs involved in safely disposing eggshells are high due to their classification as a hazardous waste product (Laca et al., 2017). The two components of the eggshell; the shell itself and the shell membrane have different properties that can be exploited as useful byproducts in a range of industries.

The eggshell is a good source of calcium which can be easily extracted and used as a powdered calcium supplement (Brun et al., 2013) for humans and in animal feed. It is also commonly used in fertilizers and as a soil stabilizer (Amu et al., 2005). Eggshells have a history of being used as an adsorbent for heavy metals and organic dyes (Guru and Dash, 2014). As well as being used as a calcium source for making bioceramics (Balázi et al., 2007), composite fillers (Toro et al., 2007) and as environmentally friendly catalysts in industrial processes such as biodiesel production (Shan et al., 2016) for example. More recently in the medical field, eggshells have been successfully used in the development of advanced materials such as hydroxyapatite for bone tissue repair (Ramesh et al., 2016). Other potential applications make use of the eggshells natural UV protection (Maurer et al., 2014), where a study by Fechey-Lippens et al. (2017) found that chicken eggshells help to protect polystyrene and nylon from UV damage.

Shell membranes mainly consist of type I, V and X collagen (Arias et al., 1992) and this can be extracted and used in a number of applications in the food, cosmetic, biochemical and pharmaceutical industries. Components of eggshell membrane have been used as biosensors (Zhang et al., 2007) and biotemplates (Liang et al., 2014) to name a few applications.

### Future Prospects of the Egg Industry

Eggs are a key part of the human diet and have a wide range of applications as a food ingredient and in other industries. Due to their popularity the increasing production of eggs to meet consumer demand has and will continue to lead to increased amounts of hazardous waste. However, the range of uses for eggshells mentioned here offers just a small insight into the endless possibilities of adding value to eggshell waste and in doing so increasing the sustainability of the egg industry. This would provide solutions for the estimated 8 million tonnes of eggshell waste that are currently produced each year (De Angelis, Medeghini, Conte and Mignardi, 2017).

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## Crustacean By-products

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### Introduction

The shellfish processing industry has seen significant expansion in the past few decades and each year production rates are several thousand tons. This industry generates a significant amount of biowaste, i.e., mainly heads and exoskeletons, that may represent around 50%–70% of the initial weight (Nguyen et al., 2017). Crustacean waste is mainly organic matter (60%) with the remaining waste being the discarded shells and carapaces of crabs, lobsters and prawns (Pfeiffer, 2003). Usually the edible part of crustaceans only represents 20%–25% which leads to the unavoidable generation of waste (Jayathilakan et al., 2012). Currently, they may be discarded at sea, burned, landfilled, or simple left out to spoil. Each of these techniques can create environmental hazards (Bellich et al., 2016). Unfortunately, this suggests that valuable compounds such as chitin, proteins, and carotenoids are lost in the absence of further processing (Jaswir et al., 2011; Gortari and Hours, 2013; Senevirathne and Kim, 2012; Lodhi et al., 2014).

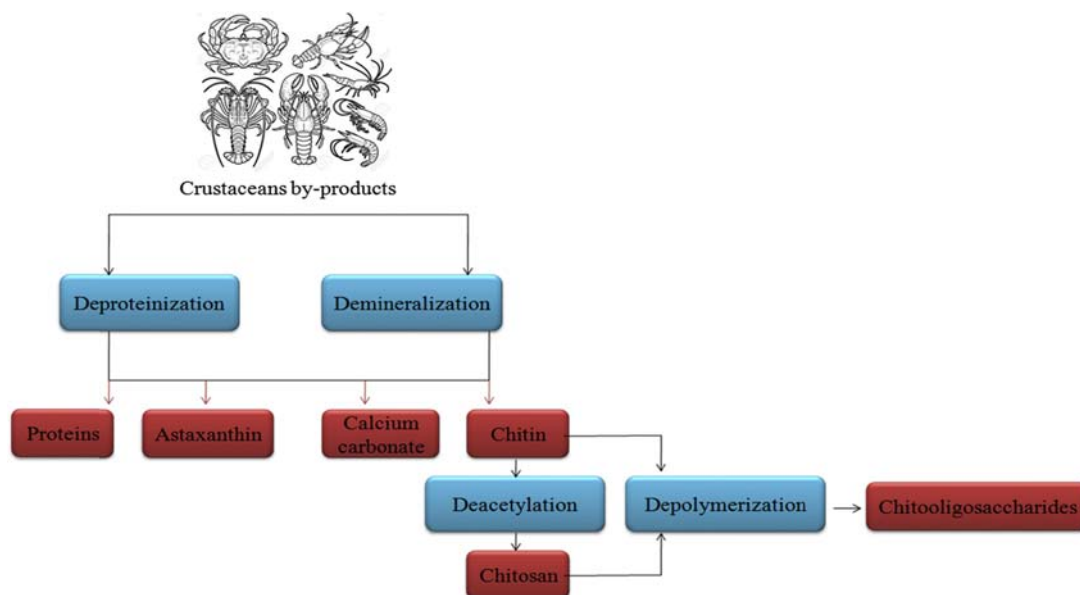
Chitin and its derivatives are non-toxic, biocompatible and biodegradable compounds that have many biological activities including antioxidant, anti-cancer, anti-microbial and anti-coagulant activities (Cheung et al., 2015; Azuma et al., 2015). In addition to the chitin, the waste stream is also rich in other compounds of high nutritional value and functionality including proteins, which can be up to 40% of the total waste weight, as well carotenoid pigments, mainly astaxanthin, which are responsible for the typical crustacean orange colour and are well known for their high antioxidant functions (Arancibia et al., 2014).

Underutilization of crustacean by-products not only leads to loss of potential profits but also requires paying disposal costs (Ospina Álvarez et al., 2014). Therefore, the industry has begun to develop technologies to make use of this waste and produce value-added compounds (Malaweera and Wijesundara, 2014).

This chapter identifies opportunities to develop value-added products from crustacean-processing by-products with different biological activity such as antioxidant, antibacterial and antifungal properties. Crustacean by-products such as chitosan are characterized by high antibacterial and fungicidal activities.

### Bioactive Compounds From Crustaceans and Their Biological Activities

From an economical point of view, it would be quite profitable to recover the by-products released from crustacean processing. The shell waste consists of the head, shell, and tail portions of crustaceans (Nguyen et al., 2017). Crustacean by-products include proteins, chitin, astaxanthin, and calcium carbonate (Fig. 1). Many studies have indicated that chitin, chitosan, and their derivatives



**Figure 1** Extraction of valuable compounds from crustacean by-products.

**Table 1** Biological properties of crustacean by-products and field of application

Bioactive compounds	Activities	Field	Applications	References
Chitin and its derivatives	Antimicrobial Antioxidant Anti-inflammatory	Pharmaceutical Biomedical Cosmetic Textiles Agriculture Ophthalmology	Non-viral vectors for gene and drugs delivery Fabrication of contact lenses Skin, hair, and oral care products Prebiotics ingredients Barrier against food spoilage (edible packaging) Seed-coating agents and protection against microorganisms and pests	Riva et al. (2011) Zeng et al. (2012) Elieh-Ali-Komi and Hamblin (2016)
Astaxanthin	Antioxidant Anti-inflammatory Anti-diabetic activity Cardiovascular disease prevention	Food Cosmetic Textile Aquaculture Pharmaceutical	Natural food colourants Colourant for cosmetic products Feed additives in aquaculture to improve the colouration of salmonids and enhance other physiological functions (growth, reproduction, and immunity)	Ambati et al. (2014) Gómez-Estaca et al. (2017) Lim et al. (2017) Rahman et al. (2016)
Proteins and hydrolysates	Antihypertensive, Hypoglycemic Antioxidant, Hypocholesterolemic, Antimicrobial Anti-coagulant	Pharmaceutical Food Cosmetic	Emulsifying agents, Prevent lipid oxidation and extend shelf-life	da Silva et al. (2017) Nasri (2017) Cheung et al. (2015)

are biologically active polysaccharides. Potential uses in agriculture, water treatment, cosmetics, and the food and pharmaceutical industries are shown in [Table 1](#) ([Cheung et al., 2015](#); [Mao et al., 2017](#)).

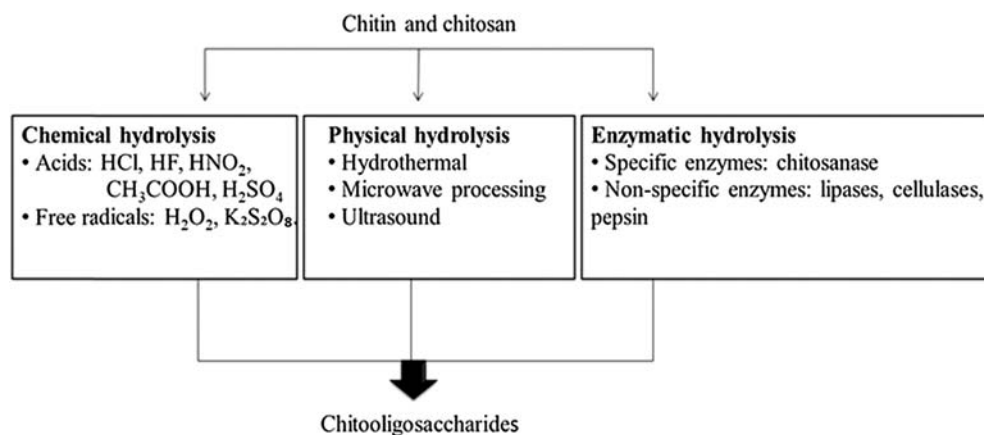
Chitin and chitosan are very abundant natural polymers with distinctive properties, such as bioactivity, biocompatibility and biodegradability ([Fenice, 2017](#)). The use of chitin and chitosan in food applications may involve the formation of biodegradable films and the preservation of food against microbial deterioration due to their antimicrobial activity. Chitosans have applications as coatings in meat and seafood products to prevent lipid oxidation, microbial growth and moisture loss. Chitosans also have pharmaceutical, medical, paper production, textile, wastewater treatment, biotechnology, cosmetics, and agriculture applications ([Bashar and Khan, 2013](#)). Currently, chitin and chitosan cannot be competitively produced using chemical synthesis or biotechnology and are, therefore, sourced from animals, usually from crustacean shells ([Vázquez et al., 2017](#)).

### Chitin and Its Derivatives (Chitosan and Chitooligosaccharides)

Chitin ( $C_8H_{13}O_5N$ )<sub>n</sub> is the second most abundant biopolymer on earth following cellulose. Chitin is a cellulose-like polysaccharide, which is a linear poly- $\beta$ -(1,4)-N-acetyl-D-glucosamine ([Anastopoulos et al., 2017](#)). It is a highly hydrophobic compound that is odourless and tasteless with a white or yellowish colour ([Kumar, 2000](#)). It can be solubilized in a few solvents such as fluorinated solvents (hexafluoroisopropanol, hexafluoroacetone), and chloroalcohols in conjunction with aqueous solutions of mineral acids, and dimethylacetamide containing 5% lithium chloride ([Sudha et al., 2017](#)). Chitin can be obtained from various sources including the exoskeletons of arthropods (insects, crustaceans, and arachnids) and molluscs (beaks and endoskeleton of cephalopods). Other organisms also produce chitin such as in the cell walls of fungi and yeasts, and the spines of diatoms ([Sharp, 2013](#); [Younes and Rinaudo, 2015](#)). The main natural sources of commercial chitin are crustacean shells including lobster, shrimp and crab shells ([Elieh-Ali-Komi and Hamblin, 2016](#)). Chitin occurs in nature in three polymorphic forms which are ordered crystalline microfibrils:  $\alpha$  chitin (anti-parallel strands),  $\beta$ -chitin (parallel chains), and  $\gamma$ -chitin (combination of  $\alpha$  and  $\beta$  structures) ([Khoushab and Yamabhai, 2010](#)).

Through partial chemical or enzymatic deacetylation, chitin can be converted to its most well-known derivative, chitosan. Chitosan is the N-deacetylated derivative of chitin. Chitosan contains copolymers of D-glucosamine (deacetylated units) and N-acetyl-D-glucosamine (acetylated units) linked by  $\beta$ (1,4)-glycosidic bonds. Therefore, the degree of deacetylation is generally defined by the glucosamine/N-acetyl glucosamine ratio. Chitosan is considered to be polycationic, nontoxic, biocompatible, and biodegradable.

Chitooligosaccharides (COS) are products of chitin and chitosan hydrolysis ([Fig. 2](#)). Depolymerisation to produce chitooligosaccharides can be achieved using different physical methods such as hydrothermal methods, microwave processing, ultrasound, chemical methods using acids, and enzymatic methods ([Montilla et al., 2013](#); [Jung and Park, 2014](#)). There is an increasing interest in COS because they are more suitable for some industrial applications because of their low molecular weights, low viscosity, and short chain lengths. Besides their water solubility properties, COS are also reported to have excellent biological properties: cholesterol lowering, antibacterial, antitumor, and immuno-enhancing effects ([Park and Kim, 2010](#)).



**Figure 2** Formation of chitooligosaccharides.

### Extraction of Chitin

The inedible parts of crustaceans (heads, shells, and tails) are considered excellent for chitin extraction (Younes and Rinaudo, 2015). Both chemical and biological methods are used to obtain chitin from crustacean waste (Pachapur et al., 2016). Crustacean by-products can be washed, dried, and size reduced (crushed or ground into a powder). The chemical method involves three steps to produce chitin (Pal et al., 2014):

- Demineralisation with an acidic treatment (generally HCl) to remove minerals (calcium carbonate and calcium phosphate), deproteinisation, and decolouration.
- Further deproteinisation with an alkali treatment (NaOH) to eliminate proteins from the demineralized shells.
- Further decolouration with acetone or organic solvent mixtures to remove the pigments including carotenoids to obtain colourless products.

The use of organic solvents can be a source of environmental concern and also damage other valuable compounds in the waste and limit the isolation of pigments and proteins (Paul et al., 2015). Therefore, enzymatic and biological treatments have been developed. The biological treatment consists of a fermentation of the crustacean biowaste using different bacteria (*Pseudomonas aeruginosa*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Bacillus subtilis*, and *Serratia marcescens*) (Vázquez et al., 2013). For the demineralisation of crustacean shells, lactic acid-producing bacteria have been used while proteases from bacteria have been employed for the deproteinisation (Kandra et al., 2012).

### Carotenoids Components From Crustacean By-Products

Carotenoids are a group of natural pigments, coloured red, yellow or orange, that are widely distributed in nature. They are responsible for the different colours of fruits (e.g., tomato, cantaloupe, mango, and pumpkin), vegetables (e.g., carrots, broccoli, and spinach), and other plants (Raja et al., 2007). There are more than 600 known carotenoid structures described in the literature. Carotenoid pigments are potent antioxidants and they have the ability to act as pro-vitamin A, i.e., they can be converted into vitamin A in the human body. Carotenoids have other bioactivities with some evidence of being protective against cancer, aging, ulcers, cataracts and macular degeneration, heart attack, and coronary artery disease (Ibañez et al., 2011; Mezzomo and Ferreira, 2016). Carotenoids are also commonly used in food products as food-colouring.

Carotenoids are lipophilic compounds that include astaxanthin,  $\beta$ -carotene, lutein, lycopene, and zeaxanthin. Carotenoids have commercial value not only as food colourants or feed additives, but also as health promoting components, which are associated with their antioxidant properties.

Astaxanthin is a red fat-soluble pigment that was approved by the United States Food and Drug Administration and the European Commission as a food dye and food colourant in animal and fish feeds (Ambati et al., 2014). Astaxanthin (3,3'-dihydroxy- $\beta$ - $\beta'$ -carotene-4,4'-dione) is the main ketocarotenoid responsible for the red-orange colour in salmonids and crustacean. Astaxanthin is also naturally present in the microalgae *Haematococcus pluvialis* and in the yeast *Xanthophyllomyces dendrorhous*. Most of the astaxanthin found commercially is produced synthetically. Therefore, crustacean by-products, which are generally discarded, represent an important potential source of natural astaxanthin (Seabra and Pedrosa, 2010). In crustaceans, astaxanthins are found in a free form or complexed to proteins known as carotenoproteins. Astaxanthin content in crustaceans varies depending on species, season, and environmental grown conditions (Cahú et al., 2012; Nguyen et al., 2017).

Carotenoids are not synthesized *de novo* in animals. Thus, they are directly accumulated through the food chain or partly modified through metabolic reactions (oxidation, reduction, and translation of double bonds). Astaxanthin can be produced by crustaceans from other carotenoids especially their main precursor  $\beta$ -carotene (Caramujo et al., 2012). Carotenoids are beneficial for human and animal health due to their antioxidant properties that quench singlet oxygen and prevent lipid peroxidation.

Carotenoids may also improve reproduction and enhance immunity in marine animals. Photoprotection, camouflage, and signalling such as breeding colour are other uses of carotenoids by marine organisms (Maoka, 2011).

#### **Extraction of Astaxanthin From Crustacean By-Products**

Conventional methods for astaxanthin extraction from crustacean shell waste use different organic solvents such as dichloromethane, methanol, ethanol, petroleum ether, chloroform, hexane, and acetone (Dalei and Sahoo, 2015). However, solvent extraction could damage the astaxanthin structure. Besides, their use is considered expensive and may cause environmental pollution (López et al., 2004). Therefore, other techniques including supercritical fluid extraction (SFE) were developed as more efficient alternatives to traditional extraction. Supercritical fluids such as carbon dioxide (CO<sub>2</sub>) are inexpensive and regarded as GRAS solvents (Radzali et al., 2014). To increase carotenoid extraction, (carotenoprotein) proteolytic enzymes could be used since astaxanthin is present in crustaceans as a protein-pigment complex (Armenta-López et al., 2002).

#### **Proteins and Hydrolysates From Crustacean By-Products**

Crustaceans are a valuable source of proteins that contain high amounts of arginine, glutamic acid, glycine, and alanine and also provide essential amino acids for human and animal feed (Nguyen et al., 2017). The protein content in crustacean shell waste depends on the species and processing methods (Mao et al., 2017). Protein hydrolysates often are more functional than proteins with respect to emulsifying properties, solubility, and texturizing capability. After further hydrolysis free amino acids can be generated from peptides of various sizes (da Silva et al., 2017).

#### **Recovery of Proteins and Hydrolysates**

Proteins in crustacean biowaste are closely associated with chitin and minerals, and are obtained by deproteinization. Protein hydrolysis, can be done enzymatically or chemically (Vijaykrishnaraj and Prabhasankar, 2015). Chemical purification can be environmentally hazardous and may destroy some amino acids. To overcome the problems with chemical treatments, other processes have been suggested including microbiological fermentation using lactic acid bacteria and proteolytic enzymes (Gortari and Hours, 2013). Enzymatic methods are advantageous as they take place in milder controllable conditions and the generation of toxic compounds is minimized (Dieterich et al., 2014). For the hydrolysis of shellfish proteins, enzymes from microbial sources such as Alcalase, Neutrase, Protamex, and Flavourzyme could be used.

#### **Future Challenges and Trends for Comprehensive Use of Crustacean By-products**

At present, world crustacean waste materials are an underutilized resource. Most interest and attention both scientifically and industrially has been focused on chitin and chitosan. Chitin, chitosan, and their derivatives are valuable compounds due to their appealing properties of biodegradability, biocompatibility, and non-toxicity. Therefore, they can be used in a wide range of industrial applications in biomedicines, pharmaceuticals, agriculture, foods, cosmetics, textiles, and enzyme-immobilization. They also have many health benefits. Recently, it has become possible to industrially produce pure chitin crystals, called “chitin nanofibrils” (CN). Due to their specific chemical and physical characteristics, CN may have a range of industrial applications, from biomedical products and biomimetic cosmetics, to biotextiles and health foods (Morganti et al., 2011). In addition, a polymeric biocide film of CN-chitosan with high tensile strength has the advantage of being elastic, chemically stable and totally bio- and eco-degradable, compared with conventional antimicrobial films made of polyethylene. In view of the chemistry and biological properties of chitin and chitosan, along with their safe toxicity profile, chitin and chitosan have the potential to be used for the biomedical industry especially in tissue engineering, wound dressing and cancer diagnosis. The numerous uses of chitin and chitosan based materials even includes cancer targeting and drug delivery systems. Chitosan has potential applications in artificial kidney, bone, liver, tendon, blood vessel, blood anticoagulation, nerve, and burn treatments (Islam et al., 2017).

Astaxanthin has also been gaining popularity with humans as a dietary supplement due to its powerful antioxidant properties. Currently, astaxanthin products are available in the market as anticancer, anti-inflammatory and immunostimulants agents. It seems possible to use waste products obtained from the crustacean processing industry to produce healthy products, thus protecting the environment and beneficially contributing to the economy.

#### **Conclusion**

Shells, heads, and tails of crustaceans, represent around 50%–70% of crustaceans and are often discarded without further processing which poses a serious environmental hazard. The underutilized crustacean by-products have been identified as an important natural source of protein, chitin, and carotenoids (mainly astaxanthin). The extraction of valuable compounds could not only minimize the impact of waste on the environment but also provide value-added products that would also be socially beneficial.

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# Microstructure of Dairy Fat Products

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## Introduction

Milk fat provides numerous benefits to food products. Besides nutritional value, flavor enhancement, and desirable mouthfeel, the crystal structure of milk fat also affects the mechanical properties of dairy fat products. The crystal structure of milk fat has been characterized at different length scales using a range of methods such as various microscopy methods, differential scanning calorimetry (DSC), pulsed nuclear magnetic resonance (pNMR), and X-ray scattering techniques (Acevedo, 2012; Acevedo et al., 2011; Marangoni et al., 2012; Peyronel and Campos, 2012). The effect of compositional changes and various environmental factors on the crystallization behavior and crystal structure of milk fat have also been extensively studied.

The thermal behavior of milk fat greatly depends on the arrangement and properties of triacylglycerols (TAGs) which constitute 98% of its composition. TAGs are composed of a glycerol backbone with three fatty acids (FAs) esterified on to it at stereospecific positions (Jensen et al., 1991; McGibbon and Taylor, 2006). The type of FAs present as well as their distribution on the glycerol backbone determine the properties of the TAG molecule (Jensen et al., 1991; Palmquist, 2009; Tzompa-Sosa et al., 2014). In milk fat, these FAs can be short, medium or long chain, and saturated or unsaturated. This diversity in TAG composition in combination with the presence of minor components such as mono- (MAGs) and diacylglycerols (DAGs) in milk fat further lead to the complex thermal behavior and broad melting range of milk fat from  $-40^{\circ}\text{C}$  to  $40^{\circ}\text{C}$  (Timms, 1980; Shi et al., 2001; Wright and Marangoni 2002, 2003; Mazzanti et al., 2004).

In this chapter, the crystal structure of milk fat at different length scales as affected by various internal and external factors will be reviewed. Furthermore, as the crystal structure of milk fat has great impacts on the microstructure and overall properties of dairy fat products, the crystal structure of milk fat within food products will also be discussed.

## Milk Fat Crystal Structure at Different Length Scales

### Molecular Level (Polymorphism)

During crystallization, milk fat melt becomes supersaturated with TAGs that have become supercooled due to high undercooling (*i.e.*, the crystallization temperature is sufficiently lower than the melting point of the particular TAGs) (Marangoni et al., 2012). The supersaturation of TAGs is very important as it determines the polymorphism of milk fat (Kloek et al., 2000; Sato, 2001; Dibildox-Alvarado et al., 2010; Marangoni et al., 2012).

Polymorphism is referred as the various conformations in which TAGs can pack and stack in a lamellar crystal forming various geometrical packing arrangements within the TAG unit cell (Marangoni et al., 2012; Sato, 2001). The more compact or dense the TAG packing, the more bonds or interactions they make and the more stable they are (D'Souza et al., 1990). In milk fat, three polymorphic forms have been identified namely  $\alpha$  ( $\alpha$ ) – hexagonal geometrical packing,  $\beta'$  ( $\beta'$ ) – orthorhombic perpendicular, and  $\beta$  ( $\beta$ ) – triclinic, in increasing order of stability (Woodrow and deMan, 1968; Sato, 2001; Lopez et al., 2001, 2005; Marangoni and Wesdorp, 2013a). Using wide-angle X-ray diffraction (WAXD), the polymorphs of milk fat have been characterized by Bragg's peaks corresponding to  $d$ -spacings of  $4.15\text{\AA}$  ( $\alpha$ ),  $3.8$  and  $4.2\text{\AA}$  ( $\beta'$ ), and  $4.6\text{\AA}$  ( $\beta$ ). Upon crystallization, the  $\alpha$  polymorph is usually formed first which eventually transforms into the  $\beta'$  or  $\beta$  form as the  $\alpha$  polymorph is thermodynamically unstable (Sato, 2001; Sato and Ueno, 2011). The order in which these polymorphs are formed can be explained using the concept of activation energies for nucleation (Marangoni et al., 2012). A lower activation energy is required for the formation of the  $\alpha$  polymorph as it is less ordered than the packing of the  $\beta'$  and  $\beta$  polymorphs. As a consequence, a faster nucleation into the  $\alpha$  polymorph is frequently observed (Grotenhuis et al., 1999; Marangoni et al., 2012; Marangoni and Wesdorp, 2013b; Martini et al., 2001; Sangwal and Sato, 2012).

At high undercooling conditions (fast cooling rate) the formation of the least stable  $\alpha$  crystals is further favored (Lopez et al., 2001, 2005). During fast cooling, TAGs do not have time to self-assemble into more compact, and stable forms, therefore they adapt a more accessible, less ordered polymorphic form -  $\alpha$  (Marangoni et al., 2012). On the other hand, at very slow cooling rates, the  $\beta'$  or  $\beta$  polymorphs of milk fat are formed because TAGs (usually uniform TAGs) have time to self-assemble into the more compact crystalline lattice (Martini et al., 2001). With the application of agitation, shear, and/or ultrasonication, a faster transition of the  $\alpha$  polymorph to more stable forms is observed due to enhanced movement of molecules or partial melting of crystals which provides a mobile phase for the recrystallization of TAGs into more stable polymorphic forms (Frydenberg et al., 2013; Mazzanti et al., 2003; Mazzanti et al., 2009).

In terms of composition, the presence of high amounts of saturated TAGs coupled with sufficient liquid milk fat result in the formation of the  $\beta$  polymorph (Tzompa-Sosa et al., 2016). Furthermore the dilution of milk fat with canola oil allows for the formation of  $\beta$  crystals, while the addition of rapeseed oil results in a faster transition of  $\alpha$  to  $\beta'$  form due to increased mobility of molecules (Kaufmann et al., 2013; Wright et al., 2005).

### Nanoscale (Crystalline Nanoplatelets)

After supersaturation of TAGs, nucleation then occurs wherein TAG molecules organize in a crystalline lattice to form TAG lamellae that further stack into crystalline domains (Marangoni et al., 2012; Marangoni and Wesdorp, 2013b; Sangwal and Sato, 2012). The stacking of several TAG lamellae, as held together by London-van der Waals forces, result in the formation of crystalline nanoplatelets (CNPs) (Acevedo and Marangoni, 2010, 2015; Ramel et al., 2016a).

The nanostructure of milk fat is greatly dependent on the properties and aggregations of CNPs. CNPs in milk fat have been visualized using cryogenic transmission electron microscopy (cryo-TEM) after extraction involving the application of controlled shear and removal of oil by washing with a cold solvent such as isobutanol (Ramel et al., 2016b). The dimensions (length, width and thickness) of CNPs have been measured and analyzed from the micrographs. The thicknesses of the CNPs, or the crystalline domain size have been confirmed through the Scherrer equation (crystalline domain size) based on lamellar thickness calculated from small-angle x-ray scattering (SAXS) data (Acevedo and Marangoni, 2015; Acevedo et al., 2011; Ramel et al., 2016b). Other properties such as surface properties and the types of aggregations of CNPs have been described through the fitting of ultra-small angle x-ray scattering (USAXS) patterns into the Unified Fit and Guinier-Porod models (Peyronel et al., 2014; Ramel et al., 2016b). The effect of various factors on the nanostructure of milk fat has not been studied.

### Mesoscale (Microstructure)

The continuous aggregation of CNPs then result in the formation of large polycrystalline aggregates that can be visualized using polarized light microscope (PLM) and scanning electron microscope (SEM). Milk fat crystal structure at the mesoscale (approximately 1–200  $\mu\text{m}$ ) have been described according to its crystal habits (size and shape) which include spherulites, needle-like crystals, platelets, disordered aggregates and fractal-like aggregates (Marangoni et al., 2012).

At fast cooling rates ( $>0.5$   $^{\circ}\text{C}/\text{min}$ ), smaller milk fat crystals are formed while at slow cooling rates ( $\leq 0.5$   $^{\circ}\text{C}/\text{min}$ ), larger crystals develop (Campos et al., 2002; Wiking et al., 2009). Furthermore, the application of controlled agitation, shear, and ultrasound during crystallization results in the formation of many small crystals. On the other hand, fewer and larger crystals are formed when milk fat is crystallized statically. Agitation and shear allows for uniform heat transfer and possible breaking of large crystals into smaller ones which allows for the formation of many nuclei that act as starting point for growing crystals (Frydenberg et al., 2013; Kaufmann et al., 2012; Martini et al., 2008; Wagh et al., 2016; Wiking et al., 2009).

In terms of composition, the microstructure formed by milk fat from the mixing of its fractions has been extensively studied. TAGs in milk fat can be categorized according to their melting points into high melting fraction (HMF), middle melting fraction (MMF), and low melting fraction (LMF) (Dimick et al., 1996; Timms, 1980; van Aken and Visser, 2000). In a recent work by Ramel and Marangoni (2016), it was found that high concentrations of HMF in binary and ternary mixtures of milk fat fractions result in the formation of many small rod or needle-like crystals (Fig. 1), while relatively higher concentrations of MMF and LMF result in the formation of large spherulites (Fig. 2). The presence of numerous saturated TAGs in HMF allows for the formation of many nuclei under high undercooling conditions. On the other hand, with high amounts of LMF, higher ratios of liquid fat are present which results in a delay in crystallization resulting in multidimensional growth (Ramel and Marangoni, 2016).

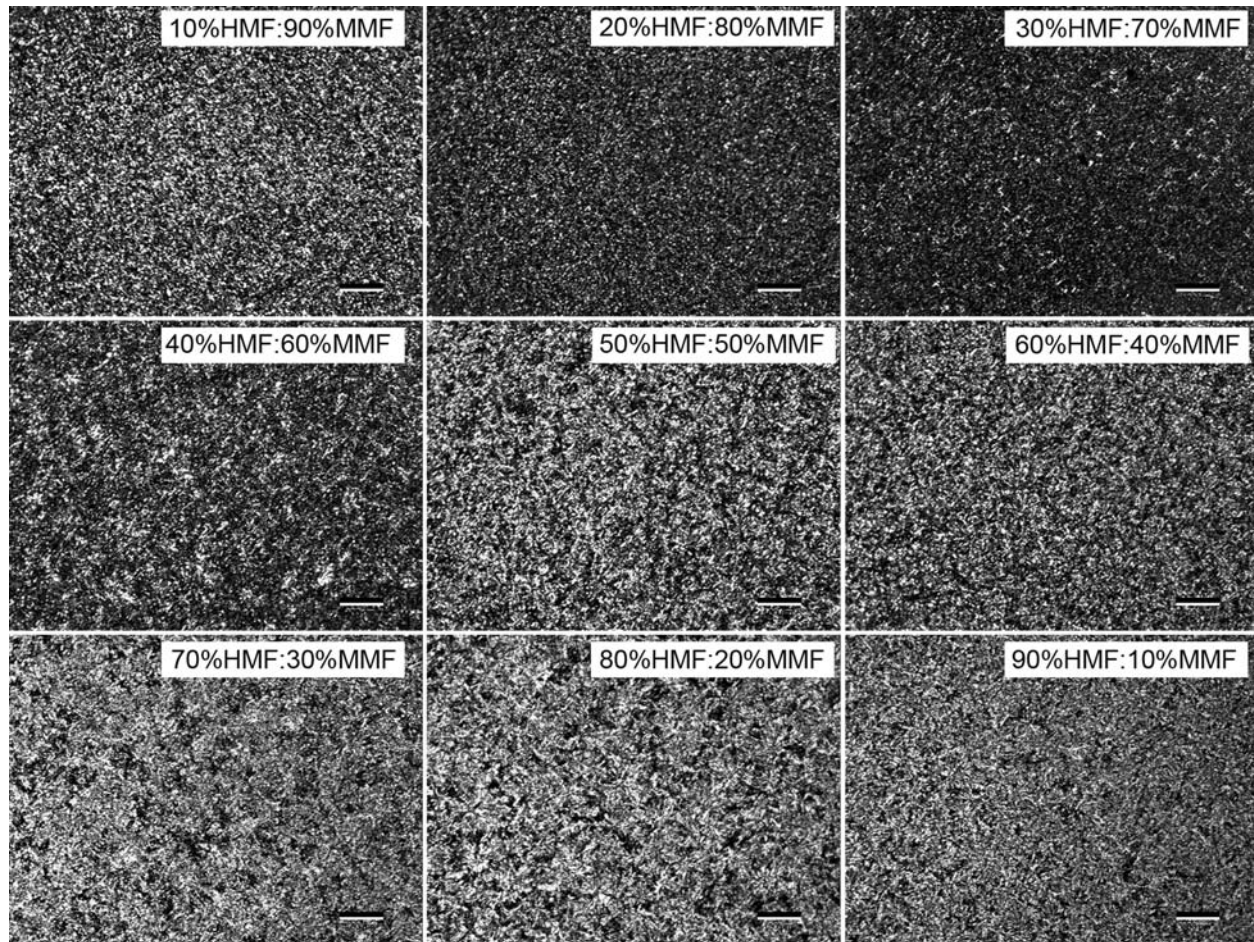
## Crystal Structure of Milk Fat Indairy Products

### Butter

Butter is an emulsion product made from cream wherein the continuous phase is fat (*i.e.*, milk fat crystals) and the dispersed phase is water. Two major processes that are important for determining the final microstructure of butter include churning and working (Wright et al., 2001). During churning at 15–20  $^{\circ}\text{C}$ , the continuous collision of fat globules results in damage of the fat globule membrane through piercing by milk fat crystals and the formation of large clumps of butter grains—partial coalescence. Working then follows to reduce moisture and water droplet size allowing increased contact between crystals which in turn strengthens the fat crystal network (Wright et al., 2001). Fig. 3 shows the resulting microstructure of butter.

Rapid cooling of cream for butter-making results in the formation of relatively small crystals that allow for the formation of a firm fat crystal network due to increase in crystalline interactions (Wright et al., 2001). Furthermore, in terms of polymorphism, the  $\alpha$  form is initially observed in cream which readily transforms into the  $\beta'$  polymorph upon churning (Buldo et al., 2013). Transformation of the  $\alpha$  form into  $\beta'$  and  $\beta$  polymorphs is also observed when cream for butter-making is matured (Ronholt et al., 2012). In margarines and shortenings, the  $\beta'$  polymorph is associated with a spreadable and elastic product, while the  $\alpha$  and  $\beta$  polymorphs have been related to a soft, and grainy or non-spreadable product, respectively (Danthine, 2012; deMan, 1992).





**Figure 1** Polarized light (PLM) micrographs of binary mixtures of high melting (HMF) and middle melting (MMF) fractions of milk fat, crystallized isothermally at 5 °C. Scale bar corresponds to 100  $\mu\text{m}$ . Reproduced from [Ramel and Marangoni \(2016\)](#) with permission from the Royal Society of Chemistry.

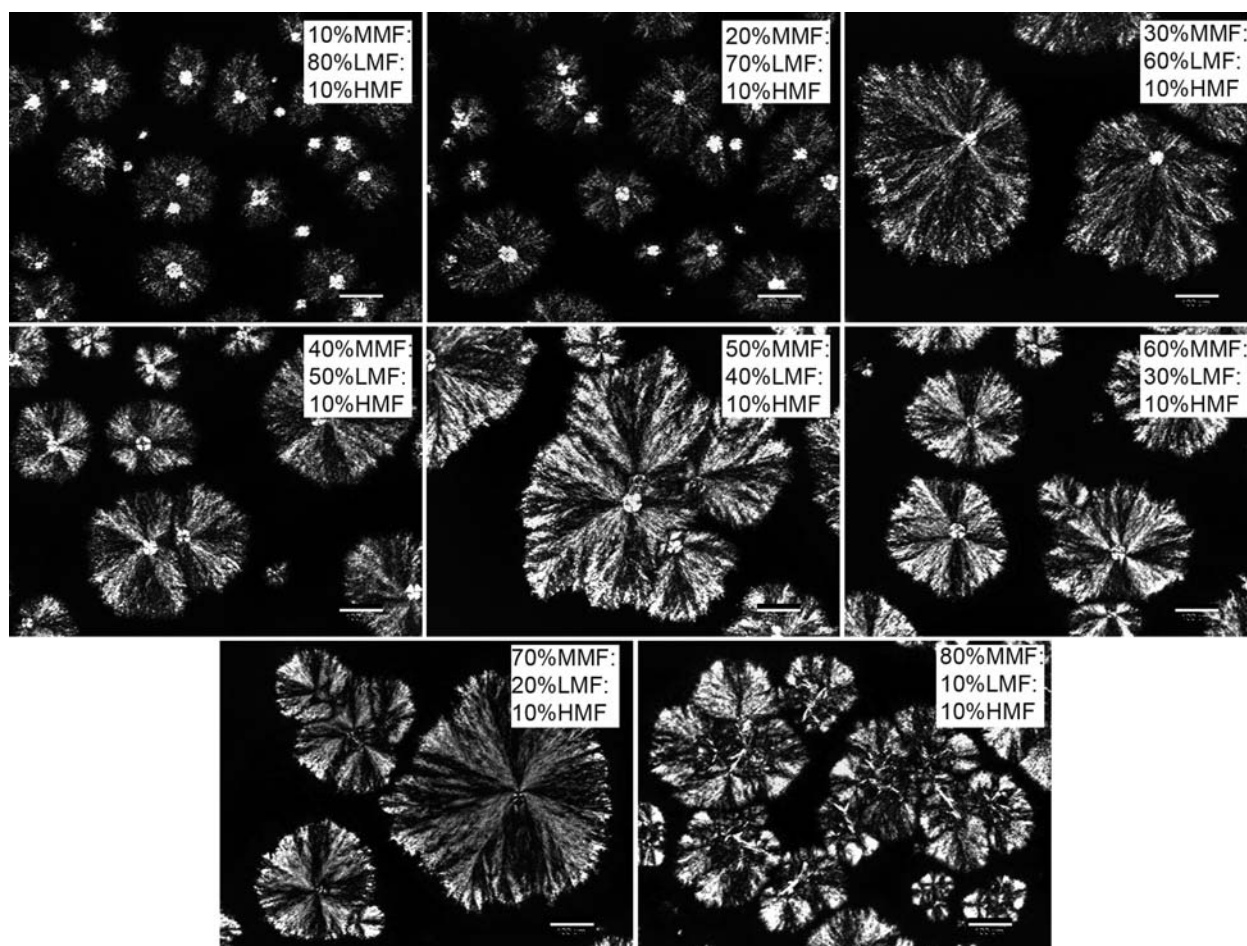
### Whipped Cream and Ice Cream

Products that are also dependent on stabilization by a continuous fat crystal network are whipped cream and ice cream. The crystal network created by milk fat globules that have partially coalesced traps air bubbles upon whipping of cream ([Fig. 4](#)). The stability of the network is affected by the amount of liquid fat and the size of milk fat crystals. Too much liquid fat in the system can cause the foam to collapse due to competitive displacement at the interface, and crystals that are too large can pierce through air bubbles. Very small globules (up to the Nano range) result in the reduction of percent overrun which is the amount of air bubbles that can be incorporated in the network ([Truong et al., 2014a,b](#)).

The microstructure of ice cream is also greatly affected by the properties of the milk fat crystal network. Homogenization is an important step in ice cream manufacture which results in small fat droplets coated with proteins becoming dispersed throughout the ice cream matrix. The addition of low molecular surfactants, or emulsifiers then displaces protein and aid in enhancing partial coalescence, *i.e.*, to increase interactions between crystals piercing through globules ([Goff, 2008](#)). The network formed by partially coalesced fat surrounded by emulsifiers or proteins then stabilize the air bubbles that are incorporated in ice cream during whipping or by vacuum or air injection under high pressure ([Goff and Hartel, 2013](#); [Goff, 1997](#)). This whipping process also introduces shear into the system which further enhances partial coalescence.

### Chocolate

In chocolate, milk fat is blended with cocoa butter for flavour and other desirable sensory qualities. When HMF is added to cocoa butter, larger crystals are formed which results in reduced crystalline interactions and a less compact network, therefore preventing extensive hardening of the product. Furthermore, addition of milk fat TAGs was found to help prevent bloom formation by delaying the transition of the  $\beta$ -V polymorph to  $\beta$ -VI polymorph which is responsible for the formation of white film on the surface of chocolate ([Sonwai and Rousseau, 2010](#)).



**Figure 2** Polarized light (PLM) micrographs of ternary mixtures of high melting (HMF), middle melting (MMF), and low melting (LMF) fractions of milk fat, showing increasing amounts of MMF, crystallized isothermally at 20 °C. Scale bar corresponds to 100 μm. Reproduced from [Ramel and Marangoni \(2016\)](#) with permission from the Royal Society of Chemistry.

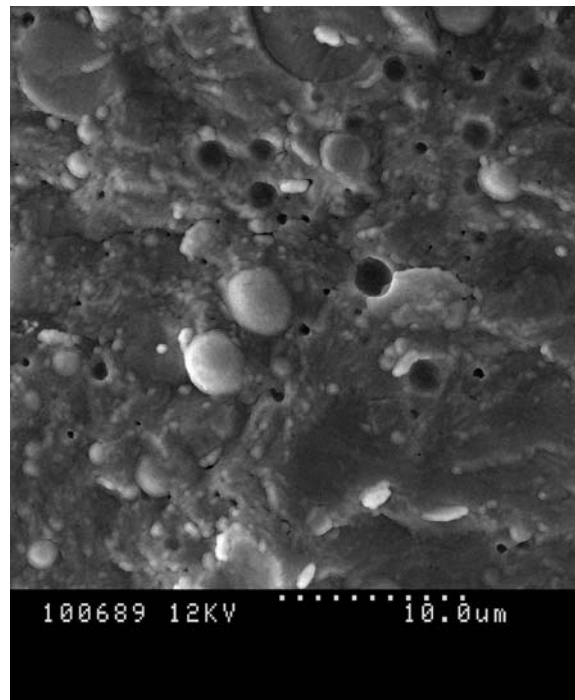
### Cheese and Processed Cheese

Milk fat globules in cheese and processed cheese products appear as spheres embedded in the spaces or pockets within the protein gel matrix formed mainly by caseins ([Fig. 5](#)). Proper emulsification of fat within the protein matrix is important for the desirable viscoelastic and melting properties of cheese and processed cheese products. The thermal history and polymorphism of milk fat was found to affect the viscoelastic properties of processed cheese ([Gluguem et al., 2009, 2011](#)). In studies by [Ramel and Marangoni \(2016 and 2017a,b,c\)](#), it was found that the embedding of milk fat within a protein matrix as that in processed cheese products results in the formation of higher ratios of the  $\beta$  polymorph to the  $\beta'$  form, while milk fat in bulk is mostly in the  $\beta'$  form ([Ramel and Marangoni, 2017a](#)). The main mechanism for the formation of the most stable crystal polymorph of milk fat in processed cheese products was found to be due to the confinement of milk fat TAGs that forces them to arrange themselves in the most compact and dense form ([Ramel and Marangoni, 2017b](#)). The microstructure of processed cheese rather than the polymorphism of milk fat was found to be greatly correlated with its rheological properties.

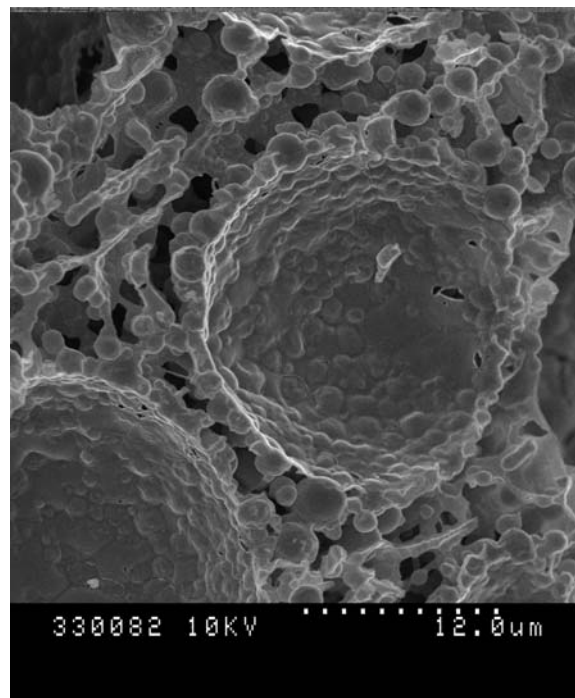
### Conclusions

In this chapter, the importance of the crystal structure of milk fat in the formation and stability of the microstructure of various dairy fat products is described. By characterizing the structure of milk fat at different length scales and studying the effect of various internal and external factors on the structure of milk fat, we now have a better understanding of how the properties of the milk fat crystal network affects the rheological and other sensory properties of dairy fat products. With the development in the methods for studying the structure of milk fat, interactions between milk fat and the different ingredients in the food matrix can be better analyzed.

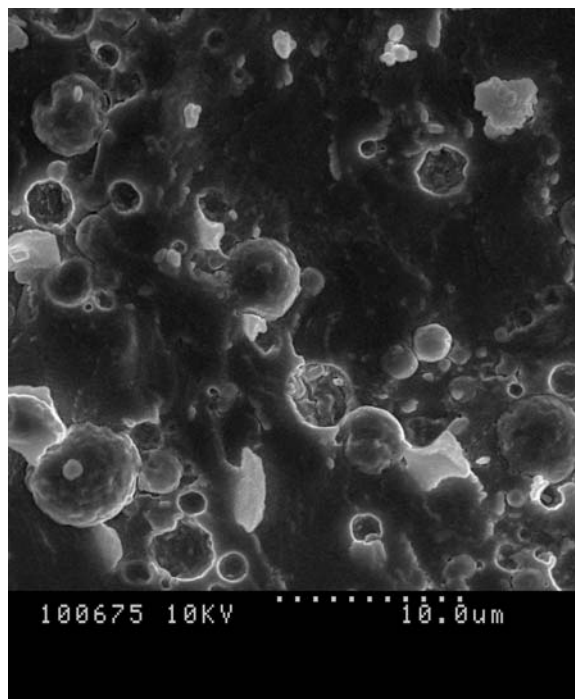




**Figure 3** Microstructure of butter under cryogenic scanning electron microscope (cryo-SEM).



**Figure 4** Microstructure of whipped cream under cryogenic scanning electron microscope (cryo-SEM).



**Figure 5** Microstructure of processed cheese loaf (PCL) produced in USA under cryogenic electron microscope (cryo-SEM). Adapted from Ramel and Marangoni (2017c).

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# The Structure and Properties of Ice Cream and Frozen Desserts

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## Glossary

**Overrun** The amount of air whipped into a product during scraped-surface freezing, as a percent of the unwhipped product, based on the equation (Vol. of ice cream – Vol. of mix)/Vol. of mix × 100%.

## Introduction

Ice cream and frozen desserts are a food category that is characterized by normally being aerated and eaten while frozen. The legal definitions of ice cream and other frozen desserts vary widely. In the United States and Canada, for example, ice cream must contain a minimum of 10% by wt. of milkfat and contain no other non-dairy fat or protein source ([United States Food and Drug Administration, 2017](#)). In these markets, the term “frozen desserts” are used to characterize both products containing non-dairy fats and non-dairy products. However, in Europe, the Euroglaces Code for Edible Ices ([Euroglaces, 2013](#)) provides a much broader definition of ice cream, essentially being an aerated emulsion, hence containing fat, that is eaten while frozen and therefore comprising both dairy and non-dairy versions. They distinguish milkfat containing products as “Dairy Ice Cream”. Also within this broad category are a range of other products, for example frozen yogurt, sherbet and sorbet. Gelato is an Italian-style ice cream with its own compositional guidelines. With gelato, whipping is done with low shear and low overrun, so there is less colloidal structure formed. There are also numerous sub-categories of ice cream, including light and lowfat versions, no sugar added versions, high-fat premium versions, etc. [Table 1](#) provides an overview of typical compositional values for ice cream and frozen desserts that all fall within the category of food products defined as above. The main focus of this chapter is the structure of these products and the properties of the products that result from structure. Structure is certainly dependent on composition, but overall there are generally four phases: fat globules, air bubbles, ice crystals and a freeze-concentrated unfrozen solution of sugars, proteins, polysaccharides and other soluble ingredients. The balance of each, and the importance of each to physical properties and product performance, will depend largely on the source and quantity of fat, proteins, sugars, etc. The first section will introduce the composition and processing steps. Then structure and properties will be divided between the colloidal structure, that arise from the presence of fat and air, and the aqueous phase structure, that arise from the presence of ice crystals and freezing point depression from solutes in the unfrozen aqueous solution. The main properties arising from each phase are creamy texture, dryness on extrusion, shape retention, resistance to melting and shrinkage dependent on the colloidal phase, and smooth to coarse texture, shelf-life, and softness/scoopability dependent on the aqueous phase.

**Table 1** Approximate composition (%) of commercial frozen desserts by formulation category

Group	Milk fat	Milk solids-not-fat	Sweeteners <sup>a</sup>	Stabilizers <sup>b</sup>	Total solids
Non-fat ice cream	<0.5	12–14	18–22	1.0	28–32
Low-fat ice cream	2–5	12–14	18–21	0.8	28–32
Light ice cream	5–7	11–12	18–20	0.5	30–35
Economy ice cream	8–10	10–12	15–17	0.4	32–36
Standard ice cream	10–12	9–10	14–17	0.2–0.4	36–38
Premium ice cream	12–14	8–10	13–16	0.2–0.4	38–40
Super premium ice cream	14–18	5–8	14–17	0–0.2	40–42
Gelato-style ice cream	4–8	9–12	15–24	0.5	32–42
Frozen yogurt: regular	3–6	9–13	15–17	0.5	30–36
Frozen yogurt: non-fat	<0.5	9–14	15–17	0.6	28–32
Sherbet	1–2	1–3	22–28	0.4–0.5	28–34

<sup>a</sup>Includes sucrose, glucose, corn syrup solids, maltodextrins, polydextrose and other bulking agents, some of which contribute little sweetness.

<sup>b</sup>Includes ingredients such as locust bean gum, guar gum, cellulose gum and carrageenan, as stabilizers, and also mono- and di-glycerides, as emulsifiers.

## Formulations, Ingredients and Manufacturing

Most companies design recipes that are unique, however there are many similarities within each category of product, as shown in [Table 1](#). The formulation defines the quantity of each component present, e.g., fat, milk solids-not-fat (MSNF), sweeteners, stabilizers, emulsifiers and water, whereas the recipe defines the quantity of each ingredient present, many of which provide more than one component of the formulation, e.g., cream, milk, condensed milk, liquid sugars, dry ingredients, etc. Flavouring materials are added to the mix components after mix processing, and it is typical to produce many flavours from the same base mix. Formulations and recipes will depend on the type of product being developed, its segmentation within the market (e.g., low-cost vs. high-cost), any health or nutrition claims being associated with the product (e.g., low-fat or no-sugar-added), the availability of ingredients (e.g., fresh cream vs. frozen butter when fresh cream is not available), and other factors that help to differentiate one brand from another.

Most formulations start off by defining the fat level. The legal minimum for ice cream in several countries is 10% by wt., but it can vary from as low as 3%–4% in low-fat products to as high as 16% in super-premium products. Milkfat is usually provided by cream, butter or anhydrous milkfat, whereas the most common non-dairy fats are from coconut or palm kernel, since fat must be partly solid at whipping temperature (as described in detail below). Fat provides smooth, creamy texture from lubrication in the mouth and provides structure, resulting in dryness, shape retention and resistance to rapid structural collapse during melting, as will be further described below.

The MSNF category provides the milk proteins, which are responsible for emulsification of fat, stabilization of air and water holding capacity, and the lactose and milk salts, which contribute to freezing point depression. Typical usage levels are 9%–12%, to provide 3%–4% protein. Concentrated sources of MSNF include condensed skim or whole milk, skim milk powder and whey-based ingredients such as whey powder or whey protein concentrates. Milk protein concentrates can also be used. In non-dairy frozen desserts, the MSNF category is usually replaced with a source of plant protein such as pea, soy, rice or other, and bulking materials such as maltodextrins, starch or inulin.

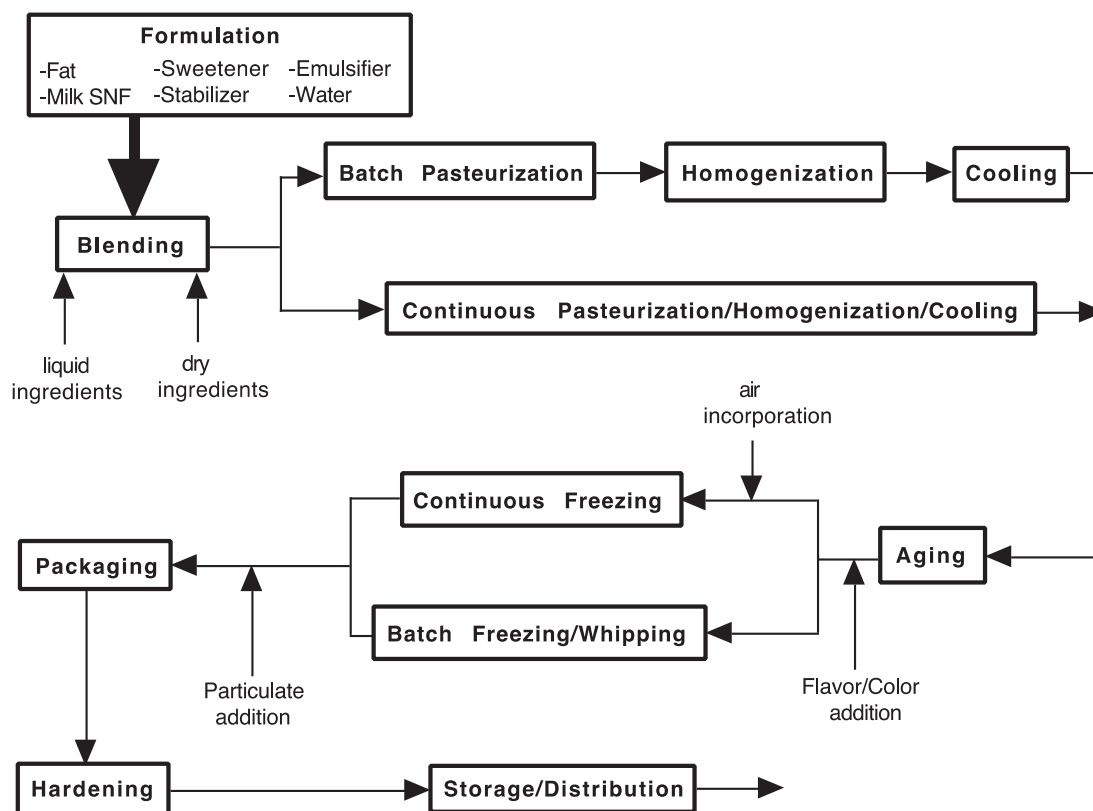
The sweetener category provides sweetness and freezing point depression. Sucrose is the most common source, and can be used alone at 14%–15%, but starch hydrolysate sweeteners (glucose solids), from corn, wheat tapioca or other starch sources, are also quite common and provide body and texture from the oligosaccharide and maltodextrin components, depending on the extent of hydrolysis (usually given as Dextrose Equivalent (DE) number). Typical recipes might replace 3%–4% of the sucrose with 4%–6% starch hydrolysates, depending on their sweetness. In No-Sugar-Added, low Glycemic Index formulations, the sugars are normally replaced by sugar alcohols, such as the disaccharide maltitol, which can replace sucrose at a 1:1 ratio for freezing point depression, or the small molecular weight polyol erythritol, which can replace sucrose at a 1:3 ratio for freezing point depression. In that case, other bulking agents may be required to provide total solids. Since the sugar alcohols are not as sweet as sucrose, a high-potency sweetener such as sucralose or the natural sweetener stevia may be used to enhance and balance the sweetness and flavour.

Stabilizers and emulsifiers are important food additives for their structural contributions, as will be discussed below. Stabilizers are normally soluble, indigestible polysaccharides such as guar gum, locust bean gum, and carboxymethyl cellulose, with a combined usage level of ~0.15%–0.25%. They interact with the water phase and can also interact with the proteins in the aqueous phase ([Cheng et al., 2015](#)). Carrageenan is often added at ~0.01% to inhibit phase separation between these polysaccharides and the casein micelles, the milk protein particle, which is incompatible in solution with these polysaccharides. The two common emulsifiers are mono-glycerides, typically at 0.15% depending on the fat content, and egg yolks at ~2%. These interact with the fat phase to provide fat structuring and air stability, as will be described in detail below.

The final category of ingredient is the source of water, to solubilize and disperse the ingredients and create the liquid mix. Total solids, the sum of the above, might range from 32%–40%, depending on formulation, which means that the water component would be 60%–68% (100-TS%). The source of water can be added water, milk or skim milk, and the contributions also coming from ingredients such as cream, condensed skim, liquid sugars, etc.

Ice cream and frozen desserts are manufactured in two main steps, mix making and freezing ([Fig. 1](#)). Mix making entails blending and solubilizing the ingredients with high shear blenders and recirculation through blending tanks, perhaps with pre-heating up to 30–40 °C depending on the choice of ingredients, pasteurizing the mix with either batch (~70 °C, 30 min) or continuous high temperature short time (HTST, ~80 °C, 30 s) pasteurizers, homogenizing the mix with a high-pressure valve homogenizer, typically two-stage at 18 MPa and 3.5 MPa, depending on the composition and fat content, cooling the mix to 4 °C or less, and ageing at this temperature for > 4 h in holding tanks.

After ageing is completed, the mix is typically sub-divided to smaller aliquots, flavoured with soluble flavours, and then either batch frozen (small-scale operations) or pumped through a continuous scraped-surface heat exchanger ("barrel" freezer), to a draw temperature of < -4 to -7 °C, depending on composition and freezer capacity. Within the freezing cylinder ("barrel"), the rapid removal of heat by the refrigerant causes the mix to freeze onto the inside surface of the barrel, where it is rapidly scraped off by the dasher and into the bulk flow ([Drewett and Hartel, 2007](#)). The minute ice crystals within this layer then mature and grow as the product freezes further to a final value of ~50% of the water frozen before exiting the barrel ([Russell et al., 1999](#); [Cook and Hartel, 2010](#)). Air is incorporated at the inlet to the barrel and the shear induced by rapid agitation from the dasher inside the barrel is responsible for the formation of small air bubbles as the mix freezes and progresses toward the exit. The air content of ice cream is defined by the overrun, the increase in volume of the mix as a result of the air incorporation ((air vol./mix vol.) × 100%). Typical overrun values vary from 25%–50% in high-quality premium products to 110%–120% in economy brands. The agitation and aeration within the barrel induce a destabilization or partial coalescence of the fat emulsion, causing some fat globules to adsorb to and



**Figure 1** Flow diagram of the processing steps in ice cream manufacture (SNF, solid-non-fat).

stabilize the air bubble interfaces and also causing some of the fat globules to develop a particle network of fat throughout the unfrozen phase of the ice cream.

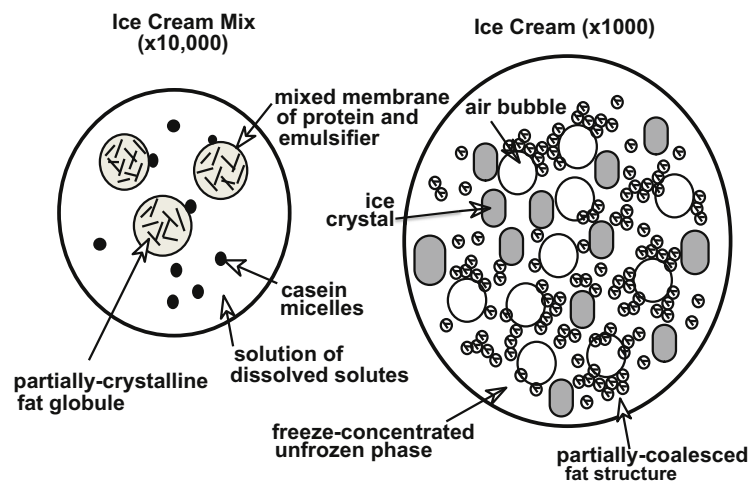
Following scraped-surface freezing, any flavouring materials that will be discrete and visible within the product, such as fruits, nuts, ripple sauces etc., are stirred into the semi-frozen ice cream through an ingredient feeder and/or ripple pump and the ice cream is then packaged and moves immediately into a hardening freezer. This second freezing step is normally done in spiral freezers with low temperature ( $\sim -30$  to  $-35$  °C) and high velocity circulating air (high convective heat transfer coefficients), with a goal to reduce core temperatures of the packages to  $\sim -20$  °C within 4 h.

## Structure and Properties

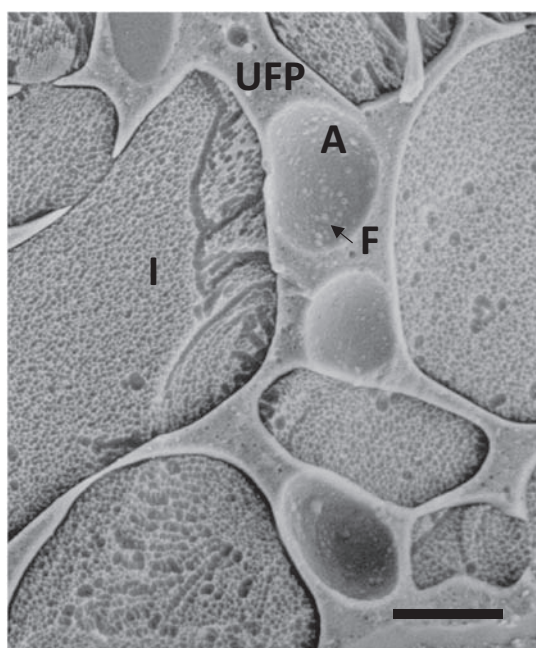
The structure of the liquid ice cream mix can be described as an emulsion of fat globules, which are partly crystalline as a result of cold ageing, a dispersion of casein micelles, if present in the recipe, and a solution of sugars, soluble proteins, salts, stabilizers and other components (Fig. 2). The freezing processes convert this structure to one comprised of ice crystals, air bubbles, fat globules, some of which are adsorbed to the air bubbles and some of which are partially-coalesced into aggregates and potentially a continuous particle network, and an unfrozen continuous phase that is freeze-concentrated compared to the mix due to the removal of water as ice (Figs. 2 and 3). These structural elements will be sub-divided into the colloidal structure and the aqueous structure and will be discussed, along with the major physical properties and performance parameters arising, in detail within each section following.

### Colloidal Structure

The colloidal structure is comprised of the fat globules and the air bubbles (Fig. 4). Homogenization of the fat creates the original size distribution of fat globules. At intermediate fat content,  $\sim 7\%$ – $12\%$ , fat globule sizes are typically normally distributed around a median of  $0.5\ \mu\text{m}$ , with none larger than  $2.0\ \mu\text{m}$ . This is the result of homogenization pressures of  $\sim 18\ \text{MPa}$  after formation of a suitable pre-emulsion when solid fat sources are used, bearing in mind that cream is already an emulsion. At higher fat levels, less pressure is used so that fat globules do not become too numerous and more prone to churning in the barrel freezer, whereas at lower fat levels,  $4\%$ – $6\%$ , fat structure can be enhanced with high pressure homogenization to enlarge its surface area. If fat levels are too



**Figure 2** Highly schematic illustration of the structure of ice cream mix and ice cream. Ice cream mix contains partially crystalline fat globules and casein micelles as discrete particles in a solution of sugars (including lactose), salts, dispersed whey protein and stabilizers, etc. The surface of the fat globule demonstrates the competitive adsorption of casein micelles, other milk proteins and added emulsifiers. Ice cream contains ice crystals, air bubbles and partially-coalesced fat globules as discrete phases within an unfrozen serum containing the dissolved material (including lactose). The partially-coalesced fat agglomerates adsorb to the surface of the air bubbles, which are also surrounded by protein and emulsifier, and link the bubbles through the lamellae between them.

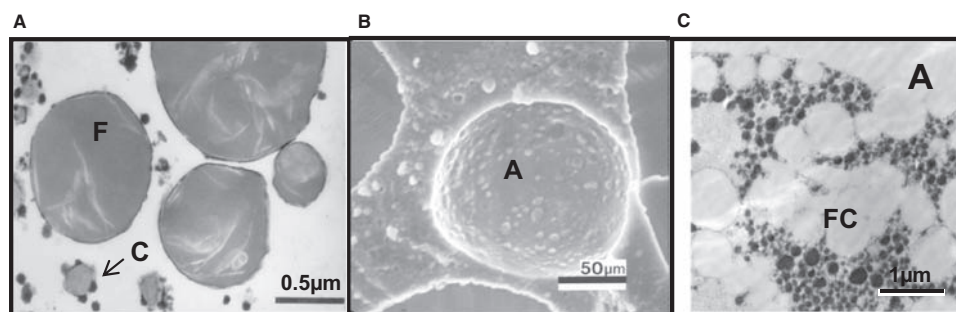


**Figure 3** Low-temperature scanning electron microscopy image of ice cream structure, showing all structural elements, the unfrozen phase (UFP), ice crystals (I), air bubbles (A) and fat globules adsorbed at the air interface (F). Bar = 20  $\mu\text{m}$ .

low, <3%–4%, very little fat structuring takes place and colloidal structure needs to be replaced with further aqueous structuring. Since milkfat, coconut oil and palm kernel oil are partially comprised of saturated fatty acids, the ageing step of <4 °C for >4 h will result in a semi-crystalline fat droplet.

The surface of the fat droplet immediately after homogenization will be stabilized by a mixture of surface-active proteins (casein micelles and whey proteins) and emulsifiers (monoglyceride or phospholipids from egg yolks). During the ageing period, the emulsifiers will continue to displace proteins from the fat surface, as they lower the interfacial tension and work towards a more thermodynamically-stable equilibrium (Goff et al., 1999). This is especially true for casein micelles, due to their bulky nature, but less displacement occurs with protein membranes comprised of whey proteins or caseinate. Non-dairy plant proteins need to be evaluated for their behaviour. The emulsifiers are much smaller in molecular weight compared to the proteins, for example





**Figure 4** The colloidal structure of ice cream mix and ice cream. (a), Fat globules (F) in mix with crystalline fat within the globule and adsorbed casein micelles (C), as viewed by thin section transmission electron microscopy. With emulsifier present, the level of casein adsorption is very low. (b), Close-up of an air bubble (A) with adsorbed fat, as viewed by low temperature scanning electron microscopy. (c), Air bubble (A) with adsorbed fat cluster (FC) that extends into the unfrozen phase, as viewed by thin section transmission electron microscopy with freeze substitution and low temperature embedding.

the polar head group of an alpha-monoglyceride is comprised only of the 2-carbon non-esterified portion of the glycerol molecule, such that their continued adsorption produces a much thinner membrane at the fat interface (Fig. 4a), and hence a fat droplet that is much more prone to shear-induced partial coalescence in the barrel freezer (Bolliger et al., 2000).

Within the barrel freezer, air is added and the dasher whips this air into progressively smaller air bubbles with progressively more surface area (Sofjan and Hartel, 2004; Xinyi et al., 2010). At the same time, the ice phase is forming, which is concentrating the fat and air and enhancing the induced shear. The combination of these actions causes the fat globules to undergo a destabilization process. The air bubble surfaces will be formed by protein from the aqueous phase (Zhang and Goff, 2004) but are rapidly stabilized by fat adsorbing at the surface (Zhang and Goff, 2005) (Fig. 4b). Concomitantly, fat droplet collisions in the shear field induce the process of partial coalescence, which is enhanced by the thin membrane induced by the emulsifiers (Bolliger et al., 2000). The solid fat crystals within the droplets are responsible for maintaining some of the spherical shape of the droplets, which allows them to form chains and clusters rather than to coalesce into larger droplets (Mendez-Velasco and Goff, 2012) (Fig. 4c). The latter happens when too much oil is present within the droplets. The liquid oil remaining in the mostly-solid droplets is responsible for helping to create fat-fat interactions through the formation of oil necks at the point of contact. If the droplet is almost completely solid, then there is less tendency for collisions to lead to aggregation (Sung and Goff, 2010). Likewise if too much protein is present at the fat interface, fat droplet collisions will not result in the same extent of fat adsorption to air bubbles or fat partial coalescence.

This structuring of fat leads to several important physical properties in the product. The first is dryness on extrusion. With optimal fat structuring, the product appears dry as it is removed from the barrel and is better able to hold flavouring inclusions. It is also able to hold its shape much better, resulting in a soft-frozen product that is able to be cut into a wide array of novel shapes, cakes, etc. Extensive fat structuring can lead to visible churning of butter particles whereas sub-optimal fat structuring leads to a wet-appearing product with little shape retention. This happens with insufficient or no emulsifier, or with fat sources that are either too solid or too liquid at whipping temperature, or with fats that have not been fully crystallized as a result of insufficient ageing or ageing too warm.

A second important manifestation of optimal fat structuring is the ability resist collapse and flow of the product during melting (Daw and Hartel, 2015). During “meltdown”, the ice crystals melt as a function of heat transfer from the ambient environment, but as the ice melts the structure can either resist collapse due to an extensive three-dimensional fat particle network or it will melt quickly if there is no underlying fat network to hold the air bubbles and aqueous phase in place (Muse and Hartel, 2004). Too much resistance leads to the “does not melt” defect in which melted ice cream resembles a stiff, dry whipped cream product, whereas too little resistance results in a product that is very prone to dripping and with a weak body and mouthfeel as the melted ice cream returns rapidly to its mix-like structure. This structural feature can also be felt as improved texture and mouthfeel due to the slower structural collapse in the mouth.

Another important manifestation of optimal colloidal structure is a stable air phase (Xinyi et al., 2010). The air bubbles need to be stabilized by adsorbed protein and fat so that they remain as distinct bubbles with an interface. Without appropriate air stability, the bubbles are prone to coalescence and channeling, and this often leads to shrinkage as some of the air escapes out of the product during storage and distribution (especially at somewhat elevated temperatures).

### Aqueous-phase Structure

The aqueous phase is comprised of the ice crystals and the freeze-concentrated unfrozen phase, which contains the solutes (sugars, lactose and salts) and the dispersed macromolecules (protein and stabilizers). Unlike the colloidal phase, which is created at the time of manufacturing and remains mostly stable thereafter, the balance between ice and unfrozen water within the aqueous phase is in thermodynamic equilibrium, defined by the solute concentration and the temperature. Thus it is continually changing post-manufacture with changes to product temperature. These changes in water:ice equilibrium can lead to rapid increases in ice crystal

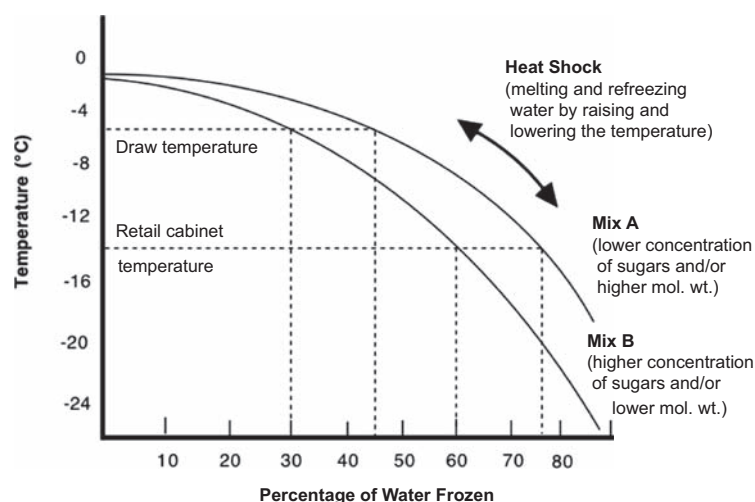
size, and hence to loss of shelf life. Thus, discussion of the aqueous phase needs to focus on ice formation and ice crystal size distribution, solute concentration and freezing point depression, leading to appropriate softness and scoopability, and finally the detrimental effects of heat shock on quality and shelf-life.

The ice crystal size and size distribution is formed by rapid heat removal in the scraped surface freezer followed by rapid hardening, together with minimizing any potential for melting of ice crystals in the delicate temperature range between these two processes. Hence addition of flavouring materials and packaging of the product should also be done rapidly and with no exposure to warm temperatures. As was discussed in the Manufacturing section above, ice crystals form initially at the barrel wall and then are rapidly scraped off into the bulk where they mature and grow as further water molecules continue to deposit on existing crystals (Cook and Hartel, 2010). Hence the barrel freezer is primarily responsible for ice crystal formation whereas the goal of rapid hardening is to maintain as many of these small crystals as possible. There may be limited further nucleation during the hardening stage but it is overwhelmingly dominated by growth (Russell et al., 1999).

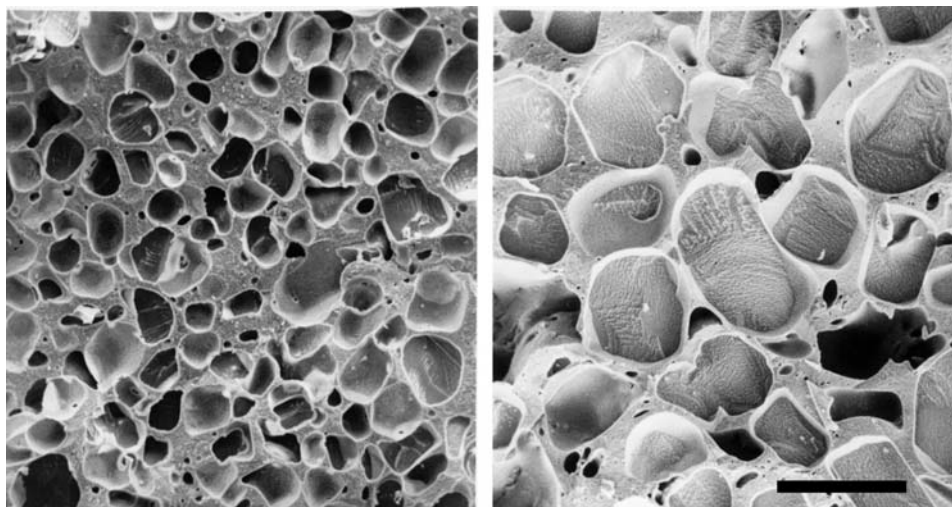
Ice crystals are pure solid water particles. During the process of crystal growth, solutes are continually excluded out of the crystalline lattice (Adapa et al., 2000). Thus, as ice crystal growth proceeds, the solutes and all other substances in the unfrozen phase become freeze-concentrated in an ever-decreasing volume with reductions in temperature. Freezing point depression is governed by Raoult's Law, which indicates that it is a molar concentration effect, hence influenced both by molecular weight and weight concentration of solutes. The principal solutes in ice cream formulations are the added sugars, lactose and milk salts. Additionally, post-pasteurization flavourings that are added also may have to be accounted for, particularly if they contain high sugar content that becomes incorporated into the bulk of the ice cream. For example, added cooked and sweetened strawberries would contribute to freezing point depression, whereas a distinct ripple sauce, even though also high in sugar, would not contribute to freezing point depression of the ice cream since it remains distinct, in that case the sugars within the ripple sauce simply keep the ripple softer than the ice cream, rather than influencing the ice cream *per se*. The combined effect of all solutes in the mix on freezing point depression can be calculated (Whelan et al., 2008) and is generally shown as a freezing curve (Fig. 5), which illustrates the initial freezing temperature (achieved within the barrel freezer) and then the amount of water frozen (ice formed) as a function of temperature for each formulation. Hence the sugars are dictating the ice phase volume at any specific subzero temperature. The freezing temperature of the unfrozen phase is equal to the ambient temperature of the product. If temperature rises or falls, the ice:unfrozen water will maintain an equilibrium balance, following this freezing curve.

If ice cream is being manufactured for scooping, it is critical that the freezing curve of all flavours overlaps in the temperature zone of the scooping cabinet freezer, otherwise some products will be much firmer than others, which makes scooping difficult and results in large product inconsistencies. Another important manifestation of the freezing curve is that ice crystals are more prone to growth and recrystallization when there is more water present in the product (Hagiwara and Hartel, 1996), i.e., on the elevated plateau portion of the freezing curve. This will be discussed further below.

If ice cream temperature is very cold and highly freeze-concentrated, the unfrozen phase can undergo a glass transition and itself become an amorphous solid. Below the glass transition temperature ( $T_g$ , usually a value of  $-28$  to  $-30$  °C is used), ice cream can be stored for many months with no change in structure. At temperatures above the  $T_g$ , mobility within the unfrozen phase is possible and recrystallization, the movement of water from one crystal to another to cause a net increase in median size, can occur (Fig. 6). At constant temperatures, this process, known as Ostwald ripening, is slow. However, with temperature fluctuations, melting and refreezing along the freezing curve occurs. Melting can cause the loss of the tiniest of the ice crystals, whereas refreezing typically



**Figure 5** Ice cream freezing curve, showing the relationship between temperature and frozen water for two mixes that vary in solute concentration. There is more water frozen at both draw temperature and retail cabinet temperature for the mix with lower concentration and/or higher molecular weight of solutes, compared to that with higher concentration and/or lower molecular weight of solutes. Heat shock occurs by melting and refreezing water, a result of raising and lowering temperature as seen by moving up and down the freezing curve.



**Figure 6** Ice crystals in ice cream as seen with low temperature scanning electron microscopy. Image on the left in freshly-frozen and hardened ice cream, image on the right in heat-shocked ice cream. Same magnification in both images, bar = 100  $\mu\text{m}$ .

causes the largest crystals to grow at a faster rate (in an effort for the system to minimize surface area and hence surface free energy; Adapa et al., 2000). Hence melt-refreeze recrystallization is much faster compared to isothermal Ostwald ripening (Regand and Goff, 2002). The rate of recrystallization is a function of the quantity of unfrozen water, hence again not just temperature but also the combined effects of the solutes (Hagiwara and Hartel, 1996). If a formulation produces a low freezing curve, it will be more prone to recrystallization at any given storage temperature compared to one with a higher freezing curve (Fig. 5). Also, at lower temperatures there is less melting and refreezing for a fixed temperature interval than for the same temperature interval at higher temperatures, due to the shape of the freezing curve (Fig. 5), hence ice cream is more prone to recrystallization at elevated temperatures than at temperatures closer to the  $T_g$ .

The size distribution of ice crystals in ice cream is directly related to its sensory quality. It has been shown that sensory perception of iciness and coarse textures correlate directly to ice crystal size (Russell et al., 1999). Usually a median size of 45  $\mu\text{m}$  is used as an upper limit. Once median size exceeds this sensory threshold, ice cream quality is noticeably affected (Fig. 6). Shelf life is mostly dictated by the development of coarse/icy textures, since there is very little enzymatic activity and no bacterial growth and there are very few, if any, chemical reactions occurring, maybe a bit of oxidation of fat, but slow, especially since mostly saturated fat is used. The polysaccharide stabilizers can help to control ice crystal growth during temperature fluctuations (Hagiwara and Hartel, 1996; Flores and Goff, 1999; Adapa et al., 2000). They do so by becoming freeze-concentrated in the unfrozen phase and, since they are large molecular weight biopolymers, exceeding their critical concentration and becoming highly viscous. Cryo-gelling can also occur in the case of locust bean gum, and protein polysaccharide interactions can also influence unfrozen phase biopolymer structure (Cheng et al., 2015). This exceedingly high viscosity or biopolymer structure in the unfrozen phase limits migration and thus tends to preserve ice crystal size distributions for longer periods (Regand and Goff, 2002). Hence maintenance of shelf life depends on freezing point depression/freezing curves, the presence of stabilizing gums and on maintenance of low and constant storage temperatures.

## Conclusions

The structure of ice cream and frozen desserts can be very complicated and prone to defects arising as a result of not optimizing structure for product performance. These products are emulsions and foams, have a temperature-dependent ice:unfrozen water equilibrium that is affected by dissolved solutes in solution, and also have dispersed macromolecules in the unfrozen phase. Physical properties arise from this complicated structure and these affect product performance. Attributes including dryness on extrusion, shape retention during melting, scoopability and texture all depend extensively on structure. Changes in structure with storage time, especially ice recrystallization, can dramatically reduce shelf life, from months to days, so a thorough understanding of the creation and control of structure is critical for successfully delivering high quality products to the consumer.

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## Further Reading

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# The Structure and Rehydration Properties of Dairy Powders

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## Introduction

Milk is a highly complex fluid, composed of multiple elements that exist in different states, such as lactose and minerals in true solution, fats in an emulsion, and proteins in a colloidal state. This makes it highly versatile beyond being consumed fresh, and it is turned into a wide variety of products including cheese, butter, fresh fermented products, and a wide range of powders. Dairy powders offer the advantage of a longer shelf life and lower shipping costs due to reduced shipping volume, however, if they are to be used in liquid form they require reconstitution.

It is desirable for reconstitution to be quick and require minimal stirring, with the end product closely resembling the liquid state prior to concentration and drying. However, reconstitution is a complex process and depends on the powder structure and the reconstitution conditions, such as temperature, stirring speed and water composition (Syll et al., 2012).

Powder structure can be defined in many ways and is determined by the processing, transport and storage history of the powder. This work focuses on the particle level related structures, which include the key properties of particle size, shape, density, and both surface and bulk composition. However, structure at the molecular level is discussed where appropriate, such as where the denaturation and interaction of proteins contributes to the reconstitution behaviour of the powder.

## Rehydration Characteristics

Rehydration is a complex process and has to be defined for the structure-rehydration relationship to be understood. Rehydration of dairy powders is usually grouped into four key steps in order of occurrence: sinking, wetting, dispersing and final solubilizing (Pisecky, 2012).

Sinkability measures the time that it takes for particles to penetrate through the surface of the water, whilst wettability is a measure of how well the powder absorbs water. In practice “wettability” is often used for industrial measurement to represent both, as they are difficult to distinguish by measurement (Pisecky, 2012). Whilst there is an ISO method for wettability (ISO/TS 17758:2014), no such standardized method for sinkability exists. Wettability is measured by weighing out a specific quantity of powder and dropping it into a beaker with water in a controlled manner. Wettability is taken as the time that it takes for all of the powder to penetrate the surface of the water. The exact measurement conditions depend on the type of dairy powder being tested (Pisecky, 2012).

Alternate measurements of wettability have also been used where the standard methods have been found to be inappropriate due to extremely poor wetting, such as of non-agglomerated casein powders (Gaiani et al., 2007). These include contact angle measurement for monitoring milk protein concentrate (MPC) wettability (Crowley et al., 2015; Fitzpatrick et al., 2016) and the initial period of turbidity measurement (Gaiani et al., 2007).

The next step in the rehydration process is dispersibility, where the powder begins to break up into individual particles. Powder is stirred by hand into a specific quantity of water for 10 to 20 s. The resulting solution is passed through a fine mesh and either the total solids of the resulting solution or the residue remaining on the mesh are measured. Any material remaining on the mesh is considered undispersed, and the larger this is the poorer the dispersibility of the powder (Pisecky, 2012).

Meanwhile, solubility, is a measure of the final solution, and is well standardised for skim and whole milk powder. It is measured using an insolubility index (IDF Standard 129A:1988) and the test is conducted using a high speed mixer followed by centrifugation to determine un-dissolvable content.

Alternative methods have been proposed for measuring rehydration properties of different dairy powders, again especially for protein powders that exhibit widely different reconstitution characteristics from whole and skim milk powders. The definitions of dispersibility and solubility have become blurred as most studies do not relate alternative methods to the standard procedures, and thus the general terms of rehydration or reconstitution characteristics are used. The main three alternative methods applied are: measuring the change in turbidity during reconstitution (Gaiani et al., 2006; Gaiani et al., 2005; Gaiani 2009); monitoring the change in particle size using laser light scattering (Mimouni et al., 2009; Syll et al., 2012; Chen and Lloyd, 1994) or focused beam reflectance measurement (Fang et al., 2011). It should be noted that in these measurements the end-point of dispersion or solubility is inconsistent between papers, thus making results difficult to compare.

## Types of Dairy Powders

A survey of literature referenced in this work and the big dairy powder manufacturers such as Arla, Fonterra and Amul, demonstrates the range of dairy powders being produced. The large range of dairy powders available has been driven by increasing demand for different nutritional and functional protein products and advances in separation technology (Tavares and Malcata, 2016; Kelly, 2011). It is not possible to have a discussion of the structure and rehydration properties of dairy powders without first knowing



**Table 1** Different types of dairy powders and typical compositions

Main type	Sub-types	Composition notes
Whole milk powder (WMP)	regular (un-agglomerated) agglomerated (AWMP)	min 26% fat
Skim milk powder (SMP)	instant (IWMP) regular (un-agglomerated) instant/agglomerated (ISMP) ultra-heat treated (UHT)	min 26% fat, 0.15%–0.25% lecithin ~1% fat
Fat filled powders	varied, depends on milkfat substitute	
Whey protein	whey protein concentrate (WPC) whey protein isolate (WPI) cheese whey powder (sweet/acid) demineralised whey	30%–80% protein >90% protein
Milk protein concentrate (MPC)	MPC40–85 milk protein isolate (MPI)	40%–85% protein >90% protein
Casein powders	micellar casein (MC) sodium/calcium caseinate (SC/CC)	84%–92% protein
Cream powder		55%–72% fat
Buttermilk powders	sweet buttermilk powders acid buttermilk powders	
Lactose		pure

exactly what type of powder is under discussion, and also how it was manufactured. Some of the key powders are summarised in **Table 1**.

The powders produced in the largest quantity are whole and skim milk powders. These are manufactured by spray drying whole and skim milk respectively, with slight variations in the processing conditions to obtain instantised and UHT versions. Instant whole milk powder (IWMP) has a high fat content and a surfactant, lecithin, added to make the powder wettable. Fat filled powders are a subset of whole milk powders (WMP) where the butterfat has been substituted with animal or vegetable fat in order to make a more economical product (Pisecky, 2012).

Historically whey powder was originally manufactured from the liquid remaining after cheese manufacture, with sweet and acidic whey being by-products of rennet-coagulated and acid-coagulated cheeses respectively (Pisecky, 2012). However, with the development of different membrane filtration and separation technologies, the range of whey protein powders has expanded significantly. Skim milk can also be used as the raw starting material, for example in the production of whey protein concentrates (WPCs) and whey protein isolates (WPIs). In addition to the whey powders shown in **Table 1** other whey products include purified proteins from fractionation and hydrolysed products. Whey protein isolate is a term used to denote very high protein content whey powders.

MPCs have been described by Kelly (2011) as “protein-enriched forms of skim milk”, as the whey to casein protein ratio is the same (Nasser et al., 2017). The acronym is often followed by a number, which specifies the total protein content of the powder, and the wide range available caters to a variety of applications, such as cheese or yogurt making. MPCs can be formed through a number of routes, such as membrane filtration of skim milk followed by spray drying, with adjustment to the protein specification taking place through the addition of WPC, WPI, or caseinates, etc (Kelly, 2011). The exact route depends on the specific protein level desired. Similar to whey protein powders, the milk protein isolate form is used to designate very high protein content milk protein concentrate (MPC).

Micellar casein is produced by microfiltration of skim milk followed by diafiltration of the retentate (Gaiani et al., 2005). Caseinate products are made from the precipitation of casein from skim milk using acid or enzymatic coagulation, followed by milling, drying and grinding. The salt form of the caseinate are prepared through a reaction with alkali solution, such as sodium or calcium hydroxide (Sarode et al., 2016).

Cream and buttermilk powders have not received much attention in literature. Similarly, buttermilk powder is not a strongly marketable product, and therefore also has not been the focus of significant research such as protein powders (Pisecky, 2012). Lactose is widely used in applications such as infant formula and pharmaceuticals (Carpin et al., 2017), however as it is a sugar, its structure-rehydration relationship is less complex and will not be discussed.

## Structure-Rehydration Relationships

Structure encompasses a large spectrum of powder parameters, and includes not only the structure of the individual proteins, fat globules and particles, but also the structure of the bulk powder. The bulk composition is discussed as the dominating structural feature driving powder rehydration characteristics due to the overriding interaction between water, proteins, fat and lactose.



However, the bulk composition does not necessarily reflect the surface composition, and addition of surfactants, such as lecithin, has been used to modify the surface of WMP particles in order to make them wettable. In addition, within the same type of powder structural features such as the density, shape and size of the particles become important in driving both the dissolution rate and in determining the voidage between the particles that is available for water penetration.

### Effect of Bulk Composition

It could be said that the largest effect on rehydration originates from the bulk chemical composition of the powder. Whilst specific structural features may give powders variability within a specific type, the composition effect is dominant and not always easy to overcome with other structural modifications.

For example lactose and minerals have to be rehydrated into true solution, fat into an emulsion and the proteins into a colloidal state; lactose and minerals are highly soluble, fat is hydrophobic and proteins are generally hydrophilic, although the casein and whey protein fractions show different properties. The relative proportion of these compounds is very important, for example [Richard et al. \(2013\)](#) found that the addition of lactose to poorly soluble MC powder improved its rehydration time by 5.2 times.

Skim milk powders rehydrate easily, with wetting taking less than 30 s and a minimum dispersibility of 90% within 10 to 20 s ([Pisecky, 2012](#)). This is because of the low fat and high lactose content. Whilst regular WMP rehydrates poorly due to the high fat content, IWMP is especially designed and can have better rehydration characteristics than SMP with a wettability of approximately 15 s and minimum dispersibility of 95% ([Pisecky, 2012](#)). Although the actual wettability and dispersibility of the powders vary depending on the powder and operational parameters during manufacture, overall the rehydration times of SMP and IWMP are very quick.

The two main types of protein, whey and casein tend to have opposing rehydration characteristics, with whey protein powders having good reconstitution ability. [Table 2](#) shows that whey permeate and WPI powders take in the order of a few minutes to rehydrate.

Meanwhile powders with a high casein content, such as MPC, MPI, and casein itself, generally have poor rehydration characteristics, which worsen with increasing protein content ([Crowley et al., 2015](#)). This is the reason that the total rehydration behaviour of high protein powders is often measured by monitoring the change in particle size using static light scattering or focused beam reflectance measurement, as opposed to using the methods described previously ([Gaiani et al., 2005](#)). Different criteria are used for classifying the powder as reconstituted, such as when the median particle size reaches an asymptote ([Syll et al., 2012](#); [Fang et al., 2011](#)) or when it reduces to a pre-specified size ([Ji et al., 2015, 2016a,b](#)).

Another key difference is that the reconstitution process of protein powders usually employs high speed mixers, as opposed to SMP and IWMPs where manual agitation is sufficient. Hence, due to the differences in the measurement techniques, the exact type of powder and the end-point of rehydration, the reported times can vary appreciably. Some example rehydration times for high casein powders are shown in [Table 2](#), and they vary from 20 minutes to over 24 hours, with 'hours' being the typical timescale, with the exception of sodium caseinate.

Although the overall bulk composition is important, how the powder is processed is as well. For example MC and WPI mixed before spray drying or after spray drying significantly affects rehydration of proteins. [Gaiani et al. \(2007\)](#) found that mixing the two proteins before spray drying significantly improved the rehydration characteristics of the powder compared with dry mixing post manufacture, possibly due to steric hindrance of whey preventing the MC folding during drying.

Another example is that excessive heat treatment and shearing during manufacture denatures proteins, imposes a high protein load on fat globules, and encourages protein–protein interactions, which impedes good solubility characteristics ([GEA, 2010](#); [McKenna, 2000](#)). A further example is the addition and removal of calcium, such as used for fortification, which has profound effects on the powder properties such as heat stability during reconstitution ([Deeth and Lewis, 2015](#)). These examples illustrate how processing affects reconstitution through changes in the molecular structure.

**Table 2** Rehydration times for different protein powders

Powder	Rehydration time
MPCs	Highly dependent on protein content, but MPC80 was not fully rehydrated after 30 minutes in the work by <a href="#">Fang et al. (2011)</a> , some MPCs shown to not be fully rehydrated after 24 hrs ( <a href="#">Crowley et al., 2015</a> ) although most formed stable suspensions, >3.5 hrs for MPC85 ( <a href="#">Mimouni et al., 2009</a> )
MPI	40–60 minutes ( <a href="#">Ji et al., 2015</a> ; <a href="#">Ji et al., 2016a,b</a> ), only 44% solubilised after 60 minutes ( <a href="#">Fitzpatrick et al., 2016</a> )
MC	from 20 minutes ( <a href="#">Ji et al., 2016a,b</a> ), to 4–35 hours depending on storage ( <a href="#">Nasser et al., 2017</a> ), to 6–8 hrs depending on stirring speed ( <a href="#">Syll et al., 2012</a> ), 9–14 hrs reported by ( <a href="#">Gaiani et al., 2005, 2007, 2009</a> ), 3–5 hrs depending on stirring speed ( <a href="#">Richard et al., 2013</a> )
SC	4 minutes ( <a href="#">Ji et al., 2016a,b</a> )
CC	>90 minutes ( <a href="#">Ji et al., 2016a</a> ), ~6 hours for four commercial products ( <a href="#">Moughal et al., 2000</a> )
Whey permeate	95% solubilisation after 1 minute ( <a href="#">Fitzpatrick et al., 2016</a> )
WPI	within 6 minutes ( <a href="#">Ji et al., 2016a</a> ), 17 minutes ( <a href="#">Gaiani et al., 2007</a> ), 4 minutes ( <a href="#">Gaiani et al., 2009</a> )

### Effect of Surface Composition

During dissolution, the surface of the particles sees the water first, and therefore the surface composition, or surface chemistry, is a factor that can be altered to change the rehydration behaviour. The addition of lecithin is a simple example of how a surface coating of a surfactant makes a hydrophobic powder wettable and easy to reconstitute, as with its application for instantising regular whole milk powder.

Some whey powders are also lecithinated and agglomerated, similar to IWMP, however in this case it is used to delay water absorption, as the proteins are very hydrophilic and instant absorption of water can interfere with dispersion (GEA, 2010).

Storage has a profound effect on the structure of dairy powders, resulting in worsening rehydration properties over time. McKenna (2000) found that it caused a 'skin' to develop on the surface of high protein particles that reduces water penetration. Mimouni et al. (2010) investigated the effect of storage on MPCs and found that interactions between casein micelles increase, resulting in a structure that prevents dispersion of the micelles and other components dispersing into the water. Similar to McKenna, the formation of a compact 'skin' on the surface of the particles caused the slowing of dissolution with storage (Mimouni et al., 2010). Increased interactions between surface casein micelles and migration of fat to the surface during storage were similarly implicated by Nasser et al. (2017).

Additionally, it has been reported that fat tends to be over-represented on the particle surface in comparison with the bulk composition (Kim et al., 2002, 2009; Gaiani et al., 2010). This was similarly found by Gaiani et al. (2006) for spray dried casein powders, in addition to the surface being depleted of minerals and lactose. The wettability of the powders was found to be correlated with the surface lactose content, whilst the dispersion of the powders was more affected by the bulk composition.

### Effect of Particle Size

Agglomeration to increase the particle size has long been investigated as a means of improving the rehydration ability of powders. Neff and Morris (1968) showed that the agglomeration of SMP, up to a limit, improved the reconstitution behaviour of SMP in 1968. Part of the instantisation process of manufacturing IWMP is the recycling of fine particles back into the spray dryer to create larger agglomerates (Pisecky, 2012). However, agglomeration only works up to a certain particle size, and above that hinders reconstitution as very large particles take a long time to dissolve. The different powder types have different 'optimal' particle sizes for reconstitution.

It is well known that IWMP rehydrates best with less than 10% particles < 125 µm and less than 10% particles > 500 µm, as the fine and coarse particles affect both the wetting and dispersing behaviour (Pisecky, 2012; Boiarkina et al., 2017). Gaiani et al. (2011) found that the optimal size for dispersion increased with increasing fat fraction, with 160 µm, 180 µm and 220 µm being optimal for SMP, semi-SMP and WMP respectively.

The effect of agglomeration on proteins is similarly complex. For example, Gaiani et al. (2007) found that rehydration time was retarded when MC powder was agglomerated, but had only a small positive effect on WPI powder. For MC the dispersibility of larger particle sizes over 350 µm decreased to 0, supporting the negative correlation. The negative effect of agglomeration on rehydration of MC powder was similarly found by Richard et al. (2013). Calcium caseinate, similar to MC, was also found to dissolve poorly with or without agglomeration (Ji et al., 2016a). One notable exception of the casein powders is sodium caseinate, which rehydrated rapidly in the work of Ji et al. (2016a), even at the smaller particle size.

Akin to the work of Gaiani et al. (2007), the negligible effect of agglomeration on WPI was also found in (Ji et al., 2016a), and the dispersibility of WPI increased slightly with decreasing particle size down to 40 µm.

Overall, powders made with hygroscopic materials such as lactose and minerals, or WPI, tend to rehydrate faster, with a favourable effect of an increase in particle size, up to a limit. But in powders where the dissolution of the individual particles are slow, like MC, increasing the particle size has the opposite effect.

### Effect of Particle Density

Although this is a structural parameter that is not often investigated in research, the higher the density of the particles the better they are able to sink and penetrate the surface tension of water for better rehydration (Birchal et al., 2005). The Handbook of Milk Powder Manufacture (Pisecky, 2012) recommend that the particle density be at least 1.15 g/cm<sup>3</sup> for fat containing powders for good wettability. The effect of particle density on other types of powders has not been investigated, and it is not known whether the recommendation of Birchal et al. (2005), that occluded air in droplets should be avoided during atomisation to increase the particle density, should apply across all spray dried dairy powders. However, the premise is sensible, although minimum or optimal particle density requirements for different powders are not known.

### Effect of Particle Morphology

Particle morphology can be measured through a number of parameters, such as convexity, solidity, circularity and elongation (Gaiani et al., 2011; Boiarkina et al., 2016). However, until recently, with advances in computing power and image processing techniques, it was not possible to quantitatively measure the morphology of a powder. Pisecky (2012) described the ideal agglomerate

shape for skim and whole milk powder dissolution as being a 'loose grape', as 'onion' shapes tend to take a long time for the particles to fully dissolve. Thus, a lot of the discussion of the shape of particles is tightly linked with powder agglomeration (Williams, 2007; Williams et al., 2009).

There has been little work looking at the link between dairy powder morphology and powder rehydration characteristics in a quantitative way. Gaiani et al. (2011) looked at morphological descriptors for understanding rehydration properties of different dairy powders. The convexity, sphericity and size were included in a partial least squares model for predicting the solubility and dispersibility of the powder, and the model performed well. However, due to the nature of the model, it is not clear exactly how these shape parameters affect the rehydration behaviour, or indeed if they are just indicators of powder type.

Boiarkina et al. (2016) investigated the difference in morphology between different spray drying plants and found that particle convexity and solidity were higher for the plant producing IWMP with the best rehydration properties, however this was also the plant that produced particles with a larger average particle size. Therefore the authors concluded that morphology is likely to be an indicator of the agglomeration state, and the size is likely to swamp any direct shape effect on dissolution.

## Conclusions

The structure and rehydration properties of dairy powders are intimately interlinked, starting with the powder composition. Skim and instant whole milk powders have been long developed to rehydrate well, through particle size enlargement and lecithination for WMP. On the other hand, the structure-rehydration relationship of the wide array of protein powder available is not well understood. Casein powders tend to have long rehydration times, unlike whey protein powders. The surface structure and composition is important for rehydration, which changes with processing and storage conditions. Any processing or storage conditions that denature proteins, encourage protein-protein interactions or protein incorporation into fat globules retard powder rehydration ability. Particle size is important for some powders, such as SMP and IWMP, but not for others, such as casein powders, where an increase in particle size generally worsens the rehydration time. Powder rehydration is highly complex, and methods for modifying the structure, such as the surface composition, can be used to modify rehydration behaviour of different dairy powders. However, in order to do this, the structure-processing relationship needs to be understood.

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## Structure and Properties of Chocolate

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Chocolate, or *Theobroma cacao*, brings a smile to faces everywhere. It has a deep history and can be traced back to the Mayan empire as the drink of the gods, but how exactly does the chocolate that we know come to be?

According to Merriam Webster, chocolate is a “food prepared from ground cacao beans” (Definition of Chocolate, 2018). The *T. cacao* tree produces pods containing cacao beans. “Cacao” is a botanical reference for the unfermented beans. Once harvested and fermented, these beans are now called “cocoa”, the major ingredient in chocolate products (Minifie, 1999).

Chocolate appears in the FDA’s Code of Federal Regulations (CFR) in 6 different categories – chocolate liquor, cocoa powder, breakfast or high fat cocoa, milk chocolate, sweet chocolate (semi-sweet or bittersweet), and white chocolate. Each type has a different “Standard of Identity” that must be met to legally be called chocolate. Chocolate can only contain cocoa butter and butter oil as fat sources and may only obtain chocolate flavor from the chocolate liquor. Additional flavors, such as dairy and those mimicking chocolate, may not be used (Food and Drug Administration, 21. C.F.R. 163.123, 2016). Table 1 lists the standards for sweet and milk chocolates. White chocolate must also only contain cocoa butter and butter oils added with dairy ingredients (21. C.F.R. 163.130, 2016), but it does not contain any cocoa particles (hence, the white color).

Chocolate processing begins on cacao plantations typically found within 15° of the equator. Cacao beans grow in pods on *T. cacao*. Once a tree reaches maturation, each pod harvested will typically have 30–40 cacao beans (Lima et al., 2011). The beans and pulp are removed from the pods and fermented for one to six days, depending on bean type (Afoakwa et al., 2008b). After fermentation, the beans are dried to a moisture content around 6%–8%, packed and shipped to bean processing centers as raw cocoa beans (Afoakwa et al., 2008b).

The fermented dried beans are cleaned and roasted before the winnowing process where the shells are separated from the nibs. The shells, about 10%–14% dry weight of the bean, are typically seen as a waste product (Afoakwa et al., 2008b). Once separated, the nibs may go through an alkalization step for additional color and flavor development or go straight to the grinding process, crushing the nib into small particles. The crushing step allows the cocoa butter to be released from the bean, creating chocolate liquor. Once ground, chocolate liquor can be pressed to separate the cocoa butter from the cocoa powder.

Chocolate liquor is the ground nib containing liquid cocoa butter with cocoa particles suspended throughout. Although some alcohol is produced during fermentation, it is quickly converted into other products, rendering chocolate liquor nonalcoholic. Liquor can either be pressed, separating cocoa butter from cocoa powder, or the liquor can go straight to chocolate production. To make chocolate, liquor is combined with additional cocoa butter, sugar, and other ingredients, such as milk products, creating a paste. This paste is then refined before conching.

Refining the chocolate paste reduces the particle size of the sugar and milk particles. Reduction of particle size impacts the texture, mouthfeel, and overall quality of the final product. Particle size in chocolate ranges from around 10–50 µm in the US and Europe (Jeffery, 1993). In production, a five-roll refiner is most common; the chocolate passes sequentially through the gaps between each roller. As the chocolate is processed, the gap thickness is reduced, creating smaller particles by attrition and breakage. Upon exiting the refiner, the cocoa mass has become solid, in the form of flakes, because of the high particle surface area. Once fully refined, the flakes are conched.

Conching is a process where shear and heat are applied to the refined mixture in two phases – dry and wet. Developed in 1879 by Rodolphe Lindt in Switzerland, conching is a key step in processing as the refined flakes become liquefied under shear. Liquefaction ensures the fat particles adequately coat sucrose, cocoa powder, and milk powder surfaces (The Lindt). During the initial “dry” conching phase, water and undesirable flavors are lost and flavor development continues. This is followed by a ‘wet’ conche, where additional cocoa butter and lecithin are added, ensuring all nonfat particles are coated with fat.

Overall, conching results in a product with desirable flavor development, and reduced water and low boiling point volatiles (Beckett, 2015). Lecithin, an emulsifier, is added to achieve desired yield stress and viscosity (Afoakwa et al., 2008a). Finally, the chocolate is ready to be tempered, formed by molding, depositing, or enrobing, and ultimately enjoyed.

**Table 1** “Standard of Identity” for sweet and milk chocolates

Chocolate type		Liquor		Milk fat		Milk components
		Minimum	Maximum	Minimum	Maximum	
Sweet	Sweet	15%	35%	–	12%	–
	Semi-Sweet	35%	–	–	12%	–
	Bittersweet					
Milk		10%	–	3%	–	12%

Source: Food and Drug Administration, 21. C.F.R. 163.123, 2016; 21. C.F.R. 163.130, 2016.

## Important Chocolate Processes

The processing of cocoa beans into bars requires several steps. Proper fermentation and drying is necessary for high quality chocolate. These processes allow valuable flavor precursors to develop within the bean.

### Fermentation and Drying

Fermentation of the beans is critical for flavor and aroma development. The beans and pulp are removed from the pod and placed in baskets, heaps, or boxes for fermentation, which lasts for one to six days depending on the bean type (Afoakwa et al., 2008b). Fermentation of the beans is still largely uncontrolled, occurring based on whatever microorganisms are present in the local environment. As the temperature during fermentation increases, the pulp drains from the beans (Schwan and Wheals, 2004). At about the third day, the beans reach between 45–50 °C and remain at this temperature until fermentation is complete.

The pulp composition surrounding the beans is about 85% water and 11% sugars (Minifie, 1999). The anaerobic conditions allow for microbial breakdown where yeast convert the sugars in the beans to ethanol. This ethanol is converted to acetic acid, carbon dioxide, and water by bacteria. During this time, the bean germinates and subsequently dies as the pulp drains off the beans (Hartel et al., 2018). Additionally, internal structural breakdown occurs, releasing enzymes and decreasing the pH from around 5.5–5.8 to 4.75–5.19 as acetic acid and lactic acid fermentation progress (Hartel et al., 2018; Lopez, 1979; Rohan and Stewart, 1967).

Numerous chemical changes take place during fermentation. Polyphenols are broken down into quinones and other compounds, while proteins and sugars are hydrolyzed into peptides and reducing sugars. These latter products serve as precursors for cocoa flavor and aroma development during roasting (Afoakwa et al., 2008b). Unfortunately, the highly nutritional polyphenols in chocolate, particularly catechin and epicatechin, are broken down during fermentation, reducing the nutritional status of the final chocolate.

Once fermentation is complete, the beans are dried. Drying the beans results in loss of moisture and, because of the warm temperatures, the start of flavor development. The desired moisture for shipping beans is around 6%–8%, which prevents the growth of molds. Drying techniques include air drying, shade drying, and sun drying, with sun dried beans having the most recognizable chocolate sensory properties (Hurst et al., 2011). Once dried, the beans are shipped to facilities to be processed and turned into chocolate products.

### Roasting

Dried cocoa beans are cleaned, sterilized and roasted upon arrival at the chocolate manufacturing plant. The beans are roasted at temperatures ranging from 120–150 °C for anywhere between 5 and 120 min, allowing further flavor development from fermentation precursors (Afoakwa et al., 2008b). Roasting also serves as an important safety step in chocolate processing. Fermented cocoa beans have high microbial counts and roasting sterilizes the beans, rendering them safe for consumption. Some chocolates on the market are 'raw', indicating that this kill step has not occurred. These products can still have high microbial contamination and be unsafe for consumption (Hartel et al., 2018).

Large scale roasting of beans is typically done in either a batch roaster or through continuous roasting. Batch roasting often occurs in a large drum with hot air blowing onto the beans to increase temperature. Several types of continuous roasting processes exist, including those that use radiation heating and heating tunnels. Overall, roasting parameters that need to be monitored are time and temperature because they drive moisture loss and govern flavor development reactions.

When roasting, it is important to ensure the beans are heated evenly but without burning the shell (Minifie, 1999). Roasting reduces the amount of moisture in the bean and allows for the shell to be loosened from the nib. This loose shell allows for easier separation from the nib during winnowing. The nib continues through production to be ground into chocolate liquor or pressed to expel the cocoa butter and cocoa powder.

## Important Chocolate Characteristics

The previously described processes develop the characteristic flavors and aromas associated with chocolate. These characteristics, along with the physical aspects of chocolate structure, will be discussed in further detail.

### Flavor and Aroma

It is important to understand how taste, flavor, and aroma differ and the roles each play when eating chocolate. Taste is comprised of the five basic attributes: sweet, salty, bitter, sour and umami. These tastes, in conjunction with trigeminal sensations, such as spicy, cooling, astringent, etc., and aroma make up the flavor of a substance. Flavor and aroma both play a key role in the quality of chocolate. Flavor is defined as "the sensation caused by and those properties of, any substance taken into the mouth which stimulates one or both of the senses of taste and smell, and/or also the pain, tactile and temperature receptors in the mouth" (American Society of Flavor Chemists). Aroma is the perception of flavor. There are over 10,000 aromas recognizable, including floral, spices, and dairy notes. Recognizing and characterizing these flavors and aromas helps to ensure chocolate quality over time.



The development of flavors and aromas from the fermentation, drying, roasting, and conching processes create the characteristic chocolatey notes. Bean origin impacts chocolate flavor and aroma; bean blending is common practice for a balanced profile although single origin chocolates are becoming increasingly popular. Other ingredients, such as milk products in milk chocolate, also contribute to the flavors and aromas of chocolates.

Alkalization is a technique commonly used to alter the flavor and color of cocoa, making it darker. Whole beans, nibs, liquor, and powder can all be alkalized using either potassium or sodium carbonate. This process results in a pH increase (hence the term alkalization) from around 5.2 to 6.8 and up (Hartel et al., 2018), which changes the pathways of the Maillard reaction and changes the volatile flavors generated. This technique can be beneficial; however, some argue that true chocolate flavors are lost.

Milk chocolates also develop flavor and aroma with the inclusion of dried milk powders, whether whole milk or nonfat milk powders. These powders are added to the liquor and sugar to form a chocolate paste that is refined. Milk powders are processed in a variety of ways, creating differences in how they affect chocolate. Particle shape, size, structure, and composition all impact chocolate processing conditions. In milk powders, the ability of milk fat to interact with cocoa butter and particles has the greatest impact, specifically on the crystallization of the cocoa butter (Liang and Hartel, 2004).

Some milk chocolates utilize the milk crumb process instead of or in addition to milk powders. Milk crumb is made by evaporating fresh milk, condensing it to about 90% total solids. During evaporation, the Maillard Reaction occurs between the milk protein and sugars, developing caramelized and creamy flavors (Minifie, 1999). The crumb is mixed with chocolate liquor and dried to a powder containing about 1% moisture for use in a similar manner as milk powders.

## Structure

The structure of chocolate is quite complex, yet these structures play an important role in chocolate quality. Various different aspects will be discussed: particle size and rheological properties, fat phase, tempering chocolate, and fat bloom.

### Particle Size and Rheology

One important differentiating factor between brand and quality of chocolate is smoothness. This attribute is governed by the size of the various particles that make up its dispersed phase. Chocolate is approximately 60%–70% dispersed particles (sucrose crystals, cocoa powders, and milk powders) contained within a fat phase, the cocoa butter. These particles have a large impact on chocolate mouthfeel (Afoakwa et al., 2009). Since humans can detect particle sizes ranging from 16–20  $\mu\text{m}$  depending on the matrix of the system (Hartel et al., 2018), the refining step, described previously, must reduce the particle size to less than this. High quality chocolates have small particles and impart a smooth, creamy mouthfeel. However, reducing particle size too much has undesirable consequences, related to how well the melted chocolate can flow.

Melted chocolate must have the proper flow properties for the intended application (Afoakwa et al., 2008a). The two parameters that govern these flow properties, or how easily the melted chocolate can be pumped or formed, are yield stress and viscosity. Yield stress is a term used to explain the “stand-up” properties of a material. Something with a high yield stress, like thick mashed potatoes, resists the force of gravity and does not flow unless some pressure is applied (as in a pump). Viscosity describes the thickness or consistency, with water having low viscosity and honey having much higher viscosity. Both yield stress and viscosity are critical to chocolate processing.

From pumping across plants, to depositing or coating, the flow properties of a chocolate are critical to efficient operation. They must also be balanced with the intended application (Hartel et al., 2018). Chocolates used for molding generally have a thinner consistency with little to no yield stress, so the fluid chocolate flows readily to fill all the nooks and crannies in the mold. Chocolates used in depositing, like the Hershey’s Kiss or chocolate chips, have a thicker consistency with a high yield stress. Such products are simply deposited onto conveyor belts and must retain their shape prior to cooling. The high yield stress of these chocolates provides the stand-up properties needed.

To control chocolate flow properties, manufacturers rely on composition factors. High fat content, for example, provides greater separation for the particles and reduces both yield stress and consistency. Lecithin, an emulsifier, is added at low levels (<0.5%) specifically to reduce viscosity. It acts by coating sugar crystals to prevent aggregation. Finally, particle size plays a key role in chocolate viscosity, with smaller particles giving higher viscosity than larger particles at a given fat content.

### Fat Phase

The main fat phase of chocolate comes from cocoa butter, although milk fat is often used in both milk and dark chocolates to moderate texture. Cocoa butter is a unique fat in nature; it is hard at room temperature yet completely melts in the mouth, with no waxy aftertaste. Rapid melting in the mouth results in rapid release of volatile aroma compounds, adding to the delightful flavor experience. But not all cocoa butters are the same. Cocoa butters from different geographical regions have varying chemical composition, giving them slightly different flavor profiles and melting characteristics (Hartel et al., 2018; Talbot, 2017).

In melted chocolate, the cocoa butter is liquid, but upon cooling, it solidifies into a crystalline form. However, cocoa butter crystallization is complex because it can take on multiple forms, depending on how it was crystallized. Cocoa butter has six known polymorphs or distinct crystalline forms, characterized by Form I–VI. Each form results in a varying degree of stability and each crystal has a distinct melting point (Wille and Lutton, 1966). Lower polymorphs, Forms I–IV, form looser crystalline structures and are quite unstable, wanting to transform into the more stable Forms V and VI (Afoakwa et al., 2009). Of these, the three main polymorphs of concern are often called  $\beta'$ ,  $\beta$ -V and  $\beta$ -VI, with their melting points shown in Table 2. The most desirable crystal form in chocolate is  $\beta$ -V based on its relatively long stability and its desirable melting point. Form  $\beta$ -VI, while technically the most

stable, is difficult to generate and has a melting point above mouth temperature. Proper tempering of chocolate is necessary to create  $\beta$ -V crystals.

### Tempering Chocolate

Tempering chocolate is a thermal treatment designed to create the proper crystalline form,  $\beta$ -V, allowing for chocolate to set properly and from a strong network (Windhab, 2017). Well-tempered chocolate results in a product with a glossy shine, an audible snap, good contraction from the mold, and resistance to bloom during storage. There are numerous methods to make tempered chocolate, although all are focused on creating the desirable  $\beta$ -V crystal (Hartel et al., 2018). The methods of tempering different chocolate types (sweet, milk, and white) are the same, with only the temperature points varying slightly.

If melted chocolate is simply allowed to cool without being tempered, the cocoa butter quickly forms unstable polymorphs. Rapid and uncontrolled transformation of these unstable crystals into more stable polymorphs leads to particle rearrangement and unsightly spots on the surface. These spots are called bloom, in this case caused by improper tempering. To accomplish good temper, melted chocolate is first cooled to rapidly promote formation of unstable polymorphs, primarily  $\beta'$ , and then heated slightly to promote transformation to the stable  $\beta$ -V polymorph. The end result of tempering is liquid chocolate with a few percent of  $\beta$ -V seeds; this liquid chocolate can then be used for molding or depositing. Upon cooling of tempered chocolate, the  $\beta$ -V seeds dominate solidification of the liquid cocoa butter, resulting in chocolates with desirable properties, including resistance to bloom during storage (Hartel et al., 2018).

### Chocolate Bloom

There are various types of bloom that can occur in chocolate. Sugar bloom occurs when moisture condenses on the surface of chocolate. A droplet of water on the surface draws sugar out of the matrix, which then recrystallizes as a white mound when the water evaporates. Sugar bloom is most common when unwrapped chocolate is removed from a freezer or refrigerator and exposed to humid air. If the surface temperature is lower than the dew point of the air, water condenses, eventually leading to sugar bloom.

Solidification of untempered chocolate also results in a form of bloom, distinguished by whitish spots surrounding a dark inner region. The whitish spots are thought to be depleted of fat and concentrated in particles (Lonchampt and Hartel, 2006).

Of most concern to the chocolate maker is long-term storage bloom. Even well-tempered chocolate is prone to gradual development of a whitish/gray surface tinge over time. Although this type of bloom looks like mold growth, it is merely the rearrangement of cocoa butter crystals at the surface that gives this appearance. Storage bloom is generally associated with appearance of the most thermodynamically stable cocoa butter polymorph,  $\beta$ -VI, although the polymorphic transition is not necessarily the cause of bloom (Bricknell and Hartel, 1998).

Chocolate makers have studied storage bloom for decades; however, the specific mechanisms that cause bloom are still unclear and the means for completely eliminating it remain elusive. It is generally accepted that storage bloom in chocolate occurs when a portion of the fat phase migrates to the surface, allowing recrystallization of cocoa butter over time, resulting in formation of a dull, whitish/gray surface (Hartel et al., 2018). Chocolate scientists continue to study bloom mechanisms in hopes of finding solutions to this centuries-old problem.

### Future of Chocolate

The future of chocolate will depend on a variety of factors, such as changes in the climate affecting the cacao growing regions of the world and developing solutions to better control and understand fermentation. Shifting temperatures and lack of humidity are already impacting cacao production. Potential solutions may include selective breeding or even genetically modified crops to help growers adapt to these continual changes (Climate and Chocolate). Controlling fermentation more carefully (rather than relying on the local microbiota) is also likely to help growers adapt to changes and lead to more controlled flavors/aromas of chocolate.

**Table 2** Melting points of cocoa butter polymorphs

<i>Polymorphic form</i>	<i>Melting point (C)</i>
$\beta'$	27–29
$\beta$ -V	34–35
$\beta$ -VI	36

Source: (Deora et al., 2013; Hartel et al., 2018)

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# Traditional African Bread and the Physicochemical Properties of Unfermented Flatbreads

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## Overview

Bread is a staple diet for communities across the globe. As humans progressed from hunter–gatherer communities to sedentary agriculture, they learned how to process bread from cereal grains grown in their area. Manual grain harvesting, threshing, grain cleaning, pounding with mortar and pestle for decortication, stone grinding to flour, mixing of flour with water to make dough, and baking on heated earthenware (clay baking griddle), on heated stone slabs, or in hot ashes were early technologies communities used in the production of their traditional/indigenous breads. In addition to wheat, barley, sorghum, maize, millets, and teff grains are widely used in the preparation of traditional breads in Africa. These cereal grains in the form of unfermented or fermented dough are baked and consumed as flat or semileavened bread. Those from wheat flour are used to process unfermented flat or fermented leavened bread. In many African countries, wheat grain production and supply are limited and because of this, composite flours from different cereal grains, legumes, and starchy food sources (tubers and roots) are also used. Common traditional breads consumed in different parts of Africa, their major ingredients, and their structural natures are described in **Table 1**. The list is by no means all inclusive for bread consumed throughout the African continent. Closely related breads or the same bread in terms of recipe and processing methods may also be called by different names in different communities. In the northern African regions, bread processed from wheat is popular, and to some degree breads processed from sorghum, millets, and maize are also consumed. In the eastern African regions, countries such as Ethiopia, Eritrea, Sudan, and parts of Somalia and Kenya, bread processed from the grains of teff, wheat, sorghum, and millets, particularly finger millet, are consumed. In the East African community countries Kenya, Uganda, Tanzania, Burundi, Rwanda, and South Sudan, breads processed from maize, sorghum, wheat, millets, and rice are consumed. In the southern African regions, bread processed from maize, wheat, sorghum, and pearl millet are consumed. In the western African regions, bread processed from sorghum, millets, fonio, cassava, wheat, rice, and maize are consumed. In the central African regions, bread processed from sorghum, millets, wheat, maize, and cassava are consumed. With the global nature of the wheat supply chain, different types of wheat breads are consumed in almost all African urban and semiurban areas.

## Bread Types Consumed in Africa

The traditional African breads may be classified based on the type of basic raw materials used and as unfermented, fermented aerated (leavened) (cross reference to article 2), flat (single layered or double layered), thin, or thick breads. The basic raw materials used are sources of carbohydrates (starches, fibers, and sugars), proteins, enzymes, minerals, lipids/fats, and bioactive/phytochemical compounds (**Figure 1**, **Table 2** (cross ref article 2) and **Table 4**). Because of differences in the chemical composition among wheat, maize, sorghum, teff, and millets, breads vary in terms of quality (nutrition, texture, and sensory properties) and cultural acceptance across regions. Furthermore, bread recipes; processing practices such as grain cleaning, soaking, decortication, pounding, milling, and dough making; fermentation conditions; the nature of fermenting microorganisms; and baking conditions can also influence the properties of the bread. In this article, the nutrient compositions of cereal grains (**Table 2**) and legumes/pulses, tubers, and roots (**Table 1** cross reference article 2) used in African traditional breads are included. The physicochemical properties inherent to bread making of cereal grains (**Table 4**) and legumes/pulses, tubers, and roots (**Table 2** cross reference article 2) are described along with the nutrient compositions of some baked breads (**Table 3**). Anatomical/histological structures of wheat (**Figure 1**), maize (**Figure 2**), and sorghum grains (**Figure 3**) are given as examples, because they are important to understand the processing of cereal grains. The methods of processing and properties of unfermented African flatbreads are described next.

## Unfermented Flatbreads

Unfermented flatbreads are probably the earliest type of processed food made by humans and predate the baking of fermented breads (Haaland, 2007). These types of bread are processed at large from wheat, maize, sorghum, teff, different species of millet, and rice grain flour. After grain cleaning, the processing of flatbread can vary depending on grain type, composition, and functionality **Table 2** (cross ref article 2) and **Table 4**. The properties of the bread can also vary, depending on flour particle size, whether the flour is refined or decorticated or whole grain flours are used, and if other ingredients (salt, oils/fats, and/or spices) are added. In addition, dough properties, dough relaxation, and baking methods all contribute. Often there are ceremonial/ritual attachments to certain breads, and in some communities bread is baked as a thanksgiving for the harvest, whereas others are attached to cultural and religious festivities.

**Table 1** Some traditional breads consumed in Africa and their common ingredients and structural features

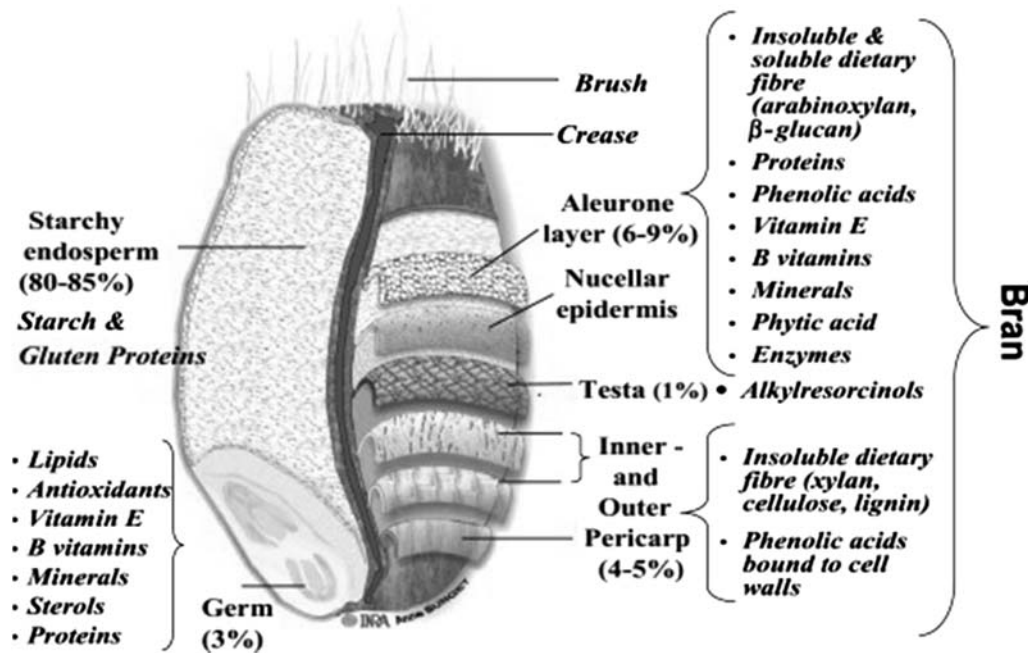
<i>Bread name</i>	<i>Ingredients</i>	<i>Fermented/unfermented/flatbread/whole grain flour product</i>	<i>Country</i>
<i>Anise bread</i>	Wheat flour, yeast, salt, water, anise seed, maize	Leavened bread	Morocco
<i>Aish merahrah</i>	Maize flour with 5%–10% ground fenugreek seeds; maize dough fermented overnight with sourdough starter	Fermented; flattened into round disk-shaped pita-type bread	Egypt
<i>Baladi/balady</i>	Coarse wheat (about 82% extraction)/nearly whole wheat flour (85%–95%), yeast or starter from natural fermentation, water, salt, and olive oil; high water absorption, dough raises in oven and separates into two thin layers from steam generated, making top and bottom crust	Fermented pocket bread/sandwich bread, rough-textured bread	Egypt, Sudan
<i>Bazin</i>	Barley flour, water, salt, with soup from date syrup, olive oil, black pepper, tomato, onion, garlic, and mint	Unfermented bread (Neolithic dish made of dough and sauce)	Libya
Cassava–wheat bread	Bread wheat flour, cassava flour/cassava starch	Fermented composite bread	Nigeria
<i>Chapati/roti/chapati-like</i>	Wheat, maize, sorghum, or millet flours, water, salt. Optional ingredients (chopped onion, chili, coriander leaves and/or grated coconut)	Unfermented/unleavened flatbread	Uganda, Kenya, Tanzania
<i>Chà-chà-bsa/ché-ché-bsa</i> (pizza-like)	Wheat flour, salt, water	Unfermented flatbread; for consumption, baked bread is broken into pieces and mixed with spiced butter, false cardamom, red pepper	Ethiopia
<i>Chikwange/yikwanga/ntuka/bugali/ kmonmogo/chawada/bobolo/ myondo/mboung/mangbele</i>	Cassava pulp fermented, ground to flour-like, sieved to produce flavor, pounded, and then steamed/cooked as bread wrapped in banana leaf	Fermented cassava bread	Congo, Tanzania, Cameroon, Gabon, Congo Brazzaville, Central African Republic
<i>Cumboo</i>	Teff flour; sometimes sorghum and millet flours are mixed with teff; baked from fermented/early fermented dough	Thick fermented bread	Ethiopia
<i>Defo dabo (difo daabo)</i>	Wheat flour	Thick fermented bread	Ethiopia
<i>Hambasha/himbasha</i>	Wheat flour	Fermented semileavened flatbread; celebratory, slightly sweet, often served at special occasions; several varieties, most distinctive flavoring is ground cardamom seeds	Ethiopia and Eritrea
<i>Harcha</i>	Coarse semolina, high fat, salt, and water	Unleavened flatbread with high fat content, rough surface, and a long shelf life	Algeria
<i>Hibist</i>	Cereal grain flours intended to be used for all purposes of baking	Steamed bread	Ethiopia
<i>Injera/enjera/caabita/budeena</i>	Teff flour, sometimes sorghum and finger millet flours	Fermented, crepe-like, soft, spongy, sour, circular flatbread, whole grain flour product	Ethiopia and Eritrea
<i>Inya</i>	Cassava alone or mixed with either sorghum or millets	Fermented flat; when mixed with millet is a thick sweet bread	Uganda
<i>Kenkey</i>	Maize flour	Fermented maize dough dumpling-like bread	Ghana

(Continued)

**Table 1** Some traditional breads consumed in Africa and their common ingredients and structural features—cont'd

<i>Bread name</i>	<i>Ingredients</i>	<i>Fermented/unfermented/flatbread/whole grain flour product</i>	<i>Country</i>
<i>Kisra</i>	Sorghum flour, also sometimes from millet flour	Fermented, sour, pancake-like flatbread, relatively thin, with no holes, and no spongy, supple, soft, and moist in texture	Sudan, Ethiopia
<i>Kitta, bixxille, daguwalo</i>	Teff flour, sometimes sorghum and finger millet flours	Thicker than injera, unleavened or slightly leavened	Ethiopia
<i>Khubz</i>	Wheat flour	Rolled like pizza	Morocco
<i>Khobz eddar</i>	Fine durum semolina, water, fat, milk, yeast, egg, salt, and sesame	Leavened flatbread	Algeria
<i>Kwon kal</i>	Millet, cassava flours	Big round lump like	Uganda
<i>Laaffisso</i>	Sorghum flour	Fermented, semileavened flatbread	Ethiopia
<i>Lahoh/lohoh</i>	Sorghum flour, warm water, yeast, and a pinch of salt; self-rising flour	Leavened, spongy, pancake-like flatbread	Djibouti and Somalia
<i>Lahooh</i>	Sorghum flour along with wheat flour and/or corn flour	Flatbread, bulkier than <i>injera</i> and <i>kisra</i>	Somalia and Djibouti
<i>Maadjouna</i>	Fine durum semolina, water, salt	Unleavened flatbread, short shelf life, for immediate consumption after baking	Algeria
<i>Mathlouaa</i>	Fine durum semolina, dry yeast as leavening agent	Leavened flatbread, light and spongy, short shelf life, to be consumed on the day baked	Algeria
<i>Mehrahras</i>	Durum wheat flour	Leavened bread, fine uniform crumb structure and somewhat yellowish color; low loaf volume compared to bread wheat	Egypt
<i>Ngome</i>	Millet flour, water, and vegetable oil; home-ground and coarse millet flour	Flatbread	Mali
<i>Pita bread</i>	Wheat flour, salt, sugar, yeast, water, with optional ingredients (sugars, oils, or improving agents)	Low specific volume, pocket-like thin bread processed from lean dough, low in fat	Morocco, Egypt, Tunisia, Libya, Algeria
<i>Potbrood</i>	Wheat flour and sweet corn flour	Leavened bread	South Africa
<i>Owambo bread or Oshikwiila</i>	Pearl millet flour, water, seasoned with salt and/or sugar	Unleavened bread	Namibia
<i>Rakhsis</i>	Fine durum wheat semolina, fat, salt, water	Unfermented flatbread, thin, soft, homogeneous surface without blisters, firm crust, and a compact crumb; relatively long shelf life	Algeria
<i>Terablesi</i>	Wheat flour of low extraction rate (70%), salt, and yeast	Leavened bread	Tunisia
<i>Ujeqe dodogoyi, madombolo, umbhako, ledombolo, sigenza, ujeqe, monepola oa poone e ncha</i>	Maize, sorghum, or wheat flour	Steamed bread, round white-gray loaf, moist, soft, fine, firm with sticky crumb; nonwheat breads are crumbly, hard, and fibrous	South Africa
<i>Wheat bread</i>	Bread wheat flour, water, salt, yeast, fat/shortening, optional various bread improvers	Leavened bread	All countries





**Figure 1** Wheat grain anatomical structure and chemical components. Adapted from Surget, A., Barron, C., 2005. Histologie du grain de blé. Ind. Des. Céréales. 145, 3–7 and Brouns, F., Hemery, Y., Price, R., Anson, N.M., 2012. Wheat aleurone: separation, composition, health aspects, and potential food use. Crit. Rev. Food Sci. Nutr. 52, 553–568; color image available online.

### Maize Bread

Maize (*Zea mays* L.), also called corn, is a tropical cereal. The grain is hull-less (grains are loosely adhered to glumes, allowing easy removal during threshing), has the largest size among the cereal grains, and comes in many colors. The accumulation of different types of anthocyanins (cyanidins, pelargonidins, and peonidins) and other polyphenols in the pericarp, testa, and aleurone layer of the grain confers a blue, purple, or red color, whereas the orange color is due to the accumulation of carotenoids (lutein, zeaxanthin,  $\beta$ -cryptoxanthin, zeinoxanthin, antheraxanthin, and  $\beta$ -carotene) (Suri and Tanumihardjo, 2016). Widely cultivated varieties are flint, dent, floury, sweet, popcorn, high lysine (high protein quality), waxy, and amylomaize, and of these, flint, dent, and floury maize grains are widely milled into maize meals and flour (Nuss and Tanumihardjo, 2010).

Prior to processing, hand-cleaned maize grains are washed and soaked in potable water (about 3–12 h) (Greiner and Konietzny, 2006) and then gently scrubbed/polished with a mortar and pestle before soaking to remove the tip cap. Soaking facilitates bran (pericarp, tip cap, and germ) separation. After soaking, the grains are decorticated by pounding with a wooden mortar and pestle. The pericarp, tip cap, and germ are detached by the impact of pounding. The process also leads to different degrees of damage to the testa, germ, and aleurone layer. The pounded mass is spread, often on plates made from a variety of materials (plastic, stainless steel, jute bags, wood, or woven grass), and dried in the sun. The dried mass is sifted or winnowed (blowing air current through the mass) to separate light bran materials from the maize meal (samp). The process may not result in complete separation of clean endosperm; hence broken pericarp, germ, testa, and aleurone layer may remain, which can be nutritionally beneficial. On soaking and pounding, some micronutrients can also diffuse into the endosperm and some water-soluble nutrients and bioactive compounds can be lost by leaching. The maize meal is milled into fine flours (about <75–212  $\mu$ m) using stone mills or cottage mills (metal plates composed of stone attrition mills or hammer mills). Stone mills generate heat (Doblado-Maldonado et al., 2012) and result in damage to the starch granules, proteins, and unsaturated fatty acids compared with roller milling technology. In ancient milling, maize grains are hand milled by using a small, light top stone to rub the grains over a large, heavy, stationary stone. The same way of milling is also used for sorghum, wheat, barley, teff, and the millet grains. The flour produced is hand mixed with water and kneaded to make a consistent dough that can be easily spread. The dough is allowed to rest for some time (approximately 10–45 min) to allow uniform hydration of the flour particles and relax and stabilize disulfide bonds in the maize zein proteins, which assists in shaping of the dough. After shaping, the dough is flattened and spread onto a lightly greased baking griddle (clay or metal baking pan), covered with a lid, and steam baked. Various heat sources are used, including electricity, natural gas, and wood- and coal-burning fires. To bake variations of the bread, open pans can also be used (without steaming) and the bread is flipped to bake both surfaces evenly. Such flatbread is not fermented and thus is not aerated and presents with a dense structure. During baking, starch granules swell and gelatinize, proteins are denatured, and the bread is set. The color and flavor of flatbreads are influenced by the Maillard and caramelization reactions as well as by the type of maize grain used. Since fermentation is not involved, antinutrients such as phytic acid and condensed tannins (proanthocyanidins) can be present in the bread (Suri and Tanumihardjo, 2016). This is due to parts of the grain rich in these compounds (e.g., aleurone layer and germ) remaining in the flour

**Table 2** Nutrient composition of cereal grains used in African traditional breads

Composition	Wheat, hard white <sup>a</sup>	Wheat, hard red, winter <sup>a</sup>	Durum wheat <sup>a</sup>	White maize <sup>a</sup>	Yellow maize <sup>a</sup>	Sorghum <sup>a,(f)</sup>	Pearl millet <sup>c,(d)</sup>	Finger millet <sup>c</sup>	Foxtail millet <sup>c</sup>	Proso millet <sup>c</sup>	Teff <sup>a,(e)</sup>	Fonio, white <sup>d</sup>	Fonio, black <sup>d</sup>	Rice, white, unenriched <sup>a</sup>
Water (%)	9.6	13.1	10.9	10.9	10.4	10.3	12.4 <sup>b</sup>	13.1 <sup>b</sup>	11.2 <sup>b</sup>	11.9 <sup>b</sup>	8.8	11.1	10.3	11.9
Protein (%)	11.3	12.6	13.7	6.9	9.4	8.4 (12.3)	14.5 (8.8)	7.3	11.7	11.0	13.3 (11)	7.1	8.9	6.0
Total lipids/fat (%)	1.7	1.5	2.5	3.9	4.7	3.3 (3.6)	5.1 (5.8)	1.3	3.9	3.5	2.4 (2.5)	3.1	3.0	1.4
Crude fiber (%)							2.0 (1.5)	3.6	7.0	9.0	(3.0)			
Total dietary fiber (%)	12.2	12.2		7.3	7.3	6.6	7.0 (9.0)	19.1	19.1	8.5	8.0	7.4	6.2	2.4
Ash (%)					1.2	(1.7)	2.0	3.0	3.0	3.6	(2.8)	2.1	2.2	0.4
CHO (%)	75.9	71.2	71.1	76.9	74.3	76.6 (70.7)	67.5 (64.8)	72.6	60.9	70.4	73.1 (73.0)	69.3	69.4	80.1
Total sugars (%)	0.4	0.4		0.6	0.6	1.9					1.8			0.12
Energy (kcal 100 g <sup>-1</sup> )	342	327	339	361	365	359 (329)	361 (364)	328	331	341	367 (335)	348	353	366
Ca (mg 100 g <sup>-1</sup> )	32.0	29.0	34.0	7.0	7.0	12.0 (15)	42.0 (14)	344.0	31.0	8.0	180.0 (165.2)	40.0	51.0	10.0
Fe (mg 100 g <sup>-1</sup> )	4.6	3.2	3.5	2.4	2.7	3.1 (4.2)	11.0 (7.6)	3.9	2.8	2.9	7.6, (5.7 <sup>a</sup> , 15.7 <sup>b</sup> )*	8.5	10.0	0.35
Mg (mg 100 g <sup>-1</sup> )	93.0	126.0	144.0	93.0	127	123 (171)	137.0 (97)	137.0	143.0	114.0	184.0 (169.8)	430.0	434.0	35.0
P (mg 100 g <sup>-1</sup> )	355.0	288.0	508.0	272.0	210.0	278.0 (352)	240.0 (207.0)	283.0	290.0	206.0	429.0 (425.4)	191.0	234.0	98.0
K (mg 100 g <sup>-1</sup> )	432.0	363.0	431.0	315.0	287.0	324.0	390.0 (401)	408.0	364.0	195.0	427.0 (380.0)	337.0	340.0	76.0
Na (mg 100 g <sup>-1</sup> )	2.0	2.0	2.0	5.0	35.0	3.0	10.0 (19)	11.0	1.3	5.0	12.0 (15.9)	7.0	8.0	0.0
Zn (mg 100 g <sup>-1</sup> )	3.3	2.7	4.2	1.73	2.2	1.6 (2.5)	3.1 (2.8)	2.3	3.5	1.7	3.6 (4.8)	1.5	3.8	0.8
Thiamine (mg 100 g <sup>-1</sup> )	0.39	0.38	0.42	0.25	0.39	0.34 (0.38)	0.38 (0.32)	0.42	0.59	0.41	0.39 (0.3)	0.25	0.26	0.14
Riboflavin (mg 100 g <sup>-1</sup> )	0.11	0.12	0.12	0.08	0.20	0.06 (0.15)	0.21 (0.27)	0.19	0.11	0.28	0.27 (0.2)	0.10	0.10	0.02
Niacin (mg 100 g <sup>-1</sup> )	4.40	5.7	6.7	1.90	3.63	4.50 (4.3)	2.8 (2.4)	1.1	3.2	4.5	3.36 (2.5)	1.7	1.7	2.6
Vitamin B6 (mg 100 g <sup>-1</sup> )	0.37	0.30	0.42	0.37	0.62	0.33	(0.74)				0.48	0.73	0.74	0.44
Folate, DFE (μg 100 g <sup>-1</sup> )	38	38	43.0	25.00	19.00	25.0	(30)					29.0	29.0	4.0
Vitamin A (IU)	9.0	9	0.0	3.00	214	0.0					9.0			
Vitamin E (α-tocopherol) (mg 100 g <sup>-1</sup> )	1.01	1.01	0.0	0.42	0.49	0.50	(0.07)				0.08	0.05	0.05	0.11

<sup>a</sup>Source: USDA (2016).<sup>b</sup>Source: Adebisi et al. (2017).<sup>c</sup>Source: Chandra et al. (2016).<sup>d</sup>Source: FAO (2012).<sup>e</sup>Source: Bultosa (2016).<sup>f</sup>Source: Kulamarva et al. (2009).

\*a=mean of iron from cleaned, acid- and/or water-washed samples and b =mean of iron from not wash cleaned samples.

**Table 3** Typical nutrient composition of breads processed from cereal grains commonly used in Africa

Composition	White bread, wheat <sup>a</sup>	Brown bread, wheat <sup>a</sup>	Ambasha bread, wheat <sup>b</sup>	Bread, maize <sup>c</sup>	Injera, teff <sup>b</sup>	Kisra, sorghum <sup>d,(e)</sup>	Kisra, millet <sup>f</sup>	Bread, sorghum <sup>g</sup>	Bread, pearl millet <sup>g</sup>	Bread, steamed <sup>h</sup>	Chapati, wheat bread <sup>i</sup>	Baladi bread (90% wheat flour extraction) <sup>g</sup>
Water (%)	39.0	39.0	41.8	31.9	59.8	52.5	6.6	27.2–35.4	25.11–28.1	51.8	33.0	36.0
Protein (%)	8.7	9.0	8.0	7.2	4.2	12.2	15.2	15.7–16.4	12.3–17.3	6.4	11.3	8.0
Total lipids/fat (%)	1.2	1.5	0.8	10.0	0.6	3.4	4.7	4.0–5.1	4.1–5.6	0.8	7.5	1.5
Crude fiber (%)						3.4		2.4–2.9	2.1–2.8			0.9
Total dietary fiber (%)	3.2	5.6	1.9	2.4	1.7		1.3			0.1	4.9	
Ash (%)	2.2	2.7	1.5	2.7	1.5	1.5	2.3	2.2–2.5	1.9–2.6	0.96		0.9
CHO (%)	45.2	42.7	48.0	48.1	33.9	79.6	70.0	73.6–75.3	50.8–71.9	37.8	46.4	53.6
Total sugars (%)							1.5			1.1	2.7	
Energy (kcal 100 g <sup>-1</sup> )	242.6	246.2	227.0	314.0	162.0				289.8–407.0	195.6	297.0	254.0
Ca (mg 100 g <sup>-1</sup> )	12.0	14.0	35.0	73.0	64.0	(204.0)	5.1	23–30		78.0	93.0	20.0
Fe (mg 100 g <sup>-1</sup> )	1.7	1.9	12.7	1.9	30.5	(48.6)	42.6	4.2–5.4		1.2	3.0	2.6
Mg (mg 100 g <sup>-1</sup> )	20.0	44.0						49–57		18.0	62.0	
P (mg 100 g <sup>-1</sup> )	53.0	90.0	219.0	376.0	129.0		180.3	187–259		69.0	184.0	134.0
K (mg 100 g <sup>-1</sup> )	133.0	223.0					935.6	300–308		78.0	266.0	
Na (mg 100 g <sup>-1</sup> )	655.0	686.0						133–174		259.0	409.0	
Zn (mg 100 g <sup>-1</sup> )	1.03	1.40		0.6		(5.2)		2.3–2.5		0.51	1.6	
Thiamine (mg 100 g <sup>-1</sup> )			0.35		0.21	(0.22)				0.11	0.55	0.22
Riboflavin (mg 100 g <sup>-1</sup> )	0.01	0.01	0.13		0.07	(0.14)				0.02	0.2	0.01
Niacin (mg 100 g <sup>-1</sup> )	4.3	5.8	2.6	2.1	0.8					0.7	6.8	1.8
Vitamin B6 (mg 100 g <sup>-1</sup> )	0.95	0.60								0.05	0.27	
Folate, DFE (μg 100 g <sup>-1</sup> )	20.0	80.0		11.0						12.0		
Vitamin A (IU)												
Vitamin A, RE (μg 100 g <sup>-1</sup> )	0.2	1.1		44.0								
Vitamin E (α-tocopherol) (mg 100 g <sup>-1</sup> )	0.06	0.12								0.16	0.88	

<sup>a</sup>Source: Danster et al. (2008).<sup>b</sup>Source: Mesfin (2006).<sup>c</sup>Source: Nuss and Tanumihardjo (2010).<sup>d</sup>Source: Abdualrahman and Ali (2012).<sup>e</sup>Source: Mahgoub et al. (1999).<sup>f</sup>Source: Mariod et al. (2017).<sup>g</sup>Source: FAO (1995).<sup>h</sup>Source: Lephole et al. (2006).<sup>i</sup>Source: USDA (2016).

**Table 4** Bread-making physicochemical functionality features of cereal grains used in African traditional breads

Cereal grain	Physicochemical functionality	References
Bread wheat ( <i>Triticum aestivum</i> )	<p>Gluten forms continuous matrix around starch granules within matured cell of starchy endosperm; less hydrophobic than either kafirin of sorghum or zein of maize; forms viscoelasticity that favors strong dough gas holding suitable for processing of leavened aerated bread.</p> <p>Protein content 11%–14%, high in carbohydrates (74%–78%), low in fat (2%) and ash (1.5%–2.0%).</p> <p>Hard grains that are high in proteins and more vitreous are suitable for leavened bread making. Soft and medium-hard grains with 10%–12% proteins are suitable for processing of unfermented chapati-like flatbreads.</p> <p>White flours are used to process white bread, reconstituted brown bran for brown bread, whole grain flours for whole grain bread.</p> <p>Gluten proteins are allergens and cause celiac disease in some individuals.</p> <p>Starches are of large bimodal size (small spherical 1–10 <math>\mu\text{m}</math>, large lenticular 10–35 <math>\mu\text{m}</math>), but trimodal sizes are also reported.</p> <p>Amylose 23%–29%.</p> <p>Starch granule damage is influenced by milling degree (2%–4% starch granule damage is essential for bread processing). For unleavened flatbread processing a high level of starch granule damage (&gt;5%–15%) is essential.</p> <p>A-type starch granules with low starch gelatinization temperature of 58°C–64°C compared with tropical cereal grains, which can favor baking of thick dough.</p> <p>Endogenous wheat enzymes play a role in the modification of dough for bread baking.</p> <p>Leavened bread has distinct crust and crumb structures.</p> <p>Color and flavor are contributed to bread by Maillard and caramelization reactions.</p> <p>Whole grain breads can supply bioactive compounds (fiber from nonstarch carbohydrates, alkylresorcinols, benzoxazinoids, lignans, phenolic acids, C-glycosides of flavones, flavonols, flavanonols, proanthocyanidins, phytosterols, and tocols). Colored compounds are from colored wheat grains.</p>	Andersson et al. (2014); Taylor et al. (2016)
Durum wheat ( <i>Triticum durum</i> )	<p>White bread processed from refined wheat flours is limited in bioactive compounds.</p> <p>Gluten forms continuous matrix around starch granules within matured cell of starchy endosperm; less hydrophobic than either kafirin or zein; forms viscoelasticity but weak compared with bread wheat, less gas-holding ability, which leads to low bread loaf volume of distinct uniform fine crumb structures with relatively short shelf life compared with bread wheat.</p> <p>Protein content high (13%–14%), high in carbohydrates (71%–73%), low in fat (2%) and ash–minerals (1.8%–2.0%)</p> <p>Whole grain or semolina can be milled to flour.</p> <p>Durum wheat is high in grain hardness resulting in higher starch granule damage than bread wheat, yellowish flour.</p> <p>A-type starch granules; starches are of large bimodal size (small spherical 1–10 <math>\mu\text{m}</math>, large lenticular 10–35 <math>\mu\text{m}</math>), but trimodal sizes are also reported.</p> <p>Amylose 26%–28%. Low starch gelatinization temperature of 52°C–64°C compared with bread wheat, which can favor baking of thick dough compared with tropical cereal grains.</p> <p>Endogenous durum wheat enzymes can play roles in the modification of dough for bread baking.</p> <p>Maillard and caramelization reactions influence bread flavor and crust color.</p> <p>Can supply bioactive compounds (fiber from nonstarch carbohydrates, alkylresorcinols, benzoxazinoids, lignans, phenolic acids, C-glycosides of flavones, flavonols, flavanonols, proanthocyanidins, phytosterols, and tocols). Carotenoids (4–8 <math>\text{mg kg}^{-1}</math>) are accumulated in the endosperm of durum wheat grains. Semolina flours can be limited with bioactive compounds derived from pericarp, seed coat, aleurone layers, and germ, however can bear carotenoid compounds from endosperm.</p>	Shewry and Hey (2015); Taylor et al. (2016); Kezih et al. (2014)

Maize (*Zea mays*)

Free of the type of gluten proteins found in wheat grains. Zein is contained in isolated storage protein bodies and is a hydrophobic monomeric polypeptide, but less hydrophobic than kafirin, highly crosslinked due to high sulfur-containing amino acids but less so than in kafirin. It is much smaller in molecular size than the HMW-GSs of wheat, with a limited degree of viscoelastic properties because of limited protein body disruption upon dough making compared with wheat, but better than sorghum and millet proteins in making weak viscoelasticity.

Low in protein content (8%–10%) and quality, high in lipids/fats (4.0%–5.0%) if germ and aleurone layers are not removed, low in ash (1.2%–1.5%)

A-type starch granules, of size 5–30  $\mu\text{m}$ ; large sizes are polygonal and small are spherical.

Amylose 25%–28%. Amylomaize (50%–70%) and waxy maize (>99%) amylopectin varieties exist. Amylomaize contributes significant resistant starches.

Bran-removed maize meal flours contain large amounts of starches followed by proteins and endosperm cell wall nonstarch polysaccharides.

High starch gelatinization temperature of 62°C–72°C limits baking of wheat-type thick bread.

Arabinoxylans (pentosans) contribute to flatbread baking; lower  $\alpha$ -amylase and  $\beta$ -amylase activities with less amylolytic activity.

Can supply bioactive compounds (fiber from nonstarch carbohydrates, phenolic acids, flavonoid phenolic compounds, phytosterols, tocots) depending on degree of pericarp, testa, aleurone layer, and germ removal. Phenolic and carotenoid compounds are variable among differently colored maize grains. Purple/blue maize grains are sources of anthocyanins and nonanthocyanin flavonoids (rutin, hirsutrin, morin, kaempferol, quercetin, naringenin, hesperitin). Yellow maize grains are sources of carotenoids: zeaxanthin and lutein. Decortication and bran removal can reduce bioactive compounds. Soaking can reduce phytic acids and soluble bioactive compounds.

Nuss and Tanumihardjo (2010); Suri and Tanumihardjo (2016)

Sorghum (*Sorghum bicolor*)

Free of the type of gluten found in wheat grains. No dough viscoelastic properties, limited gas-holding and leavening capacity. Kafirin proteins found isolated in protein bodies. They are hydrophobic monomeric polypeptides, highly crosslinked due to high sulfur-containing amino acids, much smaller in molecular size than HMW-GSs of wheat, with insignificant protein body disruption upon dough making.

Protein content is 10.0%–12.0%, protein quality is poor and even poorer when cooked/baked; high in carbohydrates (70.0%–75.0%), nonstarch polysaccharides from endosperm are predominantly insoluble; high in fat (4.0%–4.5%), low in ash (1.6%), moderate in dietary fiber (6.3%).

Bran-removed sorghum meal flours contain large amounts of starches followed by proteins and endosperm cell wall nonstarch polysaccharides.

Amylose for common varieties is in the range 19%–33%. A-type starch granules, with a high starch gelatinization temperature of 68°C–78°C.

Small starch granules present in the pericarp limit clean endosperm separation on milling.

Arabinoxylans (pentosans) contribute to flatbread baking; lower  $\alpha$ -amylase and  $\beta$ -amylase activities with less amylolytic activity compared with wheat flour. Some varieties contain condensed tannins.

Can supply bioactive compounds (fiber from nonstarch carbohydrates; phenolic acids; flavonoid phenolic compounds, notably 3-deoxyanthocyanidins luteolinidin and apigeninidin; policosanols; phytosterols; tocots) depending on degree of pericarp, testa, aleurone layer, and germ removal.

Most sorghum varieties are recognized by their high phenolic compounds, low proteins, and starch digestibility. Black sorghums are high in anthocyanins. Decortication and bran removal can reduce bioactive compounds. Soaking can reduce phytic acids and soluble bioactive compounds.

Kulamarva et al. (2009); Taylor et al. (2016); Cardoso et al. (2017)

(Continued)

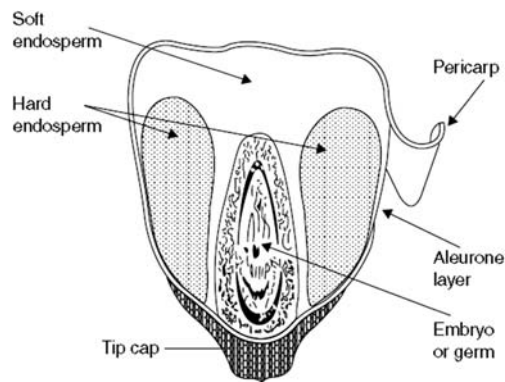
**Table 4** Bread-making physicochemical functionality features of cereal grains used in African traditional breads—cont'd

Cereal grain	Physicochemical functionality	References
Teff ( <i>Eragrostis tef</i> )	<p>Free of the type of gluten found in wheat. Prolamins of teff are dimers with no dough viscoelastic properties and a single dominant sequence. Even though they have a high degree of homology with <math>\alpha</math>-prolamins of maize and sorghum, the prolamins (eragrostins) of teff are different. Limited gas-holding and leavening capacity, insignificant protein body disruption upon dough making. <math>\alpha</math>-Globulins in teff are implicated in playing a major role in the large size protein body aggregation observed in teff influencing dough properties.</p> <p>Protein content 9.4%–13.3%, high in carbohydrates (73.0%), low in fat (2.0%–3.1%), medium in ash content (2.7%–3.0%), medium in crude fiber content (2.0%–3.5%).</p> <p>A-type starch granules, compound type. Individual starch granules 2–6 <math>\mu\text{m}</math>, high starch gelatinization temperature of 68°C–80°C. Because of its small grain size (about 0.9–1.7 mm in length, 0.7–1.0 mm in diameter, individual grain mass <math>\leq 2</math> mg) always milled to whole grain flour; low fat, and rancidity development over duration of usage insignificant (milled whole teff grain flours are used from one to two months at household handling).</p> <p>Endogenous teff enzymes play a role in the modification of dough for bread baking.</p> <p>Can supply bioactive compounds (high fiber from nonstarch carbohydrates, high phenolic acids, and low flavonoids). Relatively high in mineral contents, notably calcium, magnesium, and iron.</p>	Hager et al. (2012); Bultosa (2016); Zhang et al. (2016); Marti et al. (2017); Shumoy et al. (2017)
Rice ( <i>Oryza sativa</i> )	<p>Free of the type of gluten found in wheat. No dough viscoelastic properties, no or insignificant gas-holding and leavening capacity, insignificant protein body disruption upon dough making.</p> <p>Protein content in white rice is very low (6.3%), high in carbohydrates (mostly starches) (80.1%), very low in fat (1.4%), very low in ash–minerals (0.4%) and fiber (2.4%). A-type starch granules, polygonal compound type, individual granules 2–7 <math>\mu\text{m}</math>. Amylose percentage variable, from very low (5%–12%) to low (12%–20%), intermediate (20%–25%), or high (25%–33%); intermediate amylose content is preferred in bread baking. High starch gelatinization temperature of 65°C–78°C.</p> <p>Major proteins are globulins and glutelins, similar to oat proteins, but different from other cereal grains, in which the major proteins are prolamins. Insignificant bioactive compounds from white rice flour (small amount of fiber from endosperm nonstarch carbohydrates).</p>	Han et al. (2012); Amagliani et al. (2016)
Pearl millet ( <i>Pennisetum glaucum</i> )	<p>Free of the type of gluten found in wheat. Pennisetin protein has no dough viscoelastic properties; similar to <math>\alpha</math>-zein, but slightly smaller; limited gas-holding and leavening capacity; insignificant protein body disruption upon dough making.</p> <p>High in protein content (11.6%–11.8%), high in carbohydrates (67.0%–67.5%), high in fat (4.8%–5.0%), high in ash (2.3%), high in dietary fiber (11.3%). Shelf life for whole grain flours is poor, and can get rancid because of its high fat content.</p> <p>Amylose 20.0%–32.5%, shape polygonal with some pores on the surface and a few spherical granules with size range 3.5–23.0 <math>\mu\text{m}</math>. A-type starch granules, gelatinization temperature range 60.9°C–86.3°C.</p> <p>Higher <math>\alpha</math>-amylase activities than wheat.</p> <p>Can supply bioactive compounds (fiber from nonstarch carbohydrates, phenolic acids) depending on degree of pericarp, testa, aleurone layer, and germ removal. Low in flavonoid phenolic compounds, but significant flavones present are glucosylvitexin, glycovitexin, and vitexin.</p> <p>Decortication and bran removal can reduce bioactive compounds. Soaking can reduce phytic acid and soluble bioactive compounds.</p>	Kajuna (2001); Saleh et al. (2013); Annor et al. (2014); Kaur et al. (2014)
Finger millet ( <i>Eleusine coracana</i> )	<p>Free of the type of gluten found in wheat. No dough viscoelastic properties, limited gas-holding and leavening capacity, insignificant protein body disruption upon dough making.</p> <p>Protein content variable (6%–13%), high in carbohydrates (about 72%), low in fat (1.5%), high in ash–minerals (2.7%), high in dietary fiber (11.5%).</p> <p>Finger millet's thin membranous pericarp is loosely attached to the seed coat (utricle grain structure), and on handling and slight abrasion becomes detached. Testa is unique among millets, having five layers and strongly adhered to the endosperm, often milled to nearly whole grain flours. High in mineral content, notably calcium, potassium, phosphorus, and magnesium.</p> <p>Amylose 14.4%–16.2%. Compound-type starch granules, shape polygonal with some indentation on the surface, a few spherical, with size range 2.5–24.0 <math>\mu\text{m}</math>. A-type starch granules, high gelatinization temperature of 64.5°C–75.0°C.</p> <p>Endogenous finger millet enzymes play a role in the modification of dough for bread baking.</p> <p>Can supply bioactive compounds (high fiber from nonstarch carbohydrates, high phenolic acids: ferulic, p-coumaric, and cinnamic acids, and flavonoids such as catechin, galocatechin, epicatechin, procyanidin dimer are high since testa layer is often milled with the endosperm).</p>	Kajuna (2001); Saleh et al. (2013); Annor et al. (2014); Chandra et al. (2016); Adebisi et al. (2017)

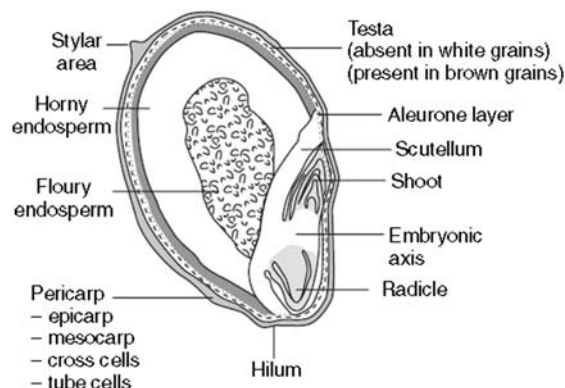


Foxtail millet ( <i>Setaria italica</i> )	<p>Free of the type of gluten found in wheat. No dough viscoelastic properties, limited gas-holding and leavening capacity, insignificant protein body disruption upon dough making.</p> <p>Protein content high (11.2%–12.3%), high in carbohydrates (60.9%–63.2%), high in fat (4.0%–4.3%), high in ash–minerals (3.3%), high in fiber (6.7%).</p> <p>A–type starch granules. Amylose 11.4%–32.0%, shape polygonal with a few spherical granules with size range 2.5–24.0 <math>\mu\text{m}</math>. Starch gelatinization temperature range 66.7°C–78.0°C.</p> <p>Utricle grain structure: pericarp and testa are loosely attached. Flour milled from unpolished grains can be high in fat unless immediately used and prone to rancidity.</p> <p>Can supply bioactive compounds (high fiber from nonstarch carbohydrates, high phenolic acids, intermediate flavonoids, and insignificant proanthocyanidins).</p>	Saleh et al. (2013); Annor et al. (2014); Kumari et al. (2017); Sharma and Niranjan (2017); Adebisi et al. (2017)
Proso, common, or white millet ( <i>Panicum miliaceum</i> )	<p>Free of the type of gluten found in wheat. No dough viscoelastic properties, limited gas-holding and leavening capacity, insignificant protein body disruption upon dough making.</p> <p>Protein content 12.5%, high in carbohydrates (70.4%), low in fat (1.1%–3.5%), medium ash (1.9%–3.1%), high in fiber (5.2%).</p> <p>A–type starch granules, amylose 17.2%–33.9%, shape polygonal with a few spherical granules with size range 2.5–24.0 <math>\mu\text{m}</math>. Starch gelatinization temperature range 62.0°C–84.4°C.</p> <p>Utricle grain structure: pericarp and testa are loosely attached.</p> <p>Can supply bioactive compounds (medium level fiber from nonstarch carbohydrates, high phenolic acids, low flavonoids, and insignificant proanthocyanidins).</p>	Kajuna (2001); Saleh et al. (2013); Annor et al. (2014); Adebisi et al. (2017)
Fonio, black or iburu ( <i>Digitaria iburua</i> Stapf)	<p>Free of the type of gluten found in wheat. No dough viscoelastic properties; prolamin proteins show some degree of hydrophobic character, but limited in gas-holding and leavening capacity; insignificant protein body disruption upon dough making.</p> <p>Protein content low (8.9%), but high in some amino acids (leucine 9.8%, methionine 5.6%, and valine 5.5%), high in carbohydrates (69.4%), medium in fat (3.0%), medium in ash (2.2%), medium in dietary fiber (8.5%).</p> <p>Amylose 19.3%–21.2%, polygonal, size range 2.0–13.5 <math>\mu\text{m}</math>. Gelatinization temperature range 62.0°C–84.4°C.</p> <p>Very small grain size (about 1.6–1.8 mm long and 0.8 mm wide, individual grain mass about 0.5–0.6 mg), often milled to whole grain flour. High viscous pentosan content limits glucose absorption.</p> <p>Can supply bioactive compounds associated with whole grains (high fiber, phenolic acids, waxes).</p>	Edema et al. (2013); Jideani and Jideani (2011).
Fonio, white or acha ( <i>Digitaria exilis</i> Stapf)	<p>Free of the type of gluten found in wheat. No dough viscoelastic properties; prolamin proteins show some degree of hydrophobic character, but limited in gas-holding and leavening capacity; insignificant protein body disruption upon dough making.</p> <p>Protein content low (7.1%), high in carbohydrates (69.3%), medium in fat (3.1%), medium ash–minerals (2.1%), medium in dietary fiber (7.4%).</p> <p>Amylose 22.6%–26.1%, polygonal, dented granules with size range 2.0–13.1 <math>\mu\text{m}</math>, high lipid in starch granules. Gelatinization temperature range 62.0°C–84.4°C. Proteins are rich in methionine and cysteine.</p> <p>Very small grain size (about 1.6–.8 mm long and 0.8 mm wide, individual grain mass about 0.5–0.6 mg), often milled to whole grain flour. High viscous pentosan content limits glucose absorption.</p> <p>Can supply bioactive compounds associated with whole grains (high fibers, phenolic acids, waxes).</p>	Jideani and Jideani (2011).

HMW-GS, high-molecular-weight glutenin subunit.



**Figure 2** Maize grain anatomical structure (Eckhoff, 2015).



**Figure 3** Sorghum grain anatomical structure (Obilana, 2004).

after milling, although soaking and decortication can reduce levels somewhat. Phytic acid has a strong binding affinity for minerals such as calcium, magnesium, manganese, iron, potassium, copper, and zinc at physiological pH and therefore can reduce absorption in the intestine (Greiner and Konietzny, 2006). This is of most concern in countries where grains form a large proportion of the daily diet. Condensed tannins, even though they are antioxidants, can inhibit mineral bioavailability and digestive enzyme activities, but can also be reduced by the decortication and winnowing actions used.

Unfermented maize flatbreads are consumed fresh on a daily basis as they do not store well because of rapid staling. Maize bread is rich in carbohydrates but can be limited in protein and micronutrient content (Table 3).

### Sorghum Bread

Sorghum (*Sorghum bicolor* (L.) Moench) is a tropical cereal with hull-less grains of different colors: white, brown, red, yellow, lemon yellow, cream, and black. Some sorghum varieties are known to accumulate condensed tannins (proanthocyanidins) in their inner integument layer around the testa and are bitter in taste. Sorghum is often marketed as an antioxidant-rich health food because of its high polyphenol content, especially 3-deoxyanthocyanins (luteolinidin and apigeninidin) (Cardoso et al., 2017). In Africa, sorghum is an ingredient in bread, porridge, and fermented beverages. To process sorghum, the grains are hand cleaned, washed, soaked in water (about 15–30 min), and decorticated by hand pounding with a mortar and pestle (Yetneberk et al., 2005). Decortication separates the fibrous pericarp and leads to some damage to the aleurone layer and germ. The decorticated mass is spread and sun dried and then winnowed to separate the pericarp and germ from the meal. The meal (mainly endosperm) is milled as described for maize. For unfermented flatbread, fine flour (about <75–200  $\mu\text{m}$ ) is required instead of the coarser grist used to make porridge.

Sorghum dough properties are influenced by the level of starch granule damage, protein content, degree of crosslinking of kafirin proteins (the major storage proteins of sorghums), and amount of arabinoxylans (pentosans) present (Kulamarva et al., 2009). The dough is worked by hand and baked as described for unfermented maize flatbread. Sorghum flatbreads are dense and unaerated, with a distinct aroma, crispiness, and chewability. Bread color is influenced by the type of sorghum grain and the polyphenol compounds remaining in the flour from remnants of the seed coat and testa and by the Maillard reaction during baking. Steam generated throughout the dough during baking can lead to a minor degree of bread expansion, as sorghum kafirin proteins form networks with damaged starch granules and cell wall polysaccharides. The baking action gelatinizes starch and facilitates digestibility of the bread. Starch and protein digestibility of the bread can be limited because of inhibition of digestive enzymes

from specific phenolic compounds such as remnants of condensed tannins and the high degree of crosslinking of kafirin protein with disulfide bonds (Cardoso et al., 2017). However, unfermented sorghum flatbread has many health-promoting properties, it is a low glycemic bread and shows the presence of phenolic compounds, policosanols, and phytosterols.

### Teff Bread

Teff (*Eragrostis tef* (Zucc.) Trotter) is a tropical cereal originating in Ethiopia; its cultivation and usage as food are also largely confined to Ethiopia, and as a result so too is the consumption of unfermented teff flatbreads (*kitta/bixxille* and *maaxino caabita*).

Teff grains are hull-less, are very small in size (0.9–1.7 mm in length, 0.7–1.0 mm in diameter, mass  $\leq$  2 mg), and range in color from white to milky white, brown, and dark brown (Bultosa, 2016). Brown teff grain is often erroneously referred to as red teff. The popularity of teff is somewhat on the rise because of its superior nutritional properties in comparison with more common cereal grains (wheat, maize, sorghum, barley, and rice) and its usability as a whole grain, and because it is gluten free. Thus cultivation is expanding into South Africa and internationally into the Netherlands and grain-growing regions of the United States, namely Montana, South Dakota, and Idaho. Because of the small grain size, decortication is impractical; instead the grains are winnowed, sieved, and milled into whole grain flour (approximately 99% extraction) using a disk attrition mill. Therefore, the pericarp, testa, aleurone layer, germ, and endosperm are all part of teff flour with different degrees of particle size reduction. In general, a flour average particle of about 90.7–98.4  $\mu$ m in diameter is achieved, with starch granule damage of approximately 5.56%–5.85% reported (Abebe et al., 2015). This increased level of damaged starch granules in the flour leads to an increase in water absorption and hydrolysis of starches into fermentable sugars by the action of  $\alpha$ - and  $\beta$ -amylases and thus affects the rheology and nutrient profiles of the dough. Fine teff flour is preferred for making teff bread (unfermented *kitta* bread, *injera*, or *cumboo*) as opposed to coarse flour (grist). The dough is worked by hand and rested for up to 1 h (before commencement of fermentation), shaped, flattened, and baked on a red-hot clay baking griddle that has been greased with a fat or oil (in traditional practice often using rapeseed meal) to avoid sticking. Bread baked this way is thick, with limited eyes (holes on the surface) and a rusty iron color on its base compared with fermented *injera*, which is thin, semileavened, with multiple eyes and a light rusty iron color on the base. The eyes on the surface of unfermented teff bread are a result of steam expansion in the dough matrix. Teff prolamin proteins have lower hydrophobicity, contain more basic polypeptides, and are less polymerized than maize or sorghum proteins, and these properties were implicated to contribute to the superior flatbread baking properties observed (Adebowale et al., 2011). Bread baked from unfermented teff grain flour is high in dietary fiber, minerals, and phytochemicals (Marti et al., 2017) such as the phenolic compounds protocatechuic, vanillic, syringic, *p*-coumaric, sinapic, ferulic, and rosmarinic acids; catechin; and naringenin (Shumoy et al., 2017). As with other unfermented breads, phytic acid can still be a concern.

### Millet Bread

Millet is a tropical cereal that produces small hull-less grains. They comprise pearl millet (*Pennisetum glaucum*), finger millet (*Eleusine coracana*), kodo millet (*Paspalum setaceum*), proso millet (*Panicum miliaceum*), foxtail millet (*Setaria italica*), little millet (*Panicum sumatrense*), barnyard millet (*Echinochloa utilis*), guinea millet (*Brachiaria deflexa*), and brown top millet (*Brachiaria ramosa*). Teff and fonio (*Digitaria exilis* and *Digitaria iburua*) are also described as part of the millet. Of these, pearl, finger, and guinea millets and fonio are important grain-producing crops for millions of smallholder farmers in semiarid dry land in Africa, used predominantly for bread, porridge, and fermented beverages. In addition to their basic nutrients, most millet grains are noted for their high levels of phenolic compounds such as phenolic acids and anthocyanins (Saleh et al., 2013). In terms of mineral nutrients, finger millet is a good source of calcium, magnesium, and potassium (344, 137, and 408 mg/100 g, respectively) (Chandra et al., 2016). Traditional unfermented millet flatbreads are made from either decorticated or nearly whole grain flour (Kajuna, 2001; Saleh et al., 2013). For the production of whole flour, millet grains are cleaned, washed (without or with conditioning: the process of adding water to adjust the moisture content of grains for optimal flour extraction), and milled (plate milling/stone milling). The milled stock is separated into flour and bran by sieving. Flour variations include different types of millet, whether the grain/kernel structure is a caryopsis or utricle, and fat content (1.5%–5.2%) (Saleh et al., 2013). In the caryopsis-type structures (pearl millet, sorghum, maize, wheat, and barley), the pericarp is adhered to the seed coat and decortication can be undertaken to remove the pericarp layer. In utricle structures (finger, foxtail, and proso millet), however, the thin membranous pericarp is loosely attached to the seed coat, which in turn is intimately attached to the endosperm; hence the seed coat is often milled with the endosperm. In finger millet, the thin layered membranous pericarp is loosely attached to and covers the entire seed and can be easily detached via abrasion or during postharvest handling (Shobana and Malleshi, 2007). Thus, because the seed coat is so intimately attached to the fragile endosperm, it is incorporated into the milled whole grain flour. The seed coat and aleurone layer contribute a significant amount of phytic acid, polyphenols, and dietary fiber and impart a chewy texture, but also contribute a dark color and somewhat musty odor to the final bread.

Pearl millet has a larger germ and so is often decorticated. If pearl millet is milled into whole flour, because of its high fat content (about 5%), rancidity can occur from the lipase activity in the germ, aleurone layer, and pericarp; thus often it must be quickly processed into bread after milling (Saleh et al., 2013). The degree to which particles are reduced and starch granules are damaged during milling is dependent on millet grain type, drying conditions, and milling force. In traditional processing, decortication is conducted by gentle pounding with a wooden mortar and pestle. The decorticated mass is milled into flour by hand using stone mills. Because millet proteins do not have the gluten proteins that are found in wheat, which favor a viscoelastic dough,

significant starch granule damage is required during milling for the dough to become cohesive. To make bread, the flour is mixed with water, salt, and other optional ingredients (oils, spices, onions, dates, eggs, milk, nuts, sesame seeds, and/or black cumin [*Nigella sativa*]) and baked on a griddle or iron pan. Freshly baked millet bread is soft and flexible but quickly stales upon storage (within a day).

### Wheat Bread

Wheat is cultivated in many parts of the world, from the north (67° N in Scandinavia and Russia) to the south (45° S in Argentina), including pocket highlands of the tropics and subtropics (Peña-Bautista et al., 2017). The predominate cultivated wheat types are hexaploid (bread wheat/common wheat, *Triticum aestivum* L. subsp. *aestivum*), followed by tetraploid (macaroni/pasta wheat, *Triticum turgidum* L. subsp. *durum* (Desf.) and rivet/poulard wheat, *T. turgidum* L. ssp. *turgidum*). Wheat grains are hull-less, with color variations in bread wheat including red, white, purple, blue, black, and light yellow, as well as endosperm textures from soft to hard. The black, purple, and blue colors are due to the occurrence of anthocyanins, and the red color is from catechins and proanthocyanidins in the pericarp, testa, and aleurone layer of the grains (Lachman et al., 2017). Hard and soft bread wheat is used in the manufacture of bread and cookies (biscuits), respectively. Durum wheat is an amber-yellow color due to carotenoids (lutein, zeaxanthin, and  $\beta$ -carotene) in its endosperm, and the endosperm texture is harder than that of bread wheat, but the strength of its gluten proteins is weaker than that of bread wheat.

Unleavened wheat flatbreads are widely consumed throughout many African countries. Traditionally, cleaned wheat grains are washed and soaked in water for approximately 30 min to 1 h. The soaked grains are decorticated with a wooden mortar and pestle to detach the beeswing pericarp layers. The pounding action damages, to different degrees, the testa, aleurone layer, and germ, while removal of the outer layers of pericarp from the crease region is challenging and may be incomplete. The pounded grain mass is spread and dried in the sun. The dried mass is winnowed and milled to different particle sizes using a traditional stone mill, plate mill, or hammer mill (Bressiani et al., 2017). Often flour produced by traditional milling methods has better nutritional and bioactive compound profiles (high extraction rate) compared with roller milled and refined wheat flour, because remnants of the testa, aleurone layer, and germ are still present in the flour. However, shelf life can be short compared with roller milled flour. The dough rheology is significantly different from that of dough from nonwheat flour because of the viscoelastic nature of wheat gluten proteins (Taylor et al., 2016; Ortolan and Steel, 2017). Flour of 10%–13% protein content, with high gliadin/glutenin ratio, and with starch granule damage (>5%–17%) is known to produce quality unfermented wheat flatbreads (Parimala and Sudha, 2015).

To make the traditional *chà-chà-bsa* flatbread from Ethiopia, wheat flour and salt are mixed and worked by hand with gradual addition of water (Mesfin, 2006). The dough is kneaded, rested (15–30 min), kneaded again, shaped into a flat disk, and baked (and flipped) on a lightly greased pan to attain a crisp texture and a golden brown color. Using durum wheat to make flat unfermented bread means that, due to the greater grain hardness and subsequently greater amount of starch granule damage during milling, there is more water absorption, limiting water for gluten network development in the dough. During baking, the bread can puff up a little, and it exhibits a dense, softer texture because of its weak gluten network. The color of the bread is cream-yellow due to its high carotenoid content. The bread stales quickly; for example, *maadjouna*, a durum wheat flatbread from Algeria, is good if consumed during the day baked (Kezih et al., 2014). Other types of flatbreads (*harcha* and *rakhsis*) are also produced from durum wheat semolina in Algeria. Mineral micronutrients can be limited because of inhibition by some phytic acids (Buddrick et al., 2014). Nevertheless, in terms of dietary fiber, phenolic acids, some B vitamins, and vitamin E, flatbread processed from whole or semiwhole wheat grains (partially decorticated grains) are superior to refined white wheat bread. Durum wheat also provides provitamin A carotenoids, and additional ingredients such as fats, other grain flours, fruit, vegetables, and spices also have the potential to improve the nutrient and bioactive profile of the bread, which is of importance in areas where bread is a dietary staple. Wheat-based flatbreads are often baked twice per day and consumed fresh because of their fast staling nature.

### Trends and Future Prospects

Flatbread is a dietary staple in some regions of Africa; in other regions porridge made from maize, sorghum, and millet grains is more commonly consumed. Traditional processing methods are still practiced for flour milling, using stone, attrition, or hammer mills. In rural villages, processing activities, such as decortication, dough processing, and baking, are at large conducted by women, and the practices are time and energy consuming. For the retail market, roller milling technology is used to mill wheat flour. However, roller milling technology for the production of maize, sorghum, teff, millets, and fonio flours for bread baking is limited. Instead, maize and sorghum grains are mostly milled to grist for the production of porridge and snacks. Thus, there is inconsistency in the commercial supply of bread-grade maize and sorghum flour. Large-scale milling of teff, millet, and fonio flour is also limited in part because supply is confined to specific geographic locations in Africa and there is limited research into processing innovations. Because such large amounts of flatbreads are consumed on a daily basis in Africa, there is great potential for innovation and to respond to the increase in nutritional awareness. Flatbreads are starting to be made with blends of flours from other sources, for example, legumes, pulses, oil seeds, starchy tubers, and roots. And many flatbreads, such as those made from maize, sorghum, teff, millets, and fonio, are naturally gluten free and thus suitable for celiac patients (Taylor et al., 2016). More research into

improved processing technologies and ingredient selection is required, as is the continuation of research into crops biofortified for iron, zinc, and vitamin A, as in some places these deficiencies are on the rise (Bouis and Saltzman, 2017).

## Conclusions

Traditional African breads are diverse in their structures, depending on their formulation and method of production, and thus have different nutritional and sensory properties. Unfermented flatbreads have not changed a great deal, considering how long humans have been making them, and in Africa, traditional bread is largely processed in the home or in cottage industries. Although there are some unwanted but naturally occurring by-products in some unfermented flatbreads, the nutritional density of bread can easily be increased by utilizing the large range of cereal grains present in Africa. Improvements in processing, such as fortifying flour with vitamins and minerals, grain modification through pretreatment (soaking and sprouting/germination), and allowing dough to ferment, can be implemented. As the food supply chain becomes more globalized, efforts to automate bread manufacture in Africa can be investigated to make these traditional breads part of the mainstream bread sector.

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# Traditional African Bread: Physicochemical and Sensory Properties of Fermented Breads

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## Overview

### Fermented Semileavened Flatbreads

Fermented breads of the semileavened variety are probably one of the most ancient forms of traditional biotechnology, learnt by humans through the mixing of cereal grain flour with water. This process involves the formation of sourdough by the action of microorganisms (lactic acid bacteria (LAB) and yeasts), which are naturally present in the raw materials, utensils used in processing, or the surrounding environment. Many different types of breads consumed in African households are made through the sourdough process often from sorghum, teff, millet, maize, and wheat (Table 1 cross ref article 1). The nutrient composition of these cereal grains and other bread components are summarized in Table 2 (cross ref article 1) and Table 1, respectively. The microorganisms responsible for fermentation are mixtures of LAB and/or yeasts that lead to the production of different organic acids, aromas, nutrient profiles, and the leavening action on the bread structure (Chavan and Chavan, 2014). Crumb and crust formation are influenced by many factors, including cereal grain type, particle size of the flour, protein and carbohydrate content, additional ingredients (bran, salt, oil/fat, dried fruit, spices), dough processing, activity of the fermenting microorganisms, and baking conditions. Sourdough is classified into three different types (Vuyst and Neysens, 2005). In Type I (traditional sourdoughs), a sourdough is restarted using portions of dough from previous fermentation (backslopping). Type II is an industrial accelerated sourdough, which uses adapted strains to start fermentation. This sourdough can be in liquid form, so it can be easily pumped through an industrial bakery. Type III sourdough (dried sourdough) is used by industrial bakeries, since the quality is constant with limited end-product variations, which may arise from the freshly produced sourdough starter. Using any one of these sourdough methods improves bread quality in various aspects, such as loaf volume, palatability, flavor generation (unique to a given sourdough), synthesis of prebiotic exopolysaccharides, reduced antinutrients, improved mineral nutrient bioavailability, decreased glycemic index, reduced bread staling, reduced ropy spoilage (bacterial spoilage caused by *Bacillus* spp. forms a sweet fruity odor of rotting pineapple or melons and a discolored, soft, sticky crumb), and extension of shelf life (Moroni et al., 2009). In Africa, *injera* and *kisra* are the best examples of Type I sourdough, semileavened flatbreads.

### Fermented Teff Bread: *Injera*

*Injera* (crepelike circular flatbread, sour, soft, and spongy) is a staple bread in Ethiopia and Eritrea (Fig. 1). *Injera* is largely processed from teff grain flour. Other cereal grains used are sorghum, finger millet, rice, barley, wheat, and maize alone or in combination with teff grain as composite flours. Of these, *injera* processed from 100% teff grain flour is superior in terms of quality and acceptance by the community that predominantly eats *injera*. Indigenous *injera* is made as follows: after cleaning the teff grain by sifting and sieving, the grain is milled into flour of fine particle size with significant levels of starch granule damage. Dough is made by combining flour with water (1:2 w/v) and worked by kneading to evenly hydrate all the flour particles. Teff has very small starch granules (2–6 µm in diameter), combined with their damage as a result of milling, and along with proteins and nonstarch polysaccharides from the pericarp and endosperm cell walls, teff flour absorbs more water compared to sorghum, maize, barley, and millet flour. The dough (or batter at this stage) is covered and left to ferment for between 24 and 48 h (Bultosa, 2016). The duration of fermentation is influenced by the environmental conditions such as ambient temperature and may take up to 72 h. Also the conditions for the fermenting microorganisms such as dough pH and degree of starch granule damage during milling will contribute to fermentation time. Teff *injera* manufacture involves two stages of fermentation, involving a complex group of microorganisms. A total of 107 LAB and 68 yeast strains have been observed in teff fermentation when making *injera* (Desiye and Abegaz, 2013). Initial fermentation lowers the pH of the batter to about 5.8 and then LAB, of which *Pediococcus pentosaceus*, *Pediococcus cerevisiae*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactococcus piscium*, *Leuconostoc mesenteroides*, and *Streptococcus faecalis* can be common, and it can take about 18 h to drop the pH further to approximately 3.8. At about 22–24 h, *Saccharomyces* and *Torulopsis* yeasts have been observed in the batter, and at the 48 h, yeasts belonging to the genera of *Candida* and *Pichia* were the dominant strains isolated from the batter. Initial fermentation is the spontaneous action of fermenting microorganisms that are present in the immediate environment. For subsequent (next batch) baking of *injera*, a small portion of fermented batter from the previous fermentation called *irsho/ersho/raacitii* (yellow liquid that accumulates on the surface of fermenting batter at the end of the first phase of fermentation and discarded) is used in a backslopping role as an inoculum. Also batter residue left at the end of second phase of fermentation is saved to serve as *irsho*. *Irsho* was reported to contain approximately 96.4% moisture and comprise of amino acids, glucose, maltose, minerals, vitamins (riboflavin and niacin), and of variable microbial flora involved in the fermentation of teff dough (Parker et al., 1989; Ashenafi, 1994). Grain amylase enzymes, together with some strains of LAB noted for the production of amylase enzymes (Lealem and Gashe, 2008), are involved in the conversion of some starch

**Table 1** Nutrient composition of legumes/pulses, tubers, and roots used in composite breads

Composition	Soybean <sup>a</sup>	Cowpeas, Black eyes <sup>a</sup>	Faba beans <sup>a</sup>	Cassava tuber flour <sup>b</sup>	Yam tuber flour <sup>b</sup>	Taro (cocoyam) corms <sup>c</sup>
Water (%)	8.5	12.0	11.0	12.7	14.0	77.5
Protein (%)	36.5	23.5	26.1	1.9	3.4	2.5
Total lipids/fat (%)	19.9	1.3	1.5	0.5	0.4	0.2
Crude fiber (%)						
Total dietary fiber (%)	9.3	10.6	25.0	3.9	11.6	0.4
Ash (%)				2.2	2.7	
CHO (%)	30.2	60.0	58.3	78.8	67.9	19.0
Total sugars (%)	7.3	6.9	5.7			
Energy (kcal 100g <sup>-1</sup> )	446	336	341	335	312	85.0
Ca (mg 100g <sup>-1</sup> )	277	110.0	103.0	138	64	32.0
Fe (mg 100g <sup>-1</sup> )	15.7	8.3	6.7	1.5	1.9	0.8
Mg (mg 100g <sup>-1</sup> )	280.0	184	192.0	45.0	31.0	
P (mg 100g <sup>-1</sup> )	704.0	424	421.0	102.0	110.0	64.0
K (mg 100g <sup>-1</sup> )	1797.0	1112.0	1062.0	587.0	2310	514.0
Na (mg 100g <sup>-1</sup> )	2.0	16.0	13.0	30.0	25.0	
Zn (mg 100g <sup>-1</sup> )	4.9	3.4	3.1	0.74	1.6	
Thiamin (mg 100g <sup>-1</sup> )	0.87	0.85	0.56	0.07	0.1	0.18
Riboflavin (mg 100g <sup>-1</sup> )	0.87	0.23	0.33	0.11	0.08	0.04
Niacin (mg 100g <sup>-1</sup> )	1.6	2.1	2.8	1.2	1.1	0.9
Vitamin B6 (mg 100g <sup>-1</sup> )	0.38	0.36	0.37	0.17	0.83	
Folate, DFE, µg 100g <sup>-1</sup>	375.0	633.0	423.0	47.0	51.0	
Vitamin A, IU	22.0	50.0	53.0			
Vitamin E (alpha-tocopherol) (mg 100g <sup>-1</sup> )	0.85	0.39	0.05	0.35	0.82	

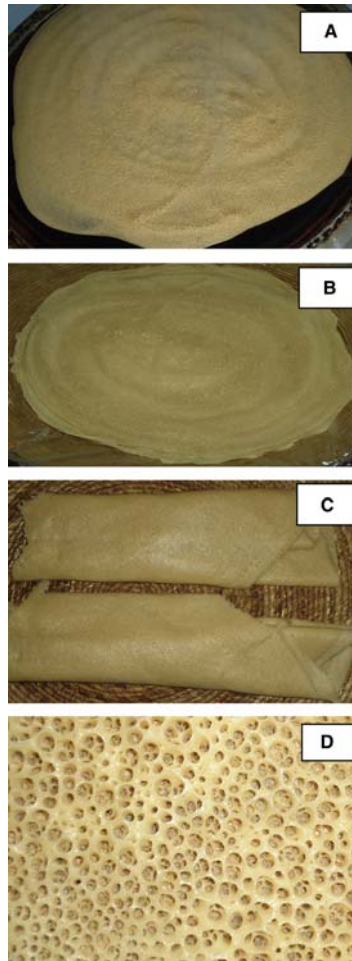
<sup>a</sup>USDA (2016).<sup>b</sup>Kaushal et al. (2015).<sup>c</sup>FAO (2012).**Table 2** Bread-making functionality features of legumes/pulses, tubers, and roots used in composite breads

Legumes/pulses/tubers/roots	Composition and functionality	References
Soybean ( <i>Glycine max</i> )	Free of wheat gluten, low in sulfur-containing amino acids, high-quality proteins with complete essential amino acids, no dough viscoelastic properties, no gas-holding and leavening capacity. High protein content (36.5%), low carbohydrate (30.2), high in fat (19.9%), including polyunsaturated fatty acids (linoleic and linolenic acids), high in dietary fiber 9.3%. Amylose 11.8%–16.2%, spherical- to polygonal-shaped starch granule, 0.7–4 µm in size, low gelatinization temperature 52.0–57.9 °C, C <sub>B</sub> -type starches (mixture of A- and B-type), starch granules more resistant to hydrolysis by alpha-amylase. Source of minerals (calcium, iron, phosphorus, magnesium) and vitamins (pro-vitamin A, thiamine, and riboflavin). Bioactive compounds in flour: fiber, phenolic acids, isoflavones, peptides, omega-3 fatty acids, leucoanthocyanins, tocopherols, phytosterols. Seed requires blanching or steam heat treatment to inactivate soybean lipoxygenase enzymes that leads to beany flavors (rancidity due to fatty acid oxidation). After seed coat removal, fat-free flours, full fat flours, and soybean protein isolates can be used for different bread character.	Garg et al. (2016); Shao et al. (2009); Liu (2004)
Cowpea ( <i>Vigna unguiculata</i> )	Antinutrients present (phytates, saponins, lectins and protease/trypsin inhibitors). Free of wheat gluten, low in sulfur-containing amino acids and high in lysine, no dough viscoelastic properties, limited gas-holding and leavening capacity. High protein content about 23.5%, medium in carbohydrate 60.0%, low in fat 1.3%, high in dietary fiber 10.6%. Amylose 25.8%–33.0%, oval- and kidney-shaped starch granules 5.0–37.5 µm in size, high gelatinization temperature 70.5–81.0 °C, C type starch granules. High starch retrogradation properties. Bioactive compounds in flour: fiber, flavonols (catechin and (epi)afzelechin), phenolic acids, anthocyanins, oligosaccharides, peptides. Depending on seed coat removal, in some varieties, there are proanthocyanidins (condensed tannins). Antinutrients present (phytates, saponins, tannins and protease inhibitors).	Ojwang et al. (2013); Hoover et al. (2010)

**Table 2** Bread-making functionality features of legumes/pulses, tubers, and roots used in composite breads—cont'd

Legumes/pulses/tubers/ roots	Composition and functionality	References
Faba (fava) bean ( <i>Vicia faba</i> )	Free of wheat gluten, low in sulfur-containing amino acids and high in lysine, no dough viscoelastic properties, limited gas-holding and leavening capacity. High protein content 26.1%, medium in carbohydrate 58.3%, low in fat 1.5%, high in dietary fiber 25.0%. Amylose 17.0%–42.0%, oval- and spherical-shaped starch granules 9.0–48.0 µm in size, high gelatinization temperature 64.3–75.3 °C, C type starch granules. Bioactive compounds in flour: high fiber, phenolic acids, oligosaccharides, peptides. Antinutrients present (phytates, tannins, saponins, lectins, protease inhibitors, vicin and convicine).	Multari et al. (2015)
Cassava ( <i>Manioc esculenta</i> )	Free of wheat gluten, no dough viscoelastic properties, no gas-holding and leavening capacity, very low protein content 1.9%, high in carbohydrate 78.8%, very low in fat 0.5%, low in dietary fiber 3.9%. Amylose 17.0%–33.0%, oval- and truncate-shaped starch granules 4.0–45.0 µm in size, medium gelatinization temperature 62.3–75.9 °C, A and C type starch granules. Bioactive compounds in flour: limited fiber and resistant starches. Treatment required (drying, fermentation) to reduce toxic cyanogen glycosides. Bland taste.	Awoyale et al. (2017); Ayele et al. (2017); Zhu (2015a)
Yam ( <i>Dioscorea</i> spp.)	Free of wheat gluten, no dough viscoelastic properties, no gas-holding and leavening capacity, low in protein content 3.4%, high carbohydrate 67.9%, very low in fat 0.4%, high in dietary fiber 11.6%. Amylose variable but for most species are 17.0%–33.0%, starch granule shape depending on species, some oval, ellipsoidal, round, triangular, or polygonal, size ranges from 7–10 µm to 28.0–49.5 µm, high gelatinization temperature 74.7–84.4 °C, C (a mixture of A and B) type starch granules. Bioactive compounds in flour: water-soluble proteins (diosgenin and dioscorine), water soluble fibers (allantoin, a steroid saponin dioscin, and resistant starches).	Amandikwa et al. (2015); Zhu (2015b)
Taro, Cocoyams ( <i>Colocasia esculenta</i> L.)	Free of wheat gluten, no dough viscoelastic properties, no gas-holding and leavening capacity, fresh corm is low in protein content 2.5%, carbohydrate 19.0%, very low in fat 0.2%, and fiber 0.4%. But on dry matter basis, taro flour can be high in carbohydrates (70%–80% starch), high in potassium. Amylose 14.0%–19.4%, starch granule shape, rounded, ellipsoidal-truncated, 0.5–5.0 µm in size, highly digestible A-type starch granules, high gelatinization temperature 81.0–85.0 °C. Good water absorption properties due to small starch granule surface area. Acridity (irritation, itching, and burning of skin) from corm oxalic acid (H <sub>2</sub> C <sub>2</sub> O <sub>4</sub> ), which binds calcium, potassium, sodium, and ammonium. Acridity removal demands peeling, grating, soaking, and fermentation. Bioactive compounds in flour: mucilage fiber, anthocyanins (cyanidin-3-glucoside, pelargonidin-3-glucoside, and cyanidin-3-chemnoside).	Kaushal et al. (2015); Odeku (2013)

into fermentable sugars. During fermentation (at a stage when the yellow liquid/*irsho* separates from the batter), to facilitate greater conversion of starch, a portion of about 10% of sediment batter is withdrawn and cooked to gelatinize the starch and make *absit* (a dough binder). After cooling to approximately 46 °C, *absit* is mixed back into the batter (initiating the second phase of fermentation, which lasts for approximately 20–30 mins) for further utilization by yeast and LAB. The fermented batter is then thinned with water to attain the desired batter consistency. Using a rotational pouring action of the batter from the peripheral to the center of baking griddle, a flat circular form is achieved (Fig. 1A). This is steam baked for approximately 2–3 mins. The gases produced during fermentation are loosely held within the batter system, and during baking, they escape leaving behind honeycomb structures on the surface of the bread called “eyes of injera” (Fig. 1B and D). The sourness of *injera*, in particular, is influenced by the duration of fermentation. *Injera* baked from shortly fermented batter is slightly sour and has a weak molasses aroma, but *injera* from batter that has been fermented for more than 24 h is markedly sour in taste. *Injera* color varies from cream white to brown and is influenced by the color of the teff grain and to some degree by the Maillard reaction that is particularly noticeable on the underside of the *injera*, which has a shiny, pale to light brown appearance. *Injera* has a spongy texture, and it can easily be rolled without fracturing (Fig. 1C). Texture can be influenced by swollen and gelatinized starch, as well as bran, embryo fragments, cell walls, protein bodies, and microorganisms that are present (Parker et al., 1989). During teff fermentation, a decrease in starch and insoluble fiber content and a limited amount of protein modification were reported (Marti et al., 2017). Starch was utilized to the extent of approximately 9% by the fermenting microorganisms (Umata and Faulks, 1988). The limited protein modification during fermentation is implicated as a contributor for protein network formation in fermented teff products. The fermentation action can reduce phytic acid content of *injera* by up to 91%–93%, due to activity of the phytase enzymes present in the fermenting batter (Umata et al.,



**Figure 1** Baked *injera* from white teff flour: (A) *injera* baking on griddle, (B) baked *injera* stacked, (C) rolled *injera* bottom side as outer surface of the roll, and (D) eyes of *injera* on the top side.

2005; Abebe et al., 2007). This reduction of phytic acid contributes toward the increased bioavailability of the minerals (iron, zinc, and calcium).

#### **Fermented Sorghum Bread: Kisra/Kissra**

*Kisra* is fermented sorghum flatbread (1.0–1.5 mm thick, 30–45 cm in diameter), similar to a thin pancake that is widely consumed in Sudan (AwadElkareem and Taylor, 2011). *Kisra* is also processed from millet grain (Mariod et al., 2017). A type of fermented flatbread, similar to *kisra*, called *laaffisso*, made from sorghum is also consumed in Ethiopia. To make *kisra*, cleaned sorghum grains are milled into whole grain flour using mechanical stone mills. For *laaffisso*, sorghum grains are washed, soaked, decorticated by hand with a wooden mortar and pestle, spread, sun-dried, and sifted/winnowed to remove the detached components (significant pericarp, germ, and seed coat). The dried processed grains are milled in a similar fashion to whole grain sorghum flour. The shelf life of these types of flour is not long because the damage to the germ leads to rancidity. Dough or batter is made by hand mixing sorghum flour with water (1:2 w/v) (Mahgoub et al., 1999). The initial fermentation is spontaneous. *Kisra* fermentation predominantly occurs through the action of LAB: *P. pentosaceus*, *Lactobacillus confusus*, *L. brevis*, *L. fermentum*, and *Lactobacillus amylovorus* (Rahman et al., 2010). *Enterobacteriaceae* has been observed initially in fermentation but becomes completely inhibited after 16 h of fermentation, due to the low pH of the batter as a consequence of LAB activity. The involvement of yeasts (*Candida intermedia*, *Candida krusei*, and *Debaryomyces hansenii*) and molds (*Aspergillus niger*, *Penicillium* sp., *Fusarium* sp., and *Rhizopus* sp.) were also reported, even though most molds were found to be inhibited after 8 h of fermentation (Rahman et al., 2010). The potential presence of *A. niger* is not of great concern as many strains of LAB inhibit mold and possible mycotoxin production (Perczak et al., 2018). Five percent of the batter is saved from the previous batch fermentation and mixed into the new batch as a starter. The batter is covered and allowed to ferment for 12–24 h to produce a fermented sorghum dough called *Aowasa* in Sudan. The thinned, fermented dough is spread in a thin layer on a heated clay hot plate and cooked for 20–40 s until the final moisture content is about 52%–53%. Baked *kisra* is described as having a nonspongy, supple, soft, and moist texture. The color, texture, and protein

digestibility of *kisra* were found to be influenced by the type of sorghum grain used (AwadElkareem and Taylor, 2011). The color can range from cream (from white sorghum grain) to dark brown (from sorghum varieties containing condensed tannins). *Kisra* from nontannin sorghum grains is flexible with an open structure of many regular gas cells, whereas bread with a more brittle, denser structure with fewer and smaller gas cells was observed in *kisra* processed from sorghum containing condensed tannins (AwadElkareem and Taylor, 2011). Variations in gas cell size and distribution are a product of the degree of fermentation. The *in vitro* protein digestibility (IVPD) was reported to be poor in *kisra* processed from sorghum that contained condensed tannins. Fermentation improved the IVPD, but cooking decreased IVPD further in *kisra* because of cross-linking among kafirin proteins (AwadElkareem and Taylor, 2011). During fermentation, many observations have been reported, including minor protein modification (proteolysis to amino acids and peptides), utilization of soluble sugars, an increase in organic acids (Osman et al., 2010), a decrease in thiamine, an increase in riboflavin (Mahgoub et al., 1999), and a reduction in tannins, phytic acid, and trypsin inhibitory activities (Rahman and Osman, 2011).

#### **Fermented Pearl Millet Bread**

Among millets, pearl millet is predominately cultivated in Africa and Asia (particularly India) where high temperature (about 30 °C) and limited rainfall (250 mm) prevails. Pearl millet grain is regarded as superior in some of its nutrient contents such as protein and fat (Taylor et al., 2010) compared to common cereal grains such as wheat, sorghum, and maize. The grain is an ovoid shape 2.0 to 3.4 mm in length and 2.5 mm in diameter and weighs 8 to 11 g (Hama et al., 2011). In Namibia, the traditional processing practice for pearl millet starts with cleaning the grains (winnowing, hand sorting, sifting), conditioning (soaking in water, 24–48 h), followed by decortication with a mortar and pestle to remove the bran (pericarp, germ, and testa layer) (Mallet and du Plessis, 2001; Taylor et al., 2010). The decorticated grains are again soaked in water for 1–2 days during which time lactic acid fermentation takes place. The fermented mass is partially sun-dried and milled with a mortar and pestle into a fine flour. Milling into a fine flour is necessary to damage the starch granules to some degree to facilitate water absorption, which leads to the production of a cohesive dough. Dough is molded into large flattened balls and is panfried or roasted or boiled in water. The processed pancakelike bread is consumed fresh or stored in plastic for a short shelf life.

#### **Fermented Maize Bread**

During traditional processing, maize grains are cleaned and soaked (3–12 h) and then decorticated with a wooden mortar and pestle. The decorticated mass is spread, dried, and sifted/winnowed to separate the bran and other lightweight materials from the maize meal. The maize meal is then milled into fine flour (<75–212 µm) with stone, disc attrition, or hammer mills. The flour is mixed with water, and dough is worked by hand. The dough is left to ferment, then thinned with water to the required consistency for baking thin or thick flatbreads. Thick bread is usually baked from early fermented dough. A portion of fermented dough is saved for sourdough processing in the next batch of bread. *Injera* can also be made from maize. When manufacturing maize *injera*, at the stage the yellowish liquid *irsho* is removed from the fermenting vat, a portion of batter (10%) is withdrawn from the sediment, cooked, cooled (about 46 °C), and then mixed back into the fermenting batter. After 20–30 mins more fermentation, the batter is thinned and baked on a baking griddle for 2–3 mins. Maize *injera* is sticky after baking compared to teff *injera*. After 1 day, maize *injera* becomes fragile and loses its rollable nature. The physicochemical properties of the dough are modified during the sourdough process by the fermentative action of LAB and yeast. Leavened wheat type bread (such as European style bread) cannot be produced from maize flour because maize zein proteins do not have the required viscoelastic properties that come from wheat gluten proteins. Because of this, wheat style bread made from maize has low loaf volumes and has a hard, dense crumb. However, sourdough technology along with selected strains of LAB was reported to improve the quality of wheat style maize bread primarily due to starch granule modification (Falade et al., 2014).

#### **Steamed Breads**

Steamed breads processed from wheat, maize, or sorghum grain flour are consumed in regions of Southern Africa (South Africa, Botswana, Lesotho). The basic ingredients for steamed bread are grain flour (wheat, maize, or sorghum), water, yeast, and salt (Manley and Nel, 1999). Other baking ingredients used are sugar, shortening, and ascorbic acid.

South African steamed bread from wheat is made from a basic dough and follows a typical bread-making methodology. Dough is left to rise (approximately 90 mins, 30 °C, 85% relative humidity), punched down, molded into the desired shape, and proved on a plate for a further 40–60 mins (30 °C, 85% relative humidity). The plate and dough is then placed on a stand inside a saucepan of shallow water and steamed for approximately 1 h. This produces a round white-gray loaf with a moist, sticky, and soft texture devoid of a crust. The crumb structure can vary from open to dense depending on the degree of leavening. The flavor of steamed bread is subtle and delicate. This type of bread has reduced lysine amino acid loss and will not lead to acrylamide formation (Ma et al., 2014). But because of its high moisture content, steamed bread is highly susceptible to spoilage and thus the shelf life is short.

#### **Ujeqe**

*Ujeqe* is a traditional steamed bread of Zulu (South Africa) origins made by boiling or steaming crushed green maize or sorghum. The green maize grains are wet milled by grinding on a stone to a thick paste. The ground mass is made from the whole grain



because the pericarp, germ, and endosperm fractions are all crushed together. Since the maize is harvested at an early maturity stage, the sugar level is higher than mature maize grains. Salt can be added during stone grinding, and then the dough is shaped and left to rise. The risen dough is wrapped with maize leaves or husks and steamed for approximately 1 h. Similar maize bread by the vernacular name of *monepola oa poone e ncha* or *malitsibana* or *mohlefe* (green mealie bread) is consumed in Lesotho (Nkhabutlane, 2014). Such breads are consumed until the harvest of the dry mature maize grain. The color of the bread is influenced by the maize grain color (white, yellow, or blue). The bread has an intense aroma with a dense and fibrous texture and is in itself satiating. Even though, no physicochemical data are available, such bread is likely to be high in fiber and bioactive compounds.

### Kenkey

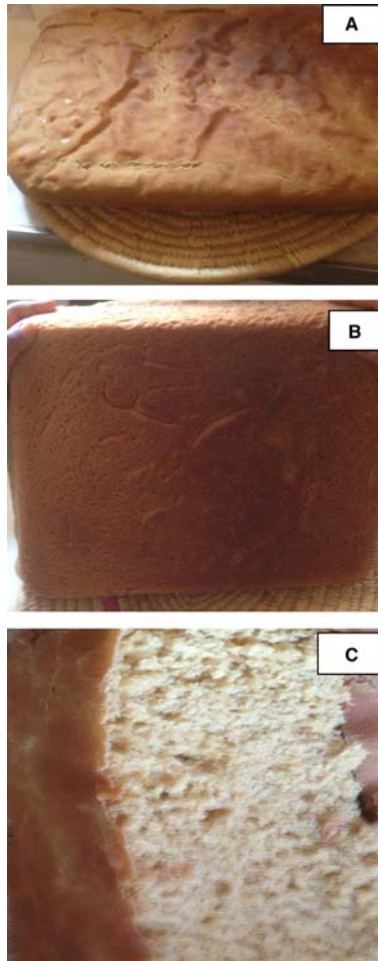
*Kenkey* is a stiff, sour, steamed dumpling type maize bread consumed in Ghana. It is fermented with LAB and yeast. Traditional processing can take up to 4–6 days and is labor and time intensive (Oduro-Yeboah et al., 2016). There are two main types of *kenkey* (Ga *kenkey* and Fante *kenkey*) originating from the Ga and Fante ethnic groups, which also differ in some of their processing techniques. In *kenkey* manufacture, whole maize grains are cleaned and steeped in water for 1–2 days, which initiates fermentation and facilitates wet milling. The steeped grains are washed with water containing clay (implicated to contribute iron) and ground (stone grinding or disk attrition mill) into wet flour. The dough is kneaded by hand (dough 50% moisture), tightly packed into containers (wooden, aluminum, enamel, or plastic), and further fermented for 2–4 days. A portion of fermented dough (about half) is cooked to gelatinize the starch, called *aflata*. The *aflata* is mixed back into the uncooked dough in a ratio that varies from 2:3 to 2:1 for Fante *kenkey* and 1:1 for Ga *kenkey*, then shaped into round balls wrapped in plantain leaves (Fante *kenkey*) or maize husks (Ga *kenkey*) and cooked in boiling water for about 3 h for Ga *kenkey* and for 3–6 h for Fante *kenkey*. Another variant, called white *kenkey* (*Nsiho*), is made from branless maize and steamed for 1–2 h. The desired texture of *kenkey* is cohesive, coarse particle bread that is not too sticky or crumbly. In addition to the quality of the maize grain, the activities of proteases, amylases, cell wall hydrolases, and lipase enzymes during steeping and fermentation are reported to impact *kenkey* quality (Oduro-Yeboah et al., 2016). Even though a reduction in phytic acid content has been observed, mineral availability such as calcium is limited in *kenkey*. Soaking is reported to reduce dietary fiber content and improve *in vitro* protein digestibility and lysine availability (Nche et al., 1996).

### Wheat Leavened Breads

Bread wheat and durum wheat flour are widely used to bake traditional African breads particularly in the northern African region, eastern African highland regions, and southern African regions where wheat is intensively cultivated (Table 1, cross reference article 1). The bread baked from wheat can be thin and aerated (still somewhat of a flatbread) or thick with greater aeration and formed into different shapes depending on the mold or pan type used. Typical traditional wheat bread (thick) is shown in Fig. 2. In traditional processing, wheat grains are cleaned, washed and soaked, decorticated, or rubbed by gentle pounding using a wooden mortar and pestle to detach the beeswing bran layer. The pounding action also damages the germ and testa layer to different degrees depending on impact applied. The decorticated grains are spread, dried in the sun, sifted to remove the detached bran, and milled into a fine flour using stone attrition/plate or hammer mills (Doblado-Maldonado et al., 2012). The flour is mixed with water, yeast, and salt. The dough is worked by hand, with the gradual addition of water to develop the gluten network (gluten polymerization). Sometimes, edible oils are worked into the dough and other ingredients such as black cumin, flax, or fenugreek seeds are spread on the dough surface after shaping. The dough is left to rise. Wheat grain flour is required to have a significant amount of starch granule damage (3%–8%) to be available for amylase enzymes to release fermentable sugars from the starch, which is then utilized by yeast. After 2–3 h, the dough is punched down, to break up any clusters of yeast and to favor yeast to access un-utilized nutrients such as sugars in the dough (Struyf et al., 2017), shaped, and transferred to lightly greased baking pan for the second proving.

The metabolic activity of yeast is one factor that influences the final loaf in terms of loaf volume, nutrient content, taste, and aroma, which in turn depends on the nature of the dough matrix, yeast strain, and fermentation conditions (Struyf et al., 2017). For example, an ideal temperature for yeast dough fermentation is from 25 to 32 °C. During the first 10 mins of baking, the dough expands further because of heat-induced water vapor, gas, and ethanol expansion in the dough matrix. The activity of the yeast is halted, proteins are denatured, and starch is gelatinized, and the dough gradually sets into an aerated foam structure. Baking is complete when the crust becomes light to medium brown in color. The crumb is predominantly influenced by the viscoelastic nature of the gluten protein network along with other trapped components (starch, endosperm cell wall, nonstarch polysaccharides, and lipids) that favor the expansion and holding of the gas formed air cells in the matrix. Expansion is influenced by gluten quality (Ortolan and Steel, 2017) and yeast fermentation (Struyf et al., 2017). Because of the weak viscoelastic properties of the gluten protein in durum wheat compared to bread wheat gluteins, a fine uniform crumb with low loaf volume and short shelf life is formed (Kezih et al., 2014). The bread crust color and flavor are mainly influenced by the Maillard and caramelization reactions and the flavor compounds generated by the method of fermentation and any additional ingredients. The type of wheat and the fiber levels also have an impact on the crumb structure (Rosa-Sibakov et al., 2015). For example, durum wheat contains carotenoids, giving a noticeable yellow tint. Bread loaf volume, softness, moistness, flavor, color, and bread staling are also influenced if an edible oil or fat is added to the dough (Pareyt et al., 2011). Antinutrients such as phytic acids are reduced by the degree of aleurone and germ removal and by the degree of phytase enzymes activated during dough fermentation. Bioactive compounds such as phenolic acids imparted to bread are variable depending on the amount of bran and endosperm cell walls in the flour used.





**Figure 2** Traditional thick wheat bread: (A) topside crust structure, (B) underneath crust structure, and (C) crumb structure.

### Composite Breads

With increasing urbanization on the African continent, bread consumption in many African communities has shifted toward leavened wheat bread (not that unlike European style wheat bread). Contemporary consensus on the definition of wheat bread is that it includes the basic ingredients: wheat flour, water, yeast, and salt. However, in many African regions, particularly in semiarid and arid climates, wheat cultivation and production are not possible. Wheat is a key global commodity, and for many African nations, the price and supply of foreign wheat are not viable or sustainable. Wheat grain flour is also limited in terms of protein quality (particularly lysine), provitamin A carotenoids, minerals (iron, zinc, and calcium), essential fatty acids, and some bioactive compounds. All these are the driving factors for composite bread to become a greater presence in African bread making. Among others, composite bread is produced from wheat and either cassava, cowpea, maize, sorghum, fava bean, millet, or fofo. A mixture of wheat with cassava and maize or soybean or just wheat and yam or wheat and taro flour is also common. When wheat bread is processed with composite flours, there are variable effects on the bread loaf volume, crumb and crust structure, sensory properties and acceptance, shelf life, and nutrients, due to the differences in the functionalities of the different types of flour used (Table 4, cross reference article 1 and Table 2). With composite flour, a decrease in bread loaf volume, an increase in the bread mass/density, a dense crumb structure, and cracked crusts have been observed in contrast to bread produced from 100% wheat flour. This is predominately due to influence on the viscoelastic properties of wheat gluten (Noorfarahzilah et al., 2014; Ayele et al., 2017). When bread wheat is blended with bland tasting flour such as cassava, the influence on the final bread flavor is negligible; however, others such as soybean flour can impart a beany flavor. Soybean flour is also higher in fat than other flours, and oxidation can occur unless the lipids are stabilized prior to processing (Shao et al., 2009; Olaoye and Ade-Omowaye, 2011). To overcome this, whole soybeans are blanched or steam treated to inhibit the soybean lipoxygenase enzyme, which can lead to the production of beany, grassy, and rancid flavors (from compounds: 1-octen-3-ol, 1-hexanol, hexanal, 1-pentanol, (E)-3-hexen-1-ol, 2-hepta-none, and 2-pentylfuran) in products.

Composite soybean flour bread (10%–17%) was found to be acceptable and improved the nutrient composition of the bread with respect to protein, amino acid, essential fatty acid, and mineral content, as well as soybean isoflavones, dietary fiber, and peptide bioactive compounds (Liu, 2004). When wheat flour is blended with starchy flours (cassava, yam, and taro), due to limited fat content, the loaf volume is decreased and moistness, flavor, color, and antistaling qualities are negatively influenced unless the fat content is supplemented during production. The addition of coarse grain flour (sorghum, maize, teff and millet) can increase bioactive compounds (dietary fiber, phenolic compounds, carotenoids, phytosterols, tocopherols, and tocotrienols) but decrease the bread loaf volume due to dilution of wheat gluten proteins. With mounting evidence that whole grain consumption can reduce the risk of some diseases such as diabetes, various cardiovascular diseases, and cancers (Benisi-Kohansal et al., 2016), bread processed from whole grains such as teff, fonio, finger millet, and sorghum are becoming increasingly appealing. However, there is some concern about the risk of surface contamination from pesticide residues, mycotoxins, and heavy metals when the outer layers of grains are not removed. Good agricultural practices, adequate grain drying, cleaning, and removal of a small portion of the outer layers can help to decrease the risk, but still preserve the health benefits associated with whole grains. Starch granule properties, including granule size, differences in gelatinization temperature, the extent of starch modification during milling, dough processing, and baking, are all factors that contribute to quality differences.

The gelatinization temperature of tropical cereal starches from maize, sorghum, rice, teff and millets are approximately 10 °C higher than wheat starch (Table 4, cross reference article 1). The gelatinization temperature of cassava, taro, yam, cowpea, and fava bean starch are also reportedly higher as well (Table 2). When composite bread is produced with the inclusion of coarse grains, there are some consumer acceptance issues. Typically, bread with greater than 30% non-wheat flour is deemed to be unsatisfactory in terms of texture and loaf volume. Bread with 5% to 20% non-wheat flour was found to be almost as acceptable as 100% wheat flour bread, even though it varies with the type of flour used. This acceptance is important for the composite bread industry to expand and deliver improved, healthful bread to the population.

### Trends and Future Prospects

Before wheat roller milling and the industrialization of baking on the African continent, bread was processed from cereal grains cultivated by communities. Bread-processing methods were also variable and uniquely adapted toward particular types of grain. However, with the spread of roller wheat milling technology throughout Africa, bread processed from refined wheat flour has also become part of many African diets. However, recent trends in promoting nutrition to prevent diet-related disease have caused a shift back to processing bread from brown (bran included), coarse grain and whole grain flour, and composite flour has become more popular. Research is ongoing globally to find ways to produce wheat-like bread from non-wheat flour through process modification, including milling variation, soaking, germination, enzyme treatments, moist heat treatments, changes in blending proportions and the inclusion of hydrocolloids.

### Conclusions

Semileavened and leavened or fermented African bread is processed from a variety of cereal grains. Those processed from teff, finger millets, and fonio benefit nutritionally from the use of the whole grain, and are also naturally free of wheat type gluten proteins. This holds great promise for the introduction of African bread into the international market, especially with the rise of gluten intolerances and coeliac disease around the world. Much of the fermented semileavened bread production still happens in the traditional style in the household or in cottage industry in Africa. Research efforts are focused on the standardization of processing methods, strain development of fermenting microorganisms, and automated technology, although there is still a way to go before these types of bread can become global commercial products.

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# Indian Flatbreads: How Structure Influences Properties

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## Introduction

Flatbreads are very popular type of bread, especially in those parts of the world where bread is consumed as a major source of dietary protein and calories (Mir et al., 2014). It is believed that people have been making flatbreads for more than 6000 years. The world's oldest baking oven was found in Babylon in 4000 BC and records of flatbread baked in the hot ashes or on heated stone slabs in the old kingdom of Egypt in 2500 BC (Kumar, 2016). Various ancient cooking methods that resemble modern day methods were used such as the tandoor, a simple wood-fired oven consisting of an earthen pot with a refractory brick wall. It originated in Persia (Iran), was brought to India by Arabs via Afghanistan in 3000 BC, and is still used today in India for the baking of tandoor bread. Small mud-plastered ovens similar to tandoors have been found in Harappa and Mohenjo Daro dating to 2500 BC in the ancient valley of India and Pakistan (Parimala and Sudha, 2015; Kumar, 2016).

Flatbreads are the most primitive type of bread prepared by humans because the ingredients were readily available and simple; flour, water, and sometimes salt were used and without any specialized tools, kneaded into pliable dough before being shaped by hand and baked. Flatbreads could be eaten at every meal, due to the ease of making them, and they also functioned as plates with other foods being served on top of them or the flatbreads could be ripped into pieces and used as an implement to facilitate eating other parts of the meal. Of great importance, among the people of India and the Middle East, forms of flatbreads are also found in Europe, Africa, Southeast Asia, and the Americas (Parimala and Sudha, 2015). The consumption of Indian flatbreads has also spread to Western countries where their popularity is increasing due to their versatility (Mir et al., 2014). In India, there are several forms of flatbreads such as *chapati*, *parotta*, *puri*, and *tandoori roti* (Fig. 1), which are consumed as part of the daily diet. Indian flatbread is made from wheat flour and water, with or without the addition of yeast or other leavening agents and ingredients such as salt. The variation comes from production and baking method (Prabhasankar et al., 2003; Kurhade et al., 2016). In contrast to European style leavened wheat bread, Indian flatbreads have little to no crumb (Fig. 2), and thus it is the crust that is of the most importance, in both functionality during the meal and sensory properties.

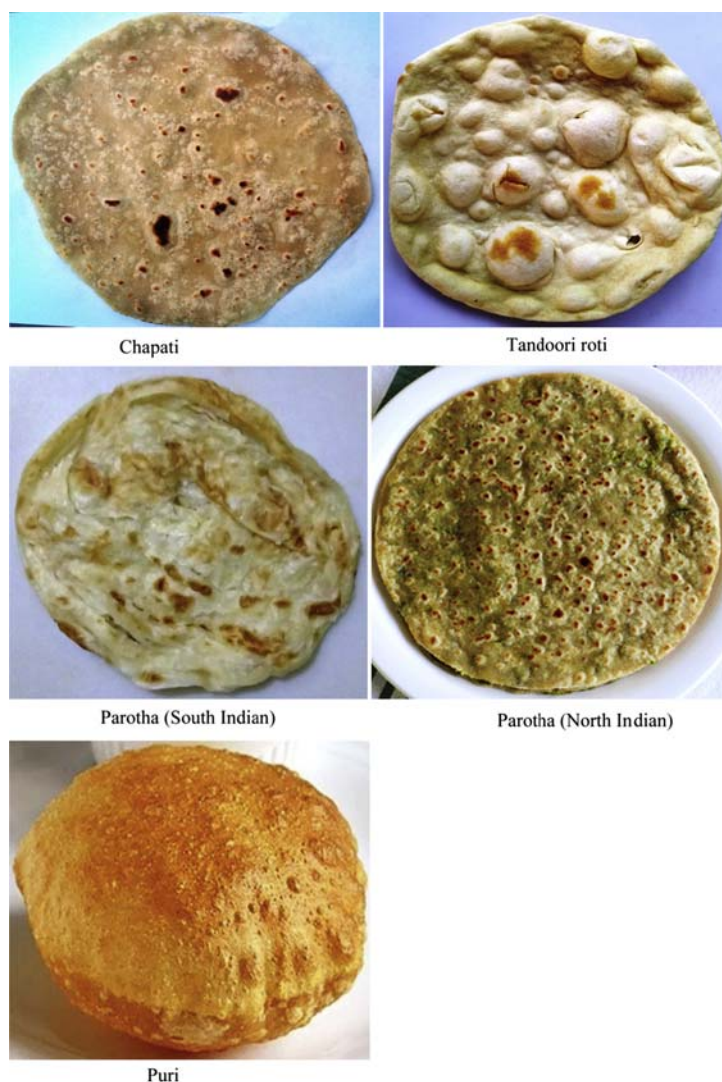
The structure of bread can be described as a multiphase colloidal system, including, but not limited to, polysaccharides, proteins, fats, salt, and water, all of which influence the final physical and sensory properties of the product. The microstructure of flatbreads is dominated by gelatinized starch granules wrapped up in a continuous matrix of denatured protein (Prabhasankar et al., 2003). Both gelatinization of starch granules and denaturation of the protein matrix occur due to the heat applied during the baking process. Porosity occurs in leavened breads, due to the production of carbon dioxide from leavening agents, such as yeast, which leads to air cavities and void structures. In contrast, in unleavened breads, gas cells are not formed in the microstructure as there are no leavening agents, and thus a closed structure is observed (Sidhu et al., 1990). Indian flatbreads are known for their special quality attributes, which have specific properties for their function as part of a meal. These properties are not only controlled by the chemical composition of the raw ingredients but also from ingredient interactions *in situ* and the processing technique. The microstructure of bread has a significant effect on its texture and sensory properties (Gao et al., 2015).

## Method of Production

In general, the flatbread-making process involves several basic steps (Fig. 3). The first step in the preparation of flatbreads is the selection of ingredients. The formulations of flatbreads vary from region to region, but the basic steps remain the same. Mixing is the important step in flatbread preparation and involves the uniform mixing and kneading of all the ingredients. During mixing, the wheat flour is hydrated and viscoelastic gluten protein network is formed. The way in which the dough is mixed and kneaded depends upon the type of method used (mechanical or by hand), water absorption, and type of bread desired (Saxena and Rao, 1996; Gocmen et al., 2009; Mir et al., 2014).

Leavened breads require fermentation as a basic requirement. The optimum temperature–time combination is necessary to produce a good quality end product with the correct aerated texture (Mir et al., 2014). The yeast or sourdough starter initiates fermentation, making the dough lighter and extensible. Normally dough is rested to allow complete flour particle hydration and the relaxation and development of the gluten network. The dough then becomes more coherent, and it achieves a point of maximum consistency. This is the point to which dough should be worked to produce a good quality bread. The dough changes from a viscous slurry into a viscoelastic mass, and the gluten forms a continuous film which enables easy sheeting with minimal stickiness (Qarooni, 1996; Parimala and Sudha, 2015). Molding is an important step in flatbread production whereby the dough is divided into 30–50 g pieces and then traditionally rounded between the palms of the hand (Kurhade et al., 2016). On a commercial level, the rounded dough is sheeted (rolled or flattened) into a circular shape. Each type of flatbread has a different shape, size, and thickness to produce variable products.



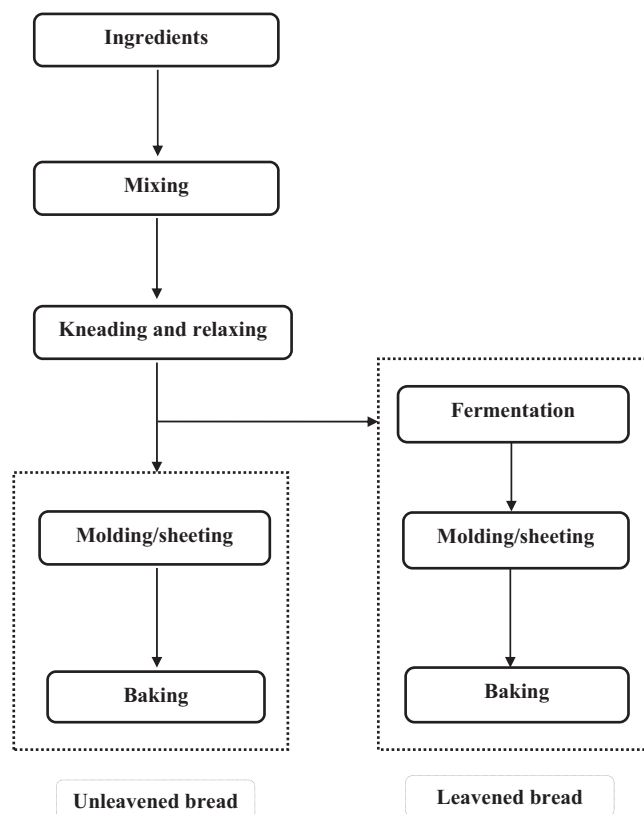


**Figure 1** Some common Indian flatbreads.



**Figure 2** Crust and crumb of flatbread (tandoori roti).

Baking is the last and the most important step in flatbread production. The flat dough is baked in various pans, in a tandoor, or an oven as per the type of bread desired. Various physical and chemical changes, including moisture loss, volume expansion, starch gelatinization, protein denaturation, crust formation, and browning, take place during baking. The extent of starch hydration, swelling, and reassociation is affected by the heating rate during baking (Sidhu et al., 1990; Mir et al., 2014). To achieve optimal baking, the common industrial practice is to bake bread in the controlled, constant temperature oven (Mondal and Datta, 2008).



**Figure 3** Typical flow sheet for the preparation of Indian flatbreads.

### Examples of Indian Flatbreads and Their Structure

In India, wheat is a dietary staple and is most commonly consumed in the form of different types of flatbreads, such as *chapati*, *parotta*, *puri*, *tandoori roti*, *phulka*, *papad*, and *nan* (Kumar, 2016). These are predominantly made from whole wheat, but refined or blended flours are also used. A number of modifications in formulations have been made to improve the quality and favorable sensory properties. These formulations vary from region to region, but the basic ingredients (wheat flour, water, and salt) are the same. Butter, vegetable shortening, or nonfat powdered milk may be added to improve the taste and aroma (Mir et al., 2014).

Variation is observed in the basic micro- and macrostructure of flatbreads. In unleavened breads, the dough is prepared from wheat flour and water without the use of yeast or leavening agent. The starch granules in the dough undergo almost no change during the short period of resting time. The steam generated during baking is responsible for the development of the matrix consisting of gelatinized starch on the inside of crust and in the crumb areas. Thus crumb of unleavened breads is much denser compared with leavened bread (Sidhu et al., 1990). The degree of deformation and folding of starch granules also varies between the crust and crumb of flatbreads. Starch granules maintain their integrity on the outside of the crust, but in the crumb, all the starch granules lost their identity and only a continuous matrix of gelatinized starch is observed. These differences also seem to be related to the water availability in the crust and crumb (Sidhu et al., 1990). On the other hand, the formation of gas cells with diverse volumes and shapes during the baking process is the major factor that determines the texture of leavened breads (Mir et al., 2014).

#### Chapati

*Chapati* is an unleavened flatbread and is the principal flatbread consumed on the Indian subcontinent. The ingredients such as flour, water, and salt are kneaded into pliable dough before being shaped and baked. The desired quality characteristics of *chapatis* are pliability, soft texture, light cream-brown color, with a slight chewiness and baked wheat aroma (Hemalatha et al., 2014). Almost 90% of the wheat produced in India is used in the production of *chapati* (Kurhade et al., 2016). The other 10% of wheat is used for making other breads, biscuits, and cake (Parimala and Sudha, 2012).



Microscopy studies have shown that the microstructure of *chapati* has gelatinized starch granules scattered throughout the crumb that lead to the formation of a continuous starch network (Fig. 4). The protein matrices are wind together to form aggregates (Hemalatha et al., 2010, 2014), and the protein matrices and starch granules overlap one another to also form aggregates (Kurhade et al., 2016). Sidhu et al. (1990) reported that the starch granules are distorted on the inner part (facing toward crumb) of the *chapati* crust. In comparison, the starch granules mostly lose their integrity in the *chapati* crumb, and only a continuous matrix is visible.

### Tandoori Roti

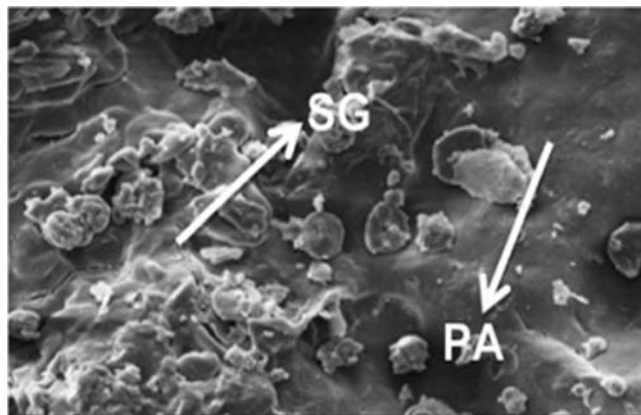
*Tandoori roti* is a popular flatbread on the Indian subcontinent. As the name suggests, it is baked in a tandoor. The *tandoori roti* is similar to a *chapati* with respect to ingredient composition but varies in size and thickness. *Tandoori roti* is somewhat thicker and larger than *chapati*; on average it is 15–20 cm in diameter and 5–8 mm thick. The crumb-to-crust ratio in *tandoori roti* is greater than that of a *chapati*. A *tandoori roti* is less pliable than a *chapati*, with a rough surface and several large blisters (Saxena et al., 2000). A literature search finds no information about the microstructure of *tandoori roti*, but one can assume some similarities with *chapati*. Generally, *tandoori roti* made from whole wheat flour has a better texture and flavor as compared to those made from refined flour (Saxena and Rao, 1995). Moisture condensation on the sheeted dough gives rise to the formation of blisters during baking. The desirable characteristics of *tandoori roti* are a typical baked aroma and slightly brittle and chewy texture (Rao and Manohar, 2003).

### Parotta

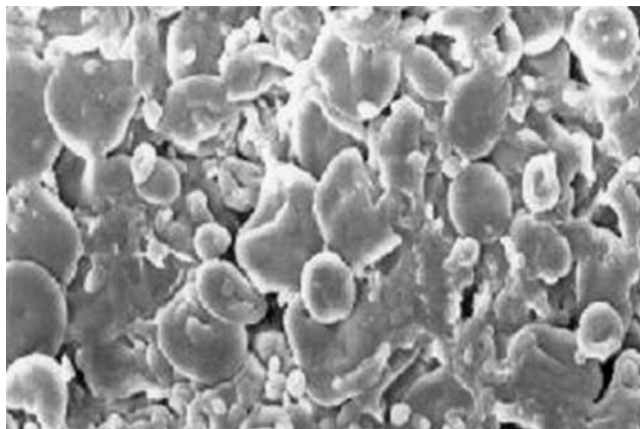
*Parotta* is another type of Indian flatbread prepared from wheat flour, water, refined oil, and salt. The optional ingredients are sugar and egg. Two different types of *parotta* are prepared in India: *South Indian parotta* and *North Indian parotta*. *South Indian parotta* containing several distinct layers is baked on a hotplate, or on a shallow iron plate heated by gas or wood. *North Indian parotta* is made by pan-frying sheeted dough on a hotplate and may be stuffed with different vegetables (Indrani et al., 2011; Sudha et al., 2015).

*Parotta* consists of several distinct layers and is slightly thicker than *chapati*. There is a slight difference between the microstructure of the outer and middle layers (Prabhasankar et al., 2003). The middle layers of *parotta* have less starch granule distortion compared to the outer layers. The microstructure of the outer layers shows a change in shape of both types of starch granules (large and small) due to gelatinization. Protein and starch molecules were observed to adhere closely (Fig. 5). However, in the middle layers, these molecules were much closer and their deformation was reduced (Prabhasankar et al., 2003; Indrani et al., 2011).

In comparison to *chapati*, the starch granules present in the crust of *parotta* are much more deformed and have a shriveled appearance but maintain a weak visible outline in the crumb under magnification. The application of fat to the outside of *parotta* during baking likely serves as an oil bath between the crust and baking pan, thus facilitating better heat transfer. The fat present between the *parotta* layers (smeared between individual layers during sheeting) may also maintain starch granule integrity during the baking process. Both of these phenomena may lead to the differences observed between the crust and crumb of *chapati* and *parotta* (Sidhu et al., 1990).



**Figure 4** Microstructure of *chapati*. PA, protein aggregate; SG, starch granule. Hemalatha, M.S., Rao, U.P., Leelavathi, K., Salimath, P.V., 2010. Influence of amylases and xylanase on chemical, sensory, amylograph properties and microstructure of chapati. LWT-Food Sci. Technol. 43, 1394–1402.



**Figure 5** Microstructure of *parotta*. Prabhasankar, P., Indrani, D., Rajiv, J., Rao, G.V., 2003. Scanning electron microscopic and electrophoretic studies of the baking process of south Indian parotta—an unleavened flat bread. *Food Chem.* 82, 603–609.

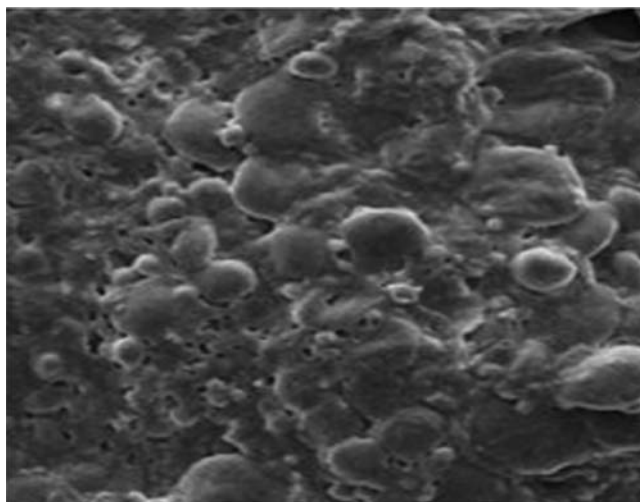
### Puri

*Puri* is an unleavened deep-fried flatbread prepared in many South Asian countries including India, Pakistan, and Bangladesh. It is consumed for breakfast, or as a snack or light meal. It is prepared from either whole or refined wheat dough containing a small amount of salt (Shaikh et al., 2007; Kumar, 2016). The dough pieces are made into round balls, rolled into circles, and deep fat fried in vegetable oil. The dough is prepared without using any leavening agent. The steam generated during frying results into puffing, thus separating the crust from the crumb. This steam is also responsible for the development of gelatinized starch matrix on the inside of crust and in the crumb areas. The crumb of *puri* appears to be very dense in comparison to bread prepared with the yeast (Sidhu et al., 1990).

In *puri*, the starch granules are partially gelatinized but retain their integrity possibly due to less water absorption, which restricts the swelling of the starch granules during frying (Fig. 6). The gelatinized starch granules were found to be wrapped up in a continuous protein matrix. The *puri* crust showed eruptions and the development of a protein matrix during deep frying. Crust eruptions are formed due to the explosion of starch granules during frying operation (Sidhu et al., 1990). The starch granules were more deformed and folded on the inside of the crust (Parimala and Sudha, 2012).

### Influence of Bread Structure on Properties

The structure of any given food product is essentially a template of the properties of that food system, representing its material behavior, physical stability, appearance, sensory performance, and other attributes related to the overall quality of that particular food (Day and Golding, 2016). Bread structure is the organization of food ingredients and their interactions during the baking



**Figure 6** Microstructure of *puri*. Parimala, K.R., Sudha, M.L., 2012. Effect of hydrocolloids on the rheological, microscopic, mass transfer characteristics during frying and quality characteristics of *puri*. *Food Hydrocoll.* 27, 191–200.

process, forming different types of structures in the end product (Heertje, 2014). The structural properties of flatbreads are controlled not only by chemical composition of the raw material but also by their interaction at the molecular level (Nishinari, 2004).

Various subjective and objective assessment systems have been used to evaluate flatbreads including physical and sensory characteristics. These include volume, crust color, presence of blisters, and uniformity of shape, as well as texture, crumb color, and flavor. Pliability, shear value, and tearing resistance are typical features used as criteria to evaluate flatbreads of Indian origin. Moreover, instrumental techniques such as texture analyzer, colorimeter, and scanning electronic microscopy are also exploited to evaluate their quality (Parimala and Sudha, 2015; Sudha et al., 2015).

Indian flatbreads are usually prepared from wheat flour, which is good source of starch and gluten. The gelatinization of starch granules and denaturation of the protein matrix are a consequence of heat to which these components are subjected during baking. Water loss and mixing of structural components that take place during baking may be responsible for the resultant chewiness, which is a typical quality attribute of flatbreads (Prabhasankar et al., 2003; Sidhu et al., 1990). Insights into how the structure of food changes during processing and during consumption are essential for producing a high-quality end product. The breakdown of food material during mastication affects the overall sensorial experience, and the breakdown during gastrointestinal transit is increasingly being used to demonstrate the role of food structure in the digestion and uptake of micro- and macronutrients (Nishinari, 2004). These microstructural properties can be used to identify processing parameters that affect the overall quality of product (Cafarelli et al., 2014).

## Conclusion

Understanding the microstructure of foods is important as it has a direct effect on the quality of the product. Many factors such as ingredients, preparation methodology, and baking procedure affect the quality of bread structure. These variables and their interactions affect the bread microstructure, which in turn influences the sensory, textural, and physical properties of the bread.

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# Tofu and Soy Products: The Effect of Structure on Their Physicochemical Properties

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## List of Abbreviations

GDL	Glucono- $\delta$ -lactone
LF-NMR	Low-field nuclear magnetic resonance
MTGase	Microbial transglutaminase
OCC	Optimal coagulant concentration
TAGs	Triacylglycerols
WHC	Water holding capacity

## Introduction

Soy products have been consumed increasingly nowadays because of its high protein content and other beneficial components. Soy products are widely distributed, such as tempeh, natto, douchi, soy milk, tofu, dried bean curd, and others. Tempeh, natto, and douchi are the same type of fermented products from soybean seeds in different countries. Soy milk is a typical colloid food consisting of water, protein, lipid, and other trace constituents. Tofu and dried bean curd are typical protein-based foods, which can be used to produce many types of other soy products. Moreover, tofu and its derived products are considered to have the similar structure. Therefore, the structure and its effect on the physicochemical properties of tofu are the main focus in this chapter.

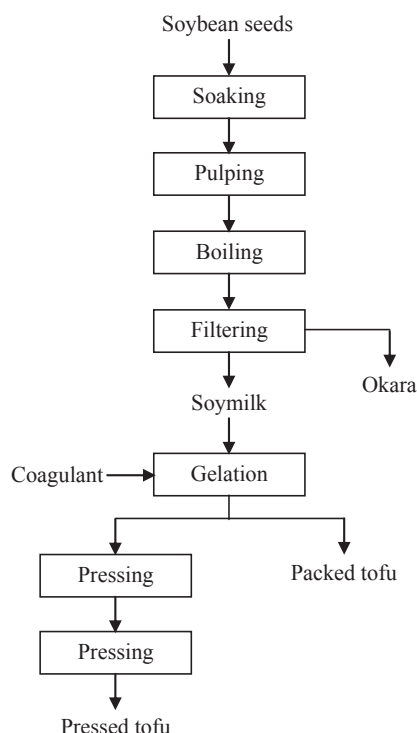
The flow diagram of tofu processing is shown in Fig. 1. As a typical gel food, the formation of tofu is mainly attributed to the gelation property of soybean proteins. Simply, thermally denatured soybean proteins aggregate to form protein aggregates because of the increased hydrophobic groups around the protein molecular surface induced by heating. When a coagulant is added, the aggregated proteins further interact with each other to form the polymerized proteins with a stable three-dimensional network structure. Other nonprotein components, such as water, polysaccharides, lipid, and other trace components, are entrapped in this network to influence the structure of tofu. The structure of tofu determines the physicochemical properties of tofu product. However, the structure formation of tofu is influenced by various factors, involving the quality of soybean seeds and processing technology, resulting in various types of tofu for satisfying different consumers (Table 1).

## Structure Formation of Tofu

The structure formation of tofu is influenced by several factors, which include the composition of soybean proteins, other nonprotein components, thermal treatment methods of soy milk, the types of coagulant, and its usage conditions. Therefore, different types of tofu structure can be obtained, resulting in different yields and edible qualities of tofu.




## Protein Composition of Soybean Seed

The composition of soybean proteins directly determines the tofu quality. Among the soybean proteins, 11S and 7S proteins, also known as the major components of glycinin and  $\beta$ -conglycinin, respectively, are most important two storage proteins that influence the structure and finally the texture of tofu. As shown in Fig. 2A, glycinin is a hexamer and each monomeric unit has an acidic polypeptide chain and a basic polypeptide chain via a disulfide bond (Malaki Nik et al., 2009). The acidic and basic subunits associate into two hexagonal rings via electrostatic interaction and hydrogen bonding (Nishinari et al., 2014). Glycinin composes of five types of subunits, which can be divided into three groups, namely group I ( $A_{1a}B_{1b}$ ,  $A_{1b}B_2$ ,  $A_2B_{1a}$ ), group IIa ( $A_5A_4B_3$ ), and group IIb ( $A_3B_4$ ) (Adachi et al., 2003). Sulfur amino acids, such as methionine and cystine, are contained in high quantity in glycinin to contribute to form the necessary disulfide bonds.  $\beta$ -Conglycinin is a mixture of heterotrimeric and homotrimeric glycoprotein, which consists of three *N*-glycosylated subunits, including  $\alpha'$ ,  $\alpha$ , and  $\beta$ , through the hydrophobic interaction and hydrogen binding



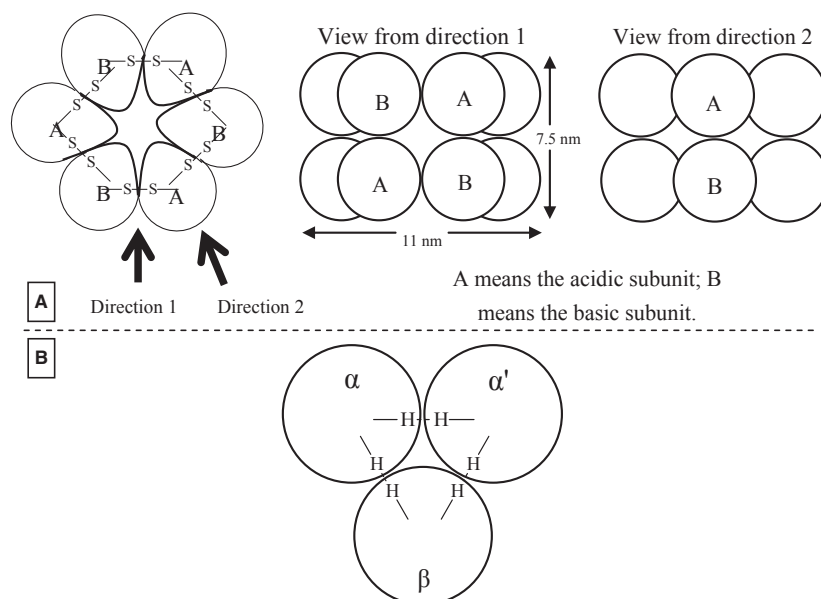
**Figure 1** The flow diagram of tofu processing.

**Table 1** The commonly consumed types of tofu and their characteristics

	Types	General characterization	Physicochemical properties	Shelf life	Syneresis	Appearance
Pressed tofu	Hard/firm tofu or momen tofu	Coagulated by many coagulants: nigari, acidic tofu whey, and GDL Pressed after tofu whey removal and stored under refrigeration No pasteurization	~80% Water content Compact internal structure; high hardness, firmness, and toughness Relatively rough taste and strong beany flavor	Several days in bulk (2–4 days)	High	
	Soft tofu	Coagulated by gypsum Processed by pressing unbroken bean curd Less removal of tofu whey No pasteurization	~90% Water content Medium firm or hard Tender, elasticity, and fine texture Light beany flavor	Several months in a pouch pack	Low	
Packed tofu	Silken tofu or kinugoshi tofu	Coagulated by GDL or dilute CaSO <sub>4</sub> solution Gelation in a pouch pack and molding without pressing No removal of tofu whey and less loss of nutrients Pasteurized after packaging	~90% water content Bland taste and fine texture Soft cheeselike texture but firm enough to retain shape after slicing Fine and smooth texture Light beany flavor	Several months in a pouch pack	Low	

GDL, glucono- $\delta$ -lactone.

(Fig. 2B) (Maruyama et al., 1998). Comparatively,  $\beta$ -conglycinin has less content of sulfur amino acids. Therefore, no effective disulfide bonds are formed to stabilize the structure of  $\beta$ -conglycinin. As reported by Bairy et al. (2010), the isolated glycinin formed a coarse gel network with a pore size of 2–3  $\mu\text{m}$  stabilized by the disulfide cross-links and noncovalent interactions. The heat-induced gels of isolated  $\beta$ -conglycinin showed a fine dispersed network with a pore size of 0.5–0.6  $\mu\text{m}$  formed from the randomly



**Figure 2** Possible schematic diagrams of glycinin (A) and  $\beta$ -conglycinin (B) molecules. Modified from Nishinari, K., Fang, Y., Guo, S.T., Phillips, G.O., 2014. Soy proteins: a review on composition, aggregation and emulsification. *Food Hydrocoll.* 39, 301–318.

aggregated assembly of clusters. However, the formation of a three-dimensional network of tofu gel is contributed to the interactions among the subunits of these two storage proteins via different types of chemical bonds (Yamauchi et al., 1991). For example, during heating, the basic subunits of glycinin react with the  $\beta$  subunit of  $\beta$ -conglycinin via a secondary bond; the acidic and  $\alpha$ ,  $\alpha'$  subunits tended to interact with each other through disulfide bonding.

As a result of discrepancies in amino acid composition and subunit composition of glycinin and  $\beta$ -conglycinin, different gelling properties can be obtained. Moreover, the special microstructures of glycinin and  $\beta$ -conglycinin determine the different types of structure transformation under various conditions, such as temperature, pH, ion type, and its strength and others.

### Thermal Treatment of Soy Milk

After the soaking and pulping processes, soy milk can be obtained by filtering the okara either before or after the heating treatment. Generally, firm tofu with higher gel strength and water holding capacity (WHC) can be obtained from the heat-treated soy milk compared with the unheated soy milk (Tang, 2007). Soy milk is stabilized by the electrostatic repulsion among the solid portions consisted of soybean protein particles and oil bodies consisting of triacylglycerols (TAGs), phospholipids, and oleosins at the ambient temperature. When raw soy milk is heated, protein particles, mainly the glycinin and  $\beta$ -conglycinin, are gradually prone to denature to expose the hydrophobic groups. The structure of oil bodies also simultaneously changes to impact on the denaturation of proteins. Consequently, significant changes are founded in the particle composition of soy milk, which is closely influenced by the heat temperature and time. The specific introduction of the changes in particle formation and gelation of soy milk induced by heat can be found in the previous review (Peng et al., 2016).

It is worth noting that the thermal denaturation temperature regions of glycinin and  $\beta$ -conglycinin are different: 65–75 °C for  $\beta$ -conglycinin and 85–95 °C for glycinin (German et al., 1997). Consequently, changes in structure of many of the protein subunits take place to form the complicated aggregates of soybean proteins using one-step heating. However, when stepwise heating of soy milk was performed to selectively denature glycinin and  $\beta$ -conglycinin, significantly increased gel strength and textural qualities of the coagulant induced tofu were obtained (Liu et al., 2004; Shin et al., 2015). Controlled heating results in effective denaturation of glycinin and  $\beta$ -conglycinin to form the protein aggregates with optimum state for the subsequent coagulation.

### Coagulation

The formation of tofu is completed by adding appropriate coagulant in appropriate concentration to the heated soy milk after heating. The aggregated soybean proteins can spontaneously assemble each other to generate polymerized proteins. However, these molecular weight-increased macromolecules are not enough for the formation of network structure needed by tofu products.

The network structure of tofu can be further modified to be coarse or fine due to the different coagulation mechanisms when different coagulants and concentrations are used. The specific coagulation mechanisms for the formation of tofu can be found in the previous review (Peng et al., 2016; Zhang et al., 2016). Based on the different coagulation mechanisms, different microstructures of tofu could be obtained, resulting in different textural properties and sensory quality. As shown in Fig. 3A, small pores



distributed uniformly in a dense and well-connected network were found in the  $\text{Ca}^{2+}$ -induced tofu; by contrast, the tofu induced by microbial transglutaminase (MTGase) showed larger pores and flakelike network structure with discontinuous and loose state (Yasir et al., 2007). As shown in Fig. 3B, for the concentration of a coagulant, the term of optimal coagulant concentration (OCC) was used to describe the most appropriate amount used for obtaining the best satisfying microstructure of tofu under the set processing conditions (Onodera et al., 2009).

### Other Components in Soybean Seeds

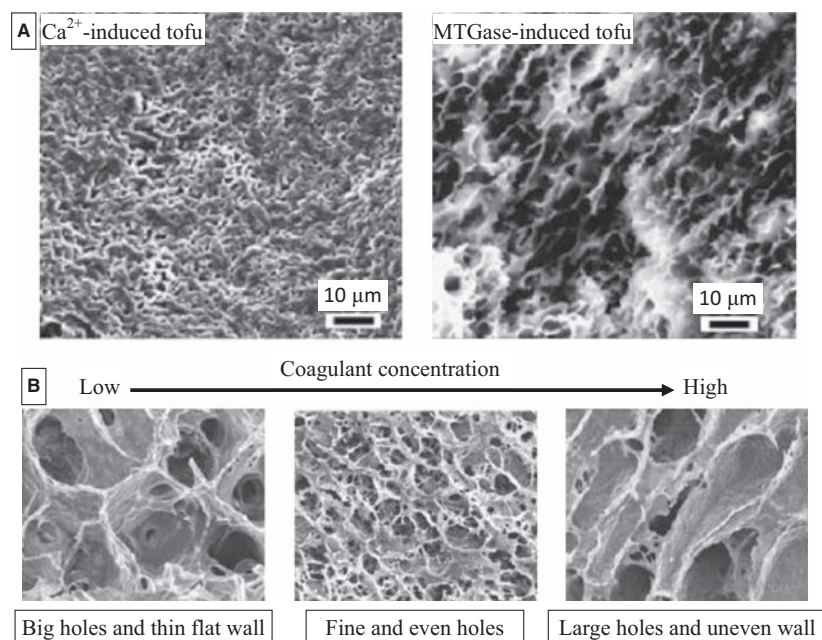
Apart from soybean proteins, water, lipids (i.e., TAGs and phospholipids), carbohydrates (i.e., sucrose, stachyose, rhamnose), phytic acid, and others are contained in the filtrated soy milk. Along with the formation of three-dimensional network these nonprotein components interacted with each other or with the aggregated proteins to be as part of the composition of tofu. Consequently, the nonprotein components are able to influence the denaturation of soybean proteins and coagulation of aggregated proteins and thereby positively and adversely influence the texture of tofu.

As mentioned above, soybean lipid is present in soy milk in the form of oil bodies. Most of these oil bodies release from the dissolved state maintained by the interactions among TAGs, phospholipids, and oleosins and finally float on the surface of soy milk accompanying with the denaturation of soybean proteins (Peng et al., 2017). However, lipids are also involved in participating in the coagulation of tofu to become a component of tofu. Similarly, water and the water-soluble carbohydrates are entrapped in the pores during the formation of three-dimensional network of tofu (Shen and Kuo, 2017). Thus, the hardness and firmness of  $\text{CaSO}_4$ - and Glucono- $\delta$ -lactone (GDL)-induced tofu were negatively related to the contents of lipids, sucrose, free sugar, and starch; however, these textural properties were positively related to the stachyose content (Poysa and Woodrow, 2002; Song et al., 2013).

As a type of metal chelator, phytic acid can bind both soybean proteins and metal ions to interfere with the coagulation by reducing the bridging action between metal ions and aggregated proteins, which is essential to the network structure of tofu (Lim et al., 1990). As a result, reduced breaking stress, hardness, and springiness of tofu made from soybean seed with high content of phytic acid were observed. However, the influence of phytic acid on the textural properties of tofu can be modified by selecting a nonsalt coagulant or increasing the OCC of the selected salt coagulant.

### Physicochemical Properties

Excellent edible acceptability and texture are important characteristics, resulting in the increasing consumption of tofu. These characteristics are always determined by the physicochemical properties of tofu. In general, WHC, texture, sensory, syneresis, and



**Figure 3** The microstructures of tofu induced by  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$  and MTGase under the same processing conditions (A) and tofu induced by GDL in different concentrations under the same processing conditions (B). *GDL*, glucono- $\delta$ -lactone; *MTGase*, microbial transglutaminase. Adapted from Onodera, Y., Ono, T., Nakasato, K., Toda, K., 2009. Homogeneity and microstructure of tofu depends on 11S/7S globulin ratio in soymilk and coagulant concentration. *Food Sci. Technol. Res.* 15, 265–274 and Yasir, S., Sutton, K., Newberry, M., Andrews, N., Gerrard, J., 2007. The impact of transglutaminase on soy proteins and tofu texture. *Food Chem.* 104, 1491–1501.

nutritional value are the most used quality attributes for evaluating the quality of tofu. The stability maintaining these physicochemical characteristics of tofu during production processes and product development is vital for keeping the market competitiveness of tofu industry. Thus, to figure out the relationship and how to relate between the network structure of tofu and its physicochemical characteristics is very important.

### Water Holding Capacity

Water plays an important role in the physicochemical properties of tofu. By using low-field nuclear magnetic resonance, three types of water are present in the tofu matrix (Li et al., 2014). Water distributed in the interspaces of network structure (such as the pores and gaps among the continuous phase) is considered the free water. Water closed to the polymerized proteins and distributed in the small meshes of tofu microstructure is regarded as the physically binding water. The third-type water is known as the chemical binding water and an integral part of the cross-linked proteins, which are distributed in the protein molecules. Based on these proposed distributions of water, the content of water retained in tofu matrix is usually evaluated by WHC influenced mainly by the free water and physically bound water. In addition, pores and meshes with different sizes exist in the three-dimensional network of different types of tofu so that WHC is usefully measured as a quality attribute for reflecting the yield, texture, color, and taste of tofu. Therefore, measurement of WHC of tofu is of practical benefit to tofu manufacturers (Cai et al., 1997).

A strong and negative correlation between water loss, indicated by WHC, and the hardness of tofu is commonly considered. When more water is bound and trapped in the network-containing proteins and other constituents, a stronger gel is formed. It is being noted that this bound water in the network structure of tofu should not be confused with the unbound water that was regarded as having a negative influence on the tofu hardness (Min et al., 2005; Yang and James, 2013). Furthermore, from the visual examination, tofu with high WHC appears smooth, whereas tofu with low WHC has a coarse texture, which is affected by the type of coagulant and its concentration. The GDL- or MTGase-induced tofu, for instance, has the relatively high WHC compared with that of the salt-induced tofu (Zhang et al., 2016).

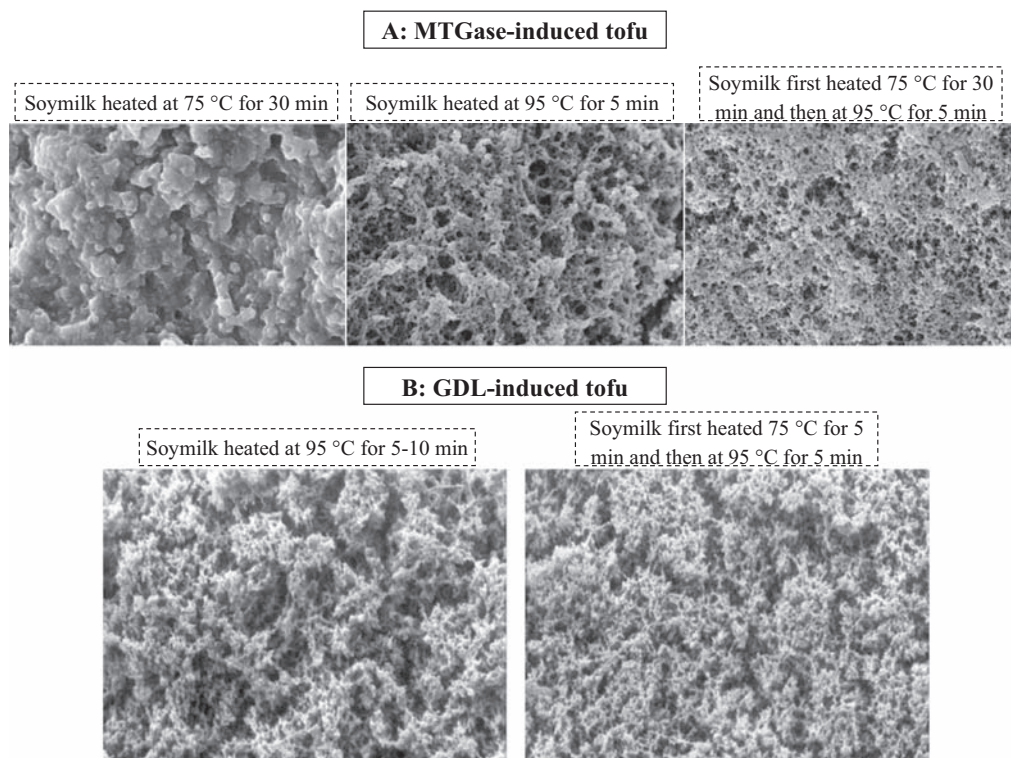
### Textural Properties

Tofu can be sliced to small pieces and shaped for foods with various forms, which is a benefit of its characteristic texture. The textural properties of tofu are the most important quality attributes used for evaluating the acceptability of tofu. For example, firm tofu often possesses greater hardness and cohesiveness so that more power is needed to break down its structure. Higher elasticity and greater chewiness are usually observed in tofu with high springiness, causing it more difficult to eat. Therefore, the factors that determine the formation of three-dimensional network structure of tofu could closely relate to the textural characteristics of tofu.

As mentioned earlier, the contents of glycinin (11S) and  $\beta$ -conglycinin (7S), content ratio (11S/7S), and their subunit profiles directly impact the formation of network structure of tofu and consequently the textural properties of tofu. In general, 11S proteins enhance the textural properties of tofu; however, 7S proteins have the opposite effect. Thus, gels made from 11S proteins have finer network structure, resulting in higher hardness, gel strength, and breaking stress than gels made from 7S proteins (Poysa et al., 2006; Renkema et al., 2001). In particular, the hardness, elasticity, cohesiveness, and chewiness of tofu have a significantly positive relationship with the 11S/7S ratio.

The hardness of gels made from the subunits of 11S proteins decreased in the order of group IIa, IIb, and I (Tezuka et al., 2000). However, regardless of the types of coagulant used, the group IIb subunit was reported to play a major role in contributing to the firmness of tofu, while the group IIa subunit showed a negative effect on the hardness and firmness of tofu (Poysa et al., 2006). Therefore, silken tofu made from soybean seeds without the group IIa subunit showed firmer structure than that made from the group IIa subunit-containing soybean variety (James and Yang, 2014). Moreover, when  $MgCl_2$  was used as a coagulant, the group I subunit was observed to have the greatest effect on the firmness of tofu than other subunits (Tezuka et al., 2004). For example, the breaking stress value of tofu made from soybean seeds with the group I subunit was twice as high as that of tofu made from soybean seeds without the group I subunit (Tezuka et al., 2000). The hardness values of gels made from  $\alpha'$ -lacking 7S proteins, 7S proteins with all three subunits ( $\alpha$ ,  $\alpha'$ , and  $\beta$ ), and  $\alpha$ -lacking 7S proteins under the same processing conditions were observed in a decreasing order (Mohamad Ramlan et al., 2004). The content of 7S $\alpha'$  subunit is known to be significantly negatively related to the hardness, elasticity, cohesiveness, and resilience of tofu. Therefore, soybean genotypes lacking both the 7S $\alpha'$  and the 11S group IIa subunits and with higher contents of 11S group I and IIb subunits can produce firmer tofu.

Denaturation of soybean proteins by heating of soy milk is the prerequisite for the formation of tofu network structure. For  $CaSO_4$ -induced tofu, the hardness, cohesiveness, and chewiness are negatively related to the heating time. For MTGase-induced tofu, more continuous and homogenous coral-like structure and higher hardness were observed in the tofu made by heating soy milk at moderate temperature and proper time, such as 75 °C for 30 min (Tang, 2007). However, this moderate temperature does not denature the glycinin completely so as to make the MTGase-induced tofu with loose or uncompact structure (shown in Fig. 4A). Therefore, as shown in Fig. 4B, it has been confirmed that a denser, finer, and more homogenous network structure was observed in GDL-induced tofu with increased apparent Young's modulus when soy milk was heated first at 75 °C for 5 min and then at 95 °C for 5 min (Liu et al., 2004). In addition, effective denaturation of both glycinin and  $\beta$ -conglycinin benefits the formation of compact and fine network structure of tofu to increase its hardness and breaking stress in spite of the type of coagulant.



**Figure 4** The microstructure of MTGase- and GDL-induced tofu when soy milk heated in different methods and heating conditions. *GDL*, glucono- $\delta$ -lactone; *MTGase*, microbial transglutaminase. Adapted from Liu, Z.S., Chang, S.K.C., Li, L.T., Tatsumi, E., 2004. Effect of selective thermal denaturation of soybean proteins on soymilk viscosity and tofu's physical properties. *Food Res. Int.* 37, 815–822 and Tang, C.H., Wu, H., Chen, Z., Yang, X.Q., 2006. Formation and properties of glycinin-rich and  $\beta$ -conglycinin-rich soy protein isolate gels induced by microbial transglutaminase. *Food Res. Int.* 39, 87–97.

Once the thermal-induced protein aggregates form, the cross-linking among them can be done by addition of coagulant. Different types of coagulant and coagulation conditions give rise to various microstructure and textural properties of tofu. Relatively slow cross-linking among the aggregated proteins takes place to form the divalent cation-bridging three-dimensional network structure with fine, homogenous, and smooth texture when sulfate salts are used. However, when chloride salts are used, relatively fast cross-linking among the aggregated proteins takes place to form the divalent cation-bridging three-dimensional network structure with rough and porous texture (Prabhakaran et al., 2006). In addition, different cross-linking sites in the denatured soybean protein molecules exist between calcium and magnesium ions because a loose network structure is observed in  $\text{MgCl}_2$ -induced tofu. Consequently, the hardness and breaking stress of  $\text{MgCl}_2$ -induced tofu are significantly higher than those of  $\text{CaSO}_4$ -induced tofu. Thus,  $\text{MgCl}_2$  is usually used in the form of modified nigari, including  $\text{MgCl}_2$  and  $\text{CaCl}_2$ , and others or by the sustained release of  $\text{MgCl}_2$ -embedding water/oil emulsions (Zhu et al., 2016). By contrast, a smoother and softer structure and greater hardness and firmness are usually observed in the GDL-induced tofu than those in the  $\text{CaSO}_4$ -induced tofu (Shen et al., 1991). The textural properties of tofu can be also modified using MTGase and other polysaccharides (such as chitosan, guar gum, and carrageenan) as a coagulant or additives (Li et al., 2015; Tang et al., 2006). Furthermore, the textural properties are also changed when different coagulation temperature, time, coagulant concentration, and stirring speed during coagulation are used (Hou et al., 1997; Kao et al., 2003).

### Sensory Properties

Because of the unique sensory properties of the white cream surface color, subtle flavor, and bland taste, tofu is commonly used to prepare various types of cuisines with different flavors.

As a type of opaque gel, tofu has a shiny appearance. The color of tofu is usually white to cream but can also be pale yellow because of the variety or quality of soybean seeds used and processing conditions. Different soybean varieties give rise to the seed coat with different shades of color, which affects the resulting color of tofu (Min et al., 2005). Tofu made from soybean seeds with a dark yellow seed coat, black hilum, or black soybean seeds produces a gray or yellow-green surface, which is less attractive to consumers (Khatib et al., 2002). Changes in color of tofu from creamy white to creamy yellow and further slightly brownish are observed when soybean seeds are stored for different storage durations (Kong et al., 2008). The heating of soy milk with high temperature and excessive time might brown the soy milk and finally, making tofu with light brown owing to the possible occurred nonenzymatic browning reaction and lipid oxidation. The coagulant concentration can also influence the tofu appearance. For

example, a whiter gel appearance was observed when high concentration of calcium salts was added to the heated soy milk with high protein content because of the formation of a more aggregated gel with denser network structure, which caused greater light dispersion (Maltais et al., 2005).

The flavor of tofu is often beany, which is an important factor contributing to its flavor. Similar to the color characteristic, the extent of the beany flavor is also related to the variety of soybean seeds and processing conditions (Kobayashi et al., 2000). The Maillard reaction between proteins and reducing sugars and oxidation of unsaturated TAGs taken place during the heating of soy milk are the major reactions for endowing tofu with a roasted nutty, fruity, light beany, and aromatic flavor. For most tofu made from the common variety of soybean seeds, the beany flavor favored by Asians originates from the oxidative decomposition products of unsaturated TAGs enzymatically oxidized by lipoxygenases, such as hexanal and (*E,E*)-2,4-decadienal (Torres-Penaranda et al., 1998). However, this beany flavor is an undesirable off-flavor for Western people, which has led to the breeding of soybean variety without lipoxygenases (Yang et al., 2015). This off-flavor can also be eliminated to a certain degree during tofu production, for instance, to select hot grinding of the soaked soybean seeds and appropriate heating temperature and time of soy milk. Tofu coagulated by most types of coagulant in its OCCs has a similar flavor.

Fresh tofu possesses a smooth, delicate, and bland taste. However, its mouthfeel is unique, and different taste properties are found in tofu induced by different coagulants (Zhang et al., 2016). For example, bitter and astringent tastes are usually shown in  $\text{CaSO}_4$ -induced tofu. Relative strong beany flavor is the characteristics of  $\text{MgCl}_2$ -induced tofu. The GDL-induced tofu has a plain taste. Moreover, according to the various different types of tofu with different textural properties, the type of coagulant has the most influence on the taste properties of tofu. Tofu with better compact structure causes a more full taste. Tofu coagulated by  $\text{MgCl}_2$  fast is prone to have a porous structure, resulting in a rough taste. Sour taste is easily perceived in acidic whey-induced tofu because of the improper fermentation of tofu whey. The water content influenced by the WHC and pressing operation also influences the final taste of tofu. Lower water content of tofu (such as dried tofu or extra firm tofu) might give a residue-like taste compared with those with high content of water (such as silken tofu and soft tofu).

### Syneresis and Cooking Loss

Generally, tofu is sold in fresh form and is eaten after certain thermal treatment, such as boiling, frying, and stewing. Thus, the ability to maintain the taste and textural properties after a fixed selling or storage duration and the thermal treatment is a very important quality of tofu, which is usually expressed as syneresis and cooking loss, respectively.

Syneresis usually reflects the liquid loss of tofu after storage at 4 °C for 24 h (Armstrong et al., 1994). The ability to retain water in the network structure of tofu is closely related to the cross-linking degree of network structure and the content of polar nonprotein compounds. The salt-induced tofu often has lower syneresis compared with that of acid-induced tofu (Murekatete et al., 2014). More water is released when the hardness of tofu is enhanced due to the subsequent bonding among the proteins and coagulants via various interactions during the storage, resulting in the tofu with more dense and compacted texture and increased syneresis (Karim et al., 1999; Murekatete et al., 2014). Consequently, reduced weight and textural properties of tofu are present since the tofu is made. Thus, the way to enhance the water retention or reduce the syneresis of tofu before the reprocessing for eating is very important for the quality maintaining of tofu. For example, the GDL-induced tofu made from soy milk treated by two-step heating showed lower syneresis compared with the GDL-induced tofu made from soy milk treated by one-step heating (Liu et al., 2004; Wang et al., 2007), and the addition of carrageenan or  $\gamma$ -polyglutamate in appropriate concentrations during the coagulation of GDL-induced tofu significantly reduced its syneresis (Lee and Kuo, 2011; Karim et al., 1999).

Cooking loss is the weight loss of tofu after boiling for certain time (Chang et al., 2011). Water and aqueous soluble compounds are released from the tofu matrix under the function of heat and concentration gradients during cooking in water, reflecting the cooking loss of tofu. Cooking loss is also closely related to the type of tofu induced by different types of coagulants. Along with the exudation of water and aqueous soluble compounds during the boiling of tofu in water, the microstructure of tofu changes to impact on its textural properties under severe heating condition. Decreased weight and more tough texture of GDL-induced tofu are usually observed after certain retort cooking treatment (Nonaka et al., 1996). Therefore, MTGase or glucose in appropriate concentrations was tentatively added to the coagulation of GDL-induced packed tofu to reduce the weight loss and retain the textural properties of tofu after retort cooking (Kwan and Easa, 2003). This treatment can be used to modify the physicochemical properties of tofu dishes and retain the quality attributes of tofu under the thermal sterilization conditions.

### Nutritional Value

As one of the best food for supplying vegetable protein with higher amino acid score, tofu also contains considerable unsaturated lipids, phospholipids, polysaccharides, minerals, and phytochemicals (e.g., soybean isoflavones). Apart from the network structure constructed by soybean proteins, other nutrients, especially the water-soluble nutrients, are also entrapped in this network structure. Therefore, the nutritional value of tofu is also related to its structure. Tofu with higher protein content was obtained by the soy milk with higher solids content (Shih et al., 1997). Coagulant used in higher concentration made tofu with decreased protein content (Sun and Breene, 1991). More content of mineral elements is observed in the salt-induced tofu compared with tofu induced by acidic coagulants and enzymatic coagulants (Obatolu, 2008). Therefore, the formation of network structure of tofu influences the nutrient composition of tofu products.



## Conclusions

Based on the influence of various factors on the structure formation of tofu, three-dimensional network structure formed by the coagulation of aggregated proteins is the fundamental skeleton of tofu; other nonprotein components, including water, carbohydrates, and phospholipids, are randomly retained in the spaces among this network structure. Furthermore, other aqueous soluble components (i.e., water-soluble vitamins and metals) and lipid-soluble components (i.e., TAGs and carotenoids) are interacted with water and phospholipids, respectively, to be indirectly contained in this network structure. Consequently, a compact, uniform, and orderly microstructure of tofu is formed. Therefore, even though the factors influencing on formation of tofu texture are various and interaction effect among these factors exists, the expression of tofu texture is based on the three-dimensional network structure of soybean proteins. In other words, the three-dimensional network structure of soybean proteins is the basis for formation of tofu textural and sensory properties, and the nonprotein components spread over the network structure make these textural and sensory properties be more abundant, such as juiciness, smooth, soft, and refreshing. Understanding the close relationship between the structure of tofu and its physicochemical properties promotes the production of tofu products with more edible and nutritional qualities.

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## The Structure of Meat Analogs

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### Introduction

Meat is considered as a complete protein source as it contains all 9 of the essential amino acids that cannot be synthesized *de novo* by human beings and are thus must come from the diet alone. Furthermore, the functional attributes of proteins in meat such as gelation, emulsification and water holding capacity are unmatched to any other protein source (Xiong, 2005). In developing countries, consumption of meat per capita has rapidly increased in recent years, primarily due to rapid industrialization, urbanization, increasing population size and rise in income (Kumar et al., 2017). However, there are many issues associated with meat production in its present form and these are leading the search for suitable and sustainable alternatives.

Rearing of animals for meat production involves converting vegetable protein into animal protein, which is highly intensive process. Production of vegetable crops requires comparatively less water, land and energy per unit as compared to per unit production of meat and thus the transition of proteins can be considered inefficient (Steinfeld et al., 2006; Bruinsma, 2009).

Meat production is often associated with environmental strain due to emission of greenhouse gases and water pollution (streams and rivers) as well as rampant deforestation for the expansion of livestock farms that has all put additional pressure on natural resources (Steinfeld et al., 2006; Aiking, 2011).

From a dietary point of view, the consumption of meat delivers essential nutrients and energy but a direct relationship with the overconsumption of meat and the incidence of certain diseases has been established (Mehta et al., 2015). Larsson and Wolk (2006) suggested that the consumption of 120 g red meat/day or 30 g processed meat/day would significantly raise the risk of colorectal cancer in human beings. Certain people avoid the overconsumption (or consumption altogether) of specific types of meat due to high saturated fat and cholesterol content as well as various unrelated reasons such as religious views or animal welfare concerns. All these concerns have combined together to spur the debate on the sustainability of meat as a diet for humans. For years, the fabrication of protein rich foods using plant sources has been a research interest amongst food scientists; the major challenge being the simulation of texture, taste, colour, flavor and nutritive value equitable to meat. Commonly known as meat analogs, meat substitutes, meat alternatives or faux meat, these products have had a surge in popularity in recent years, though presently still only account for a marginal share of the total meat market (De Bakker and Dagevos, 2010).

### Defining Meat Analogs

A product that can closely approximate the characteristics and quality of meat can be termed a meat analog. They are primarily vegetable protein based products developed from pulses, fungi or cereal proteins. Though the term meat analog is frequently used for products based on spun protein filaments, it may also include various other products such as textured vegetable proteins. These can be seen as a good medium through which vegetable and unconventional proteins can be integrated into the diet (Kumar et al., 2017). The key drivers for establishment and growth of this sector is food safety concerns amongst consumers regarding animal products, increasing promotion of the vegetarian or vegan diet, demand for variety by consumers, health concerns surrounding the overconsumption of meat and the increased use of convenience foods (Sadler, 2004).

Some traditional foods are already meat analogues in their own right as they are used as a meat substitute in meals, especially in countries where meat may have been scarce or expensive in the past. Some examples of these types of meat analogues are Tofu and Tempeh, both produced from soy beans. Tofu is already extensively covered in this section (cross reference to tofu article).

Tempeh or tempe is a traditional fermented soybean food widely popular in South-east Asia. It is prepared by using *Rhizopus* strains and known to have superior nutritive qualities and regulation of body metabolism. It is commercially available in strip and cake form with denser texture and can be used in many of the culinary contexts as tofu. During fermentation process, various volatile compounds such as free amino acids, fatty acids, are generated by enzymatic digestion of substrates chiefly lipids and proteins, leading to development of characteristic flavor (Jeleń et al., 2013). Tempeh is less prone to lipid oxidation and has higher amount of isoflavones than other soy products. Dehulled soy seeds are cooked and cooled at room temperature and inoculated with spore suspension and kept for fermentation for 5 days at 37 °C. The fermented soy beans containing overgrowth of *Rhizopus* strains are appear similar to cake which is consumed by roasting, frying, etc.

## Key Ingredients in Meat Analogs

A variety of ingredients can be utilized for the formulation of meat analogs, though plant based derivatives are dominant. At present, the bulk of production is based on soybeans as they provide the cheapest and most common source of alternative proteins. However, algae and fungi have also been used for development of analogs and insect proteins are becoming increasingly likely in future applications. Typical meat analog ingredients and their functions can be found in [Table 1](#).

Soy proteins are known to have various functional and health benefits. Soy protein isolate is the most common soy derivative used in meat analogs along with textured soy protein and tofu ([Sadler, 2004](#); [Kumar et al., 2017](#)). Soy protein isolate consists of approximately 90% protein and is separated from the carbohydrate and fat fraction. Textured soy protein is produced by the extrusion of defatted soy flour, whereas tofu is manufactured from pressing soy curd. As far as the nutritional value is concerned, [Hoffman and Falvo \(2004\)](#) reported that on the PDCAAS (Protein Digestibility Corrected Amino Acid Score) scale, soy protein was comparable to animal protein with a score of 1.0, the highest possible rating. Soybeans are also a good source of calcium and linoleic acid. As soy protein is seen as a viable alternative to animal protein, much research has been conducted. [Ishizuka et al. \(1986\)](#) patented the development of meat analog through decreasing the pH of soy milk to 5.6–6.2, forming tofu; coagulating and dehydrating it to obtain a solid content of 35%–50% and shaping to simulate ground meat. [Lin et al. \(2000\)](#) developed a soy protein meat analog, which was extruded at high temperature and varying moisture content (60%–70%) and concluded that it was moisture that acted as an important factor in determining texture. Low moisture content resulted in higher die pressure for extrusion, a firmer texture and consequent lower protein solubility.

The use of fungi, particularly mushroom varieties for development of meat analogs has been proven popular due to their characteristic chewability ([Naylor et al., 2001](#)). The cell walls of the hyphae are a rich source of dietary fiber whereas; cell membranes are a source of polyunsaturated fat, which can in reducing reduce LDLs (low density lipoproteins) and increase beneficial HDLs (high density lipoproteins) in the body ([Rodger, 2001](#)). While developing analogs from fungi and to attain meat-like textures, the fungal biomass is generally mixed with flavoring agents and protein binders such as egg albumin (although this makes the product no longer solely plant based) which on heating form a gel and bind the product ([Denny et al., 2008](#)). The addition of egg white also increases the total protein content of the product. [Kumar et al. \(2010\)](#) reported a significant improvement in the physico-chemical properties and sensory attributes of meat analogs upon incorporation of egg white.

Flavor is improved by the incorporation of fungi, for e.g. *Fusarium graminearum* was employed for first time in 1984 the commercial development of meat analogs and it provides a meaty flavor due to the presence of sulfur containing amino acids ([Trinci, 1994](#); [Angold et al., 1989](#)). This is an advantage of using fungi compared to textured soy protein as the latter gives a beany flavor rather than a flavor associated with meat. [Kim et al. \(2011\)](#) used *Agaricus bisporus* mycelium to produce a meat analog with distinct umami flavor characteristics and greater textural scores for hardness, springiness and chewiness compared with soy based meat analogs.

A variety of texture modifiers such as carbohydrates and gums are frequently used to develop meat analogs. They can equally be used as fat substitutes for the development of low-fat products as on hydration, starches and maltodextrins form gels that can mimic the fat-like texture in meat analog products.

The use of gluten as a meat analog ingredient has been long established owing to its multi-functional properties of solubility, viscosity, swelling, nutritional qualities, etc. A meat analog resembling chicken breast was developed using wheat gluten in the 1980's by [Nguyen \(1988\)](#) and the formula and processing method were patented. The product included a dry powder blend consisting of 80% wheat gluten, 11% wheat flour and 9% vegetable oil that was mixed and sprayed with water. The prepared dough was

**Table 1** Typical meat analog ingredients and their purpose

Ingredient	Purpose	Usage level (%)
I. Water	Ingredient distribution, Emulsification, juiciness, low cost	50 to 80
II. Textured vegetable proteins: textured soy flour, textured soy concentrate, textured wheat gluten, textured protein combinations such as soy and wheat	Water binding, Texture and mouthfeel, Appearance, protein fortification/nutrition	10 to 25
III. Non-textured proteins: isolated soy proteins, functional soy concentrate, wheat gluten, egg whites, whey proteins	Source of insoluble fiber Water binding, emulsification Texture/mouthfeel Protein fortification/nutrition	4 to 20
IV. Flavors/spices	Flavor, savory, meaty, roasted, fatty Flavor enhancement (for example, salt) Mask cereal notes	3 to 10
V. Fat/oil	Texture/mouthfeel, Succulence	10 to 15
VI. Binding agents: wheat gluten, egg whites, gums and hydrocolloids, enzymes, starches	Texture/"bite," water binding, may contribute to fiber content, can determine production processing conditions	1–5
VII. Coloring agents: caramel colors, malt extracts, beet powder, Food drug and cosmetics (FD and C) colors	Appearance/eye appeal, Natural or artificial	0 to 0.5

Adopted from [Malav et al. \(2015\)](#).

heated by conventional or microwave means. The major advantage was that resultant product lacked the unpleasant flavor of soy based meat analogs. Further, Kumar et al. (2012) reported that the incorporation of wheat gluten in analog meat nuggets improved almost all sensory traits.

### Analogs Versus Real Meat: A Key Comparison

The aim of a meat analog is to make the consumer think that they are consuming meat in all sense of the meaning including mimicking structure, composition and organoleptic properties. However, meat has a complex structure, which is difficult to reproduce. Redman (2008) filed a patent for a meat analog containing 5%–40% protein with a characteristic cross sectional contraction resembling the skin of meat and imparting appearance of cooked meat. Similarly, Kumar et al. (2011) compared chicken nuggets with meat analog nuggets prepared by incorporating texturized soy protein, mushroom, wheat gluten, etc., for their various physico-chemical and sensory attributes. They reported higher sensory scores in chicken nuggets as compared to analogs however, the lipid profile was comparatively better in the latter due to less saturated fats. The scores for the textural properties of real meat were significantly higher than the meat analog in terms of hardness, chewiness and cohesiveness. From a food safety point of view, the microbiological profile of analogs is found to be comparatively better than meat products in many studies (Talabi et al., 1986). However, this is largely determined by the initial load as well as processing conditions adopted. Though, meat analogs are seen as a promising candidate for the replacement of meat in the future, for now a comparative assessment almost always gives better scores to real meat.

### Health Considerations of Meat Analogs

Havlik et al. (2010) determined total purine content in 39 commercially available meat analogs and analyzed them for their protein content. They reported that mycoprotein and soy protein based meat alternatives had significantly higher ( $p < 0.05$ ) purine content than wheat and egg white based products. Therefore, the latter has potential for use in meat analogs for people who need to follow low-purine diets. The high purine diet is associated with high uric acid in the blood and can cause causing crystal formation and accumulation around the joints leading to gout, a problem in populations with high meat consumption. Lousuebsakul-Matthews et al. (2014) conducted a prospective cohort study on meat analogs and legume intake in relation to incidence of hip fractures amongst Caucasian men and women and concluded that hip fracture incidence was inversely proportional to high intake of meat analogs due to high protein content containing lysine which promoted greater absorption of calcium through the intestine and increased bone mineral density. van Nielen et al. (2014) studied the effect of the replacement of meat proteins with soy on insulin resistance and blood lipid profile in post-menopausal women with abdominal obesity and found that replacement lead to the prevention of metabolic syndrome and had clear advantages regarding insulin sensitivity and total and LDL cholesterol.

### Consumer Acceptability of Meat Analogs

Any product can be a success but only if it is widely accepted by a range of consumers. Despite many positive features, the overall acceptability of most of meat analogs is lower than traditional meat products. As a remedial measure, the main emphasis lies on increasing the acceptance and recognition of these products as a partial or total replacement of meat and ensuring, acceptable sensory properties such as appearance, taste, juiciness, texture and even the ability to be cooked in a variety of methods. Common meat analogs available on the market have been listed in Table 2. Currently there is a Dutch research programme, Protein Foods, Environment, Technology and Society (PROFETAS) that is dedicated to pursuing options to increase the sustainability of food production and consumption worldwide ([www.profetas.nl](http://www.profetas.nl)). For what concerns protein, they are examining the difference between animal and vegetable protein production, “pigs v peas” and reporting on a range of issues from the prospect of pro-environmental protein consumption to the habits and motivation of young vegetarians, and groups who consume different quantities of meat in their diet in order to understand the most effective ways to move towards a future of reduced meat consumption (de Boer et al., 2017). As part of this research programme, Elzerman et al. (2011) examined the acceptance and appropriateness of meat substitutes in a meal context and concluded that appropriateness was majorly influenced by the appearance of the meat substitute-meal combination rather than flavor and texture. This provided a major insight into gaining wider acceptability of meat analogs by designing meal combinations for these products rather than serving them alone. Long-term acceptance of environmentally sustainable meat alternatives was studied by Hoek et al. (2013) and they found that initial positivity towards meat substitutes was significantly less than meat and boredom to eat the substitutes occurred more frequently. However, on repeated exposure, the likability tends to increase over time. Furthermore, they also concluded that meal context needs to be considered for such consumer studies. Hartmann and Siegrist (2017) revealed that the general consumer has low awareness about the detrimental effect on the environment as a result of protein production from animal sources. Also, they are least willing to shift dietary preference from meat to a sustainable alternative. Thus, more studies exploring the motivational aspects of food choices need to be conducted to give even more insight into what is required to enhance the acceptability of meat alternatives.

**Table 2** Common meat analogs available in market

Name of product	Introduction/first reported	Main ingredients/origin	Characteristics/remarks	References
Tofu	Japan	Pressed soy curd prepared from coagulated soy	Most widely recognized meat alternatives, bland taste, can impart flavor by smoking/marinating	Sadler, 2004
Tempeh	1851 in Indonesia	Fermented soy based cake	Controlled fermentation of soy	Malav et al., 2015
Tivall	1997	Soy based fibrous vegetable protein	Simulate meat muscle and provide a different eating texture to other soy formats	Sadler, 2004
Wheat Pro™	1992	Wheat protein	Texture resembling meat manufactured by Kerry Ingredients	Sadler, 2004
Arrum™	1995	Wheat and pea protein (1:1)	Resembles chunks of meat	Sadler, 2004

Adopted from Kumar et al. (2017).

## Conclusions

Ease of availability and sustainability of quality protein is a target for achieving nutritional security worldwide. Over reliance on animal based foods has resulted in un-sustainable farming practices and environmental problems. Thus, the search for suitable alternatives has zeroed in on meat analogs. They can provide a choice for health conscious, food source conscious and also economically poor consumers. In recent years, the development and popularization of meat alternatives has resulted in a greater choice of products on the market, which use a variety of different ingredients to mimic and/or replace meat altogether. Time will tell if meat analogs can truly compete with the popularity of meat and earn a substantial share of the food market.

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# Nanomaterials in Food: An Overview

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## Introduction

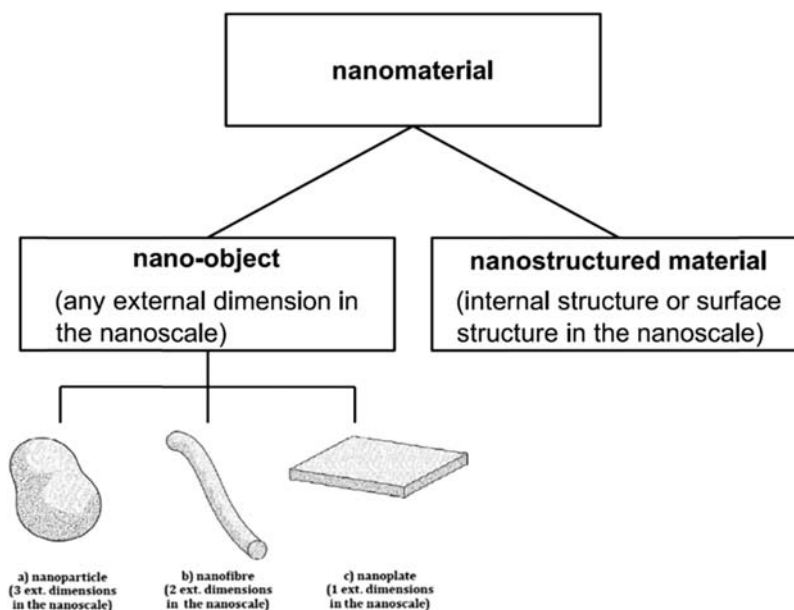
Nanomaterials (NM) utilization gain more and more importance in industrial applications, including food sector, as a route to provide new consumer benefits and added functionality. These applications are often linked to improved properties as bioavailability or active substance release (He and Hwang, 2016). As a prerequisite for the use of such technology, and above all philosophical and consumer acceptance considerations (that are linked to country considered, to socio-demographic factors and knowledge about this field (NanOpinion, 2014; Giles et al., 2015; Yue et al., 2015)), all aspects related to safety and risk linked to usage of any nanomaterials have to be specifically addressed (Cockburn et al., 2012; EFSA Scientific Committee, 2011).

Discussions about nanoparticles and nanomaterials in food and food packaging are continuously increasing in the public and is often associated with a perceived risk to health (as shown in recent reports Illuminato, 2014; Behar et al., 2013), risk that is not always substantiated by scientific evidence and often in contradiction with risk assessments performed by authorities (EFSA, 2016a; EFSA, 2018; EFSA Scientific Committee, 2011). For this reason, Szakal et al. recently highlighted that: “As food products using new nanotechnologies reach commercialization, there is a need to anticipate, to understand, and to manage both potentially positive and negative effects that might result from nanomaterial consumption.” (Szakal et al., 2014).

In order to increase clarity and alignment between all stakeholders, a clear definition about what is a NM has to be accepted.

The International Organization for Standardization (ISO) defines nanomaterials as materials with any external dimension in the nanoscale (approximately from 1 nm to 100 nm) or having internal structure or surface structure in the nanoscale, which includes nano-objects (discrete piece of material with one, two or three external dimensions in the nanoscale, see Fig. 1) and nanostructured materials (material having internal nanostructure (composition of inter-related constituent parts in which one or more of those parts is a nanoscale region) or surface nanostructure) (ISO, 2015).

Strictly speaking, this definition would include most materials, natural and manmade. A classification of these materials is therefore generally made, by defining the origin of the material, either natural, engineered, manufactured or incidental. Regarding these classes, ISO defines engineered nanomaterial as being designed for specific purpose or function, manufactured nanomaterial



**Figure 1** Nanomaterial Framework. Adapted from ISO 2015. ISO/TS. ISO/TS 80004-1:2015, Nanotechnologies – Vocabulary – Part 1: Core terms. 3. 2015. <https://www.iso.org/obp/ui/#iso:std:iso:ts:80004:-1:ed-2:v1:en>; ISO 2015. ISO/TS. ISO/TS 80004-2:2015, Nanotechnologies – Vocabulary – Part 2: Nano-objects. 10. 2015. <https://www.iso.org/obp/ui/#iso:std:iso:ts:80004:-2:ed-1:v1:en>.



as being intentionally produced to have selected properties or composition and incidental nanomaterial as those generated as an unintentional by-product of a process.

While engineered nanomaterials are currently mainly used in food contact material applications, the other classes can be found in food. Examples of the different classes and their origin and use are given further in this paper. Nanomaterials generally used in food, feed and packaging sector can be roughly divided in three main categories: inorganic NM (e.g. metals, metals oxides), organic NM (e.g. micelles, liposomes, emulsions, nano-encapsulates ...) and combined inorganic/organic NM (e.g. modified clays) (Peters et al., 2016).

In addition to the ISO definition, some regulatory bodies refer to specific definitions, often with a limited scope. In the European Union, several definitions coexist in different legislative texts. There is however a desire to harmonize these definitions by aligning them on the European commission recommendation on the definition of nanomaterials which is currently under revision (European Commission, 2011a, 2013). In summary, this definition currently states that nanomaterial means a material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm. The scope of the definition is therefore limited to materials composed of a majority of nano-objects, either in isolation, or attached together, whatever the strength of the link between these particles. Contrarily to the ISO definition, this approach limits the definition only to solid particles, i.e. minute piece of matter with defined physical boundaries (European Commission, 2011a). The limitation of the current definition to solid particles is underlined in a working document of the European Commission (European Commission, 2012).

While natural and incidental nanomaterials are present in foodstuffs and packaging as a consequence of the natural structure of certain ingredients and components or as by-products of certain processes, manufactured and engineered nanomaterials are added to the food for their specific properties (obtained either through the design of the materials or as a consequence of the production process used) or to impart specific properties to the food or packaging material.

This chapter gives an overview of such applications and occurrences and presents the analytical challenges and opportunities to assess the properties of nanomaterials, their presence in food and their potential migration from food contact materials.

## Nanomaterials in Food

Consumers' food can potentially contain NM from various sources:

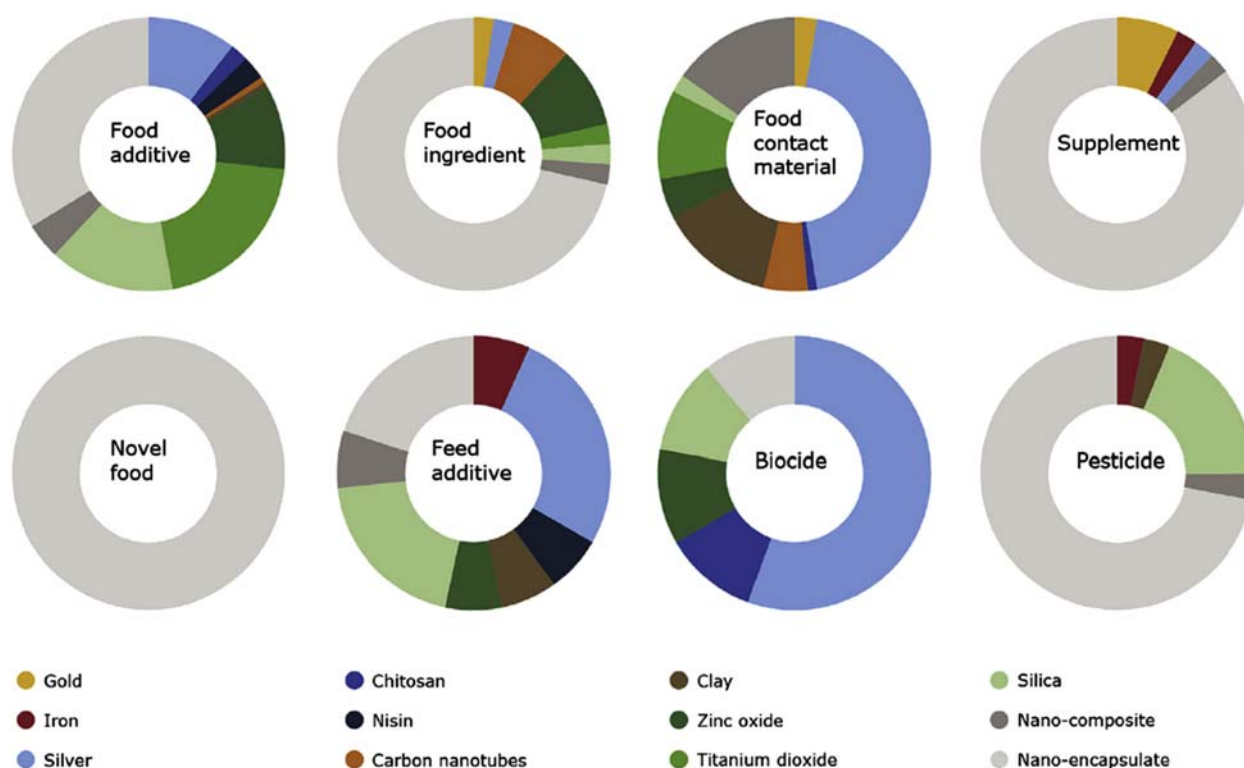
1. Ingredients or components naturally contain nanomaterials as self-assembling nano-fibers and micelles (Rogers, 2016) or NM formed during cooking process which is mostly the case for carbon dots nanomaterials (Li et al., 2017; Mandani et al., 2017; Palashudin et al., 2012).
2. Ingredients or a fraction of ingredients in the nanoform used in the finished product.

This case is the most common case. A distinction is to be made between materials engineered to be under the "nano" form (engineered nanomaterials) and the ones that are "nano" due to the formation process (manufactured nanomaterials).

When NM are voluntary engineered as such, it is generally to benefit from specific attributes: better physico-chemical properties of material (agglomeration state, surface functionality, surface charge ...), better absorption of active ingredients for consumers, enhanced color or flavor, better preservation properties. This is for example the case of novel ingredients that are developed under the nanoform to deliver specific active ingredients thanks to nano-encapsulation (He and Hwang, 2016).

At the opposite, some well-known ingredients are under the nano form due to the production process (manufactured nanomaterials). As an example the approved additive E551 (silicon dioxide, SiO<sub>2</sub>) which has been extensively used in a variety of products for many decades is nanostructured and composed of nano-sized primary particles. In a similar way ultrafine titanium dioxide, TiO<sub>2</sub>, is generated by a precipitation process that generates particles in the nanorange. The size of these particles induce a specific interaction with light which make ultrafine TiO<sub>2</sub> a good barrier to UV light, while maintaining a high level of transparency in the visible range. These optical properties make ultrafine TiO<sub>2</sub> a perfect ingredient to block harmful sunlight wavelength in the UV range and is therefore used in cosmetics such as sunscreens as a physical light filter. In parallel, TiO<sub>2</sub> is also authorized in food as a white coloring agent (E171), application for which ultrafine TiO<sub>2</sub> is not suited. E171 production process forms primary particles in the range 200–400 nm where the coloring property is optimal. Even though the average particle size increases, a small fraction of nanoparticles remains in the final product. In this context, E171 is not a nanomaterial according to European commission recommendation on the definition of nanomaterials as confirmed by EFSA evaluation (EFSA, 2016a) but contains nanoparticles according to ISO definition. For this reason this additive is included in studies on the usage of NM in food as shown in Fig. 2.

As all ingredients, nanomaterials can be present in the food to fulfil a specific function, e.g. anticaking or coloring but also be carried over by some components without having a function in the final product. An example of this is the use of SiO<sub>2</sub> as carrier for some flavors to which it imparts anticaking and free flow properties during their fabrication but has no function in the final product.



**Figure 2** Most frequently used types of NMs in agriculture, feed and food sector. From Peters, R.J.B., Bouwmeester, H., Gottardo, S. et al., 2016. Nanomaterials for products and application in agriculture, feed and food. *Trends Food Sci. Technol.* 54, 155–164.

3. Finally, NM can be present as a result of a contamination, at any step of the value chain, from agriculture where the use of nanomaterial is increasing to improve the efficiency of phytosanitary products (Fraceto et al., 2016), as micro- or nano-sized plastic residues, generated by the slow degradation of littered plastic (EFSA, 2016b), or as migration from packaging and other Food Contact Material (FCM) (Wyser et al., 2016). The specific point of nanomaterials in packaging is developed in next chapter.

Fig. 2, extracted from a review on Nanoparticles for products and applications in agriculture, feed and food from Peters et al., presents the most frequently used types of NMs in agriculture, feed and food sector (Peters et al., 2016):

## Nanomaterials in Packaging

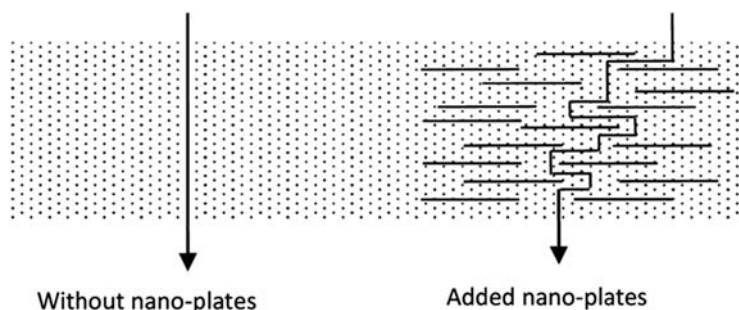
Compared to food industry, the packaging industry has been an early adopter of nanotechnology and nanomaterials, as potential routes to:

- improved properties related to food preservation such as barrier to gasses and vapor, light, improved mechanical properties, active packaging,
- new consumer functionality, such as intelligent and connected packaging.

While some applications of nano-enabled properties are already on the market, some of which since decades, the development of solutions for new functionalities is still ongoing.

An example of nano-enabled packaging are materials coated with thin layer of metals (mainly aluminum) or metal oxides, deposited under vacuum to decrease gas and vapor uptake into the packaging.

To achieve the same goal, engineered nanomaterials, such as exfoliated clay particles or more recently graphene or graphene oxide flakes are added into polymer matrices. Both of these materials are in the form of nanoplates, which are, due to their nature, efficient barrier to the diffusion of dissolved compounds such as oxygen or water molecules. By achieving a good distribution and orientation of the platelets, a tortuous path is created for the migrating species, increasing drastically the diffusing distance, and hence reducing the permeability (see Fig. 3).



**Figure 3** Addition of nanoplates increases tortuosity and decreases permeability. From Wyser, Y., Adams, M., Avella, M., Carlander, D., Garcia, L., Pieper, G., Rennen, M., Schuermans, J., Weiss, J., 2016. Outlook and challenges of nanotechnologies for food packaging. *Pack. Technol. Sci.*, 29, 615–648.

These developments allow the extended use of light weight materials in rigid and flexible packaging, e.g. removing 99% of the aluminum in high barrier metallized packaging materials compared to classical alufoil or replace glass bottles in the range of 300–500 g per liter by coated plastic bottles at least 10 times lighter.

With the recent developments in active and intelligent packaging, the use of nanomaterials in packaging applications will definitely rise (Wyser et al., 2016). A common example for this is the use of silver nanoparticles which are known to impart antimicrobial properties to packaging materials (Mihaly Cozmata et al., 2015). Printed electronics, which will probably be an enabler of intelligent and connected packages, will most probably be based on nanomaterials. However, as mentioned in the next section, their use will depend on their authorization by regulatory bodies, which, together with the low level of public acceptance mentioned previously, might be the main barrier to their implementation in real life applications.

In order to be authorized, the use of nanomaterials in FCM has to go through a risk assessment, i.e. the evaluation of the hazard of an exposure to a specific nanomaterial combined with the expected exposure of the consumer to this material. In this sense, most authorization of nanomaterials in food contact materials have been based on the conclusion that the exposure to them would be negligible. There is indeed increasing evidence that nanomaterials embedded in polymer matrices are not released to the food as nanomaterials (Störmer et al., 2017; Wyser et al., 2016). In some cases, migration through dissolution of the material can be observed. In such cases however, the safety of the substance itself is considered for risk analysis, and not that of the nanoparticles. One exception to the absence of particulate migration, are those active applications where the release of the particles is desired in order to create the functionality, as in the case of nanosilver in anti-microbial materials (Echegoyen and Nerin, 2013).

## Legislation and Analytics

The most advanced part of the world regarding legislation about nanomaterials in food is Europe.

EU regulation 2011/1169 (European Parliament and Council, 2011) on food information to consumers and EU regulation 2015/2283 (European Parliament and Council, 2015) on Novel Food both refer to nanomaterials regarding their labelling and classification as novel foods, respectively. The definition contained therein is due for revision which is expected to be based on the European commission's recommendation on the definition of nanomaterial (European Commission, 2011a).

Although the definition recommended by the European Commission is mainly based on the size of the material, it also mentions that: "material should be considered as falling under the definition where the specific surface area by volume of the material is greater than  $60 \text{ m}^2/\text{cm}^3$ ."

Several other countries (Turkey, Morocco, Switzerland, Ukraine) are aligning their regulation with the EU approach.

For packaging, only nanomaterials (according to European recommendation) authorized and specifically mentioned in Annex I of Regulation 10/2011 can be used in plastic packaging (European Commission, 2011b). In its first release, the later contained three authorized nanomaterials, namely  $\text{SiO}_2$ , carbon black and titanium nitride. These were included in the list of authorized substances as their initial authorization was based on a risk assessment, which evaluated material that were already specified as nanomaterials. In terms of functionality,  $\text{SiO}_2$  is mainly used in flexible packaging as anti-blocking agent (upon unwinding), carbon black as a pigment and titanium nitride as a heat absorption enhancer in PET, to improve the re-heating of preforms prior to the blowing process. The annex has been amended numerous times, including with the addition of additional nanomaterials such as montmorillonite clays and zinc oxide.

European regulation 10/2011, as well as regulation 450/2009 on active and intelligent packaging both specify that the concept of functional barrier (which allows the use of some substances that are separated from the food by a material layer that would theoretically not allow its migration to the food) cannot be applied to nanomaterials (European Commission, 2009, 2011b).

Regulatory definitions imply that analytical methodologies applied to identify or detect nanomaterials must be deployed and able to give information about the size (or specific surface area by volume) but also about the number of particles for each size class (number based distribution).

In the last years, most analytical efforts were put on solid nanomaterials (generally inorganic, if we exclude carbon based NMs).

This topic was reviewed in an important report dealing with “Requirements on measurements for the implementation of the European Commission definition of the term nanomaterial” that has been published by the European Joint Research Center (JRC) in 2012 (Linsinger et al., 2012).

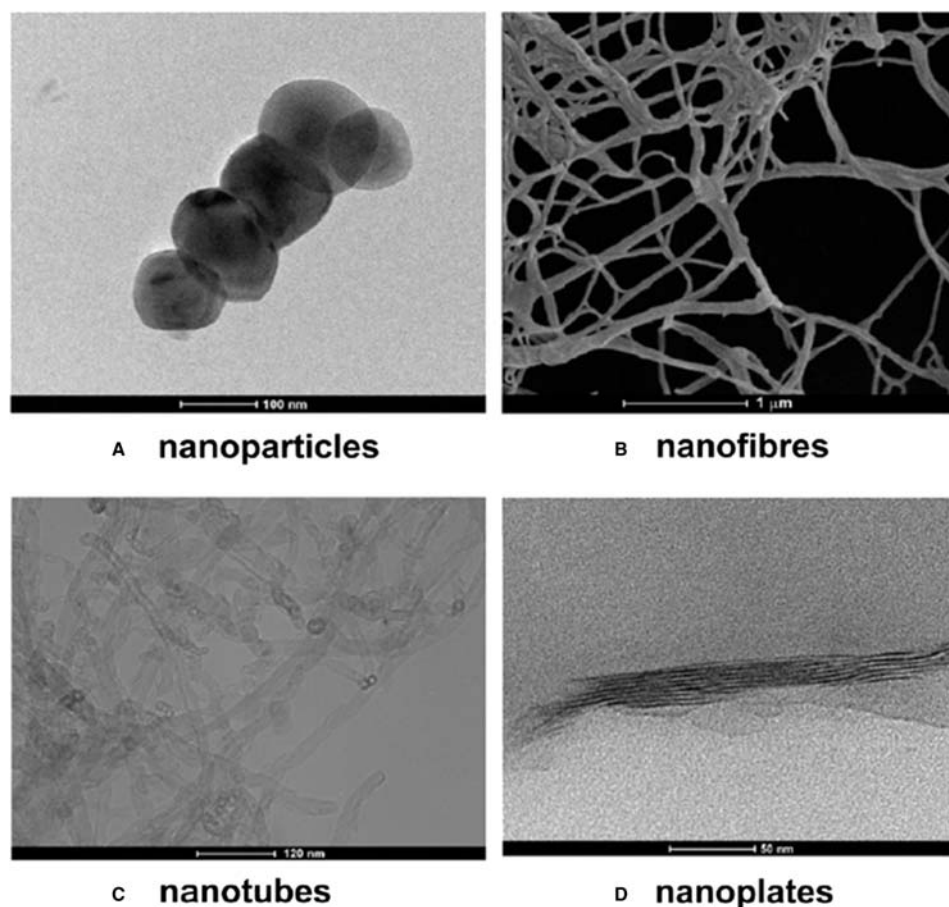
Among all existing analytical methodologies adapted for Nanomaterials analysis, some techniques appear more adapted, mature and promising:

- Microscopy and imaging techniques are considered as the “gold standards” for identification and characterization of nanomaterials. Among them, the most pertinent are Electron Microscopy (EM) based techniques (Scanning Electron Microscopy - SEM and Transmission Electron Microscopy – TEM). Nanoprobes technics as Atomic Force Microscopy (AFM) can also be used. Electron microscopy imaging technics are based on the interaction, in vacuum, of a high energy and accelerated electron beam with the sample. The magnified image is obtained by classical optical physic principles. In the TEM, the image is obtained by the transmission, or not, on the beam through the sample. In the SEM, the focused electron beam is scanning the sample surface and the scattered signal is recorded to create the image. The size range of the imaged sample lies between few hundred micrometers to subnanometric depending on the microscope performances. EM is today the only technique able to cover the full range (1–100 nm) and to give visual aspects of nanomaterials as shown in Fig. 4.

EM will also easily provide a visual evaluation of the aggregation/agglomeration status of nanomaterials (see Fig. 5).

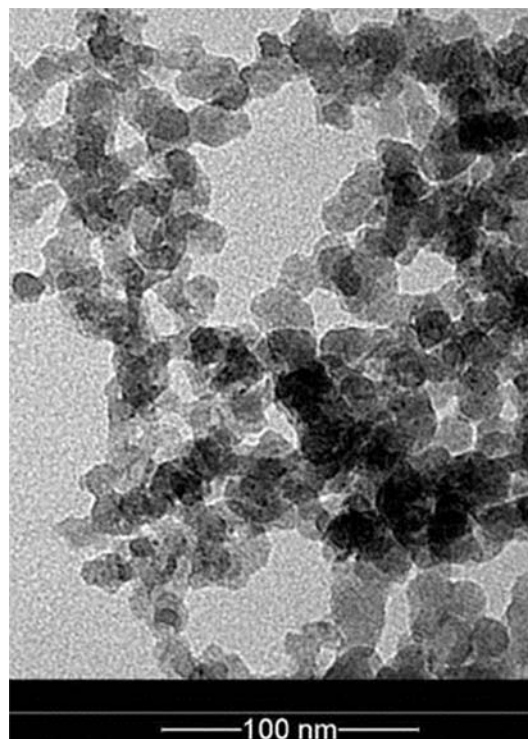
As the imaging is done under high vacuum conditions, samples have to be dried, dehydrated or cryoprepared. It has to be noted that some samples are beam sensitive and therefore can be damaged during the imaging (organic particles or organic-coated particles).

Finally, Energy-dispersive X-ray spectroscopy (EDX) can be associated to either SEM or TEM in order to give information about elemental composition and thus to distinguish between particles of different chemical composition.



**Figure 4** Nanomaterials images obtained from EM. From Wyser, Y., Adams, M., Avella, M., Carlander, D., Garcia, L., Pieper, G., Rennen, M., Schuermans, J., Weiss, J., 2016. Outlook and challenges of nanotechnologies for food packaging. Pack. Technol. Sci. 29, 615–648.





**Figure 5** Example of aggregated nanoparticles.

- **Light Scattering:** among light scattering techniques (including Static or Multi-Angle Laser Light Scattering - MALLS - and dynamic light scattering), only Dynamic Light Scattering (DLS) also known as Photon Correlation Spectroscopy (PCS) or Quasi-Elastic Light Scattering (QELS) has a sufficient dynamic range to cover almost the full size spectrum of nanomaterials definition. In ideal conditions, MALLS can reach tens of nanometers whereas DLS's resolution is nanometric ([Brar and Verma, 2011](#)).

The principle of DLS is based on the evaluation of the short timescales intensity fluctuation of scattered light of particles, nanomaterials or molecules in solution when illuminated with a laser. This fluctuation is due to the Brownian motion of particles caused by thermally induced collision between suspended particles and solvent molecules. These fluctuations are dependent of the size of the particles.

DLS measures particles in suspension in a liquid and gives a light scattering intensity weighted average value which means that results can be strongly affected by the presence of aggregates/agglomerates or by poly-modal distributions and does not give information about primary particles.

For these reasons, DLS can be a powerful tool used for known relatively monodisperse raw materials but can also be associated to nanomaterial separation/fractionation techniques such as Field Flow Fractionation (FFF) for difficult samples in order to introduce a first fractionation step before DLS measurement.

- **Brunauer-Emmet-Teller (BET):** BET method is based on the measurement of gas adsorption (usually  $N_2$  is used as adsorbate) on material surfaces at defined temperature and pressure. The number of adsorbed molecules or atoms on the surface of the particles provides the information of the absolute surface area of the material. Moreover, the knowledge of the particle density allows the determination of the Volume-Specific Surface Area (VSSA) which is used in the recommendation for definition with a threshold at  $60 \text{ m}^2/\text{cm}^3$  ([Wohlleben et al., 2017](#)).

BET measures particles as dry powders and this measurement is affected by the global available surface of the particles meaning that an open porosity in the material will affect the measurement. Some methods (called t-plot methods) appear to be able to separate the surface contributions of the outer particle surface and of the open porosity surface ([Wohlleben et al., 2017](#)).

- **ICP-MS based methods**
  - **Single-particle ICP-MS (sp ICP-MS):** ICP-MS is an elemental detector which allows to quantify elements with very low detection limits. When liquid samples containing a low concentration of nanomaterials/nanoparticles in suspension are introduced at a low flow rate, the assumption that each ICP-MS pulse represents a single particle can be made. Thus, each pulse can be linked to the size and mass fraction of the single nanoparticle. The frequency can thus be linked to the number concentration. This methodology is so called single-particle ICP-MS ([Sannac et al., 2013](#); [Laborda et al., 2014](#)). The size range accessible is 10 nm to micrometers (depending on nanomaterial nature) but even if this technique allows to distinguish polymodal distributions, it is currently limited to nanomaterials with known elemental composition and geometry and to

elements not suffering from current classical ICP-MS limitations and interferences (Laborda et al., 2014; Pace et al., 2011; Loeschner et al., 2018).

- Hyphenation of fractionation techniques with ICP-MS and other detectors: very often, ICP-MS is only used as elemental detector and is coupled with fractionation techniques such as Field Flow Fractionation (FFF) based technologies and other detectors such as Static Light Scattering (SLS) and/or DLS (Pornwilard and Siripinyanond, 2014; Dubascoux et al., 2010). FFF based techniques are separation techniques allowing the fractionation of nanomaterial according to their sizes. The main developed FFF technique is nowadays Asymmetrical Flow Field Flow Fractionation (AF4). The association of AF4 with SLS/DLS and ICP-MS allows to introduce a first fractionation step before DLS measurement and thus to decrease polydispersity while ICP-MS gives information about the chemical composition of fractionated nanomaterials. This kind of coupling is increasingly used due to the sensitivity of ICP-MS and the combination of information given by associated detectors (size, composition). Such coupling and off line sp-ICP-MS analysis of FFF fractions have been notably tested by Loeschner et al. on food samples for the analysis of Ag nanoparticles in chicken meat (Loeschner et al., 2013). In this study, where enzymatic digestion was used as sample preparation, the development of preparation/extraction step appears critical.

Numerous European initiative have been launched in order to address analytical challenges (e.g. European NANOLYSE project from 2010 to 2012 [www.nanolyse.eu](http://www.nanolyse.eu), European NANODEFINE project from 2013 to 2017 [www.nanodefine.eu](http://www.nanodefine.eu)) and have evaluated main techniques and development/optimization for the implementation of the EC Recommendation.

Even if, for pure powdered substances, some methods such as EM, DLS and BET seem to be well fitted and could be applied without too much obstacles, numerous studies highlight the difficulty to evaluate nanomaterials in complex matrices such as food products, due to the difficulty residing in the sample preparation:

- Stamm et al. underline the fact that: “nanomaterials are likely to form part of complex mixtures which may naturally contain particles at the nanoscale (e.g. liposomes, micelles, colloids, etc.), thus making it much more difficult to discriminate intentionally added nanoparticles from natural nanoscale structures (which may also include large organic molecules). Therefore the development of analytical methods, which can be applied routinely by enforcement bodies, is particularly challenging.” (Stamm et al., 2012).
- A review from Calzolari et al. on measurement of nanoparticles size distribution in food and consumer products (Calzolari et al., 2012) explains that: “One recurring theme in the available literature is the need for extensive, carefully executed and documented sample preparation” and “At the moment there is no single technique that can by itself provide a robust analytical method, especially considering the need to measure the number size distribution of nanoparticles introduced by the definition of nanomaterials. The most likely solution to this problem will be to use combinations of instruments, each with a different physical principle of operation, and in this way complement the weaknesses of each instrument with the strengths of another”

It's worth stressing out that, in finished products, since 5–10 years, strong efforts of the analytical community have been put to develop knowledge and to go beyond analytical issues. Some validated work (notably in the frame of European NANOLYSE project) on food matrices have already been conducted for specific combination of nanomaterial/matrix (e.g. SiO<sub>2</sub> in tomato soup (Wagner et al., 2015), Ag nanoparticles in chicken meat (Loeschner et al., 2013), silica nanoparticles in coffee creamer (Heroult et al., 2014) or silicon dioxide (SiO<sub>2</sub>) and titanium dioxide (TiO<sub>2</sub>) nanostructures in commercial dietary supplements (Lim et al., 2015)) but here again sample preparation have to be adapted for each combination.

## Conclusion

While nanotechnology and nanomaterials are generally recognized to be a route to added consumer benefits and functionality for foods and food packaging, their implementation is currently limited by remaining uncertainties on the consequences of their use in such applications.

While nanomaterials manufactured since decades and declared as safe by food safety authorities can be found in current food products, engineered nanomaterial have for now mainly found their way in food packaging applications, in cases where the absence of exposure has been demonstrated.

Further work on all aspects of the technology, i.e. safety assessment, legislative framework, analytical evaluation and a clear identification of consumer benefits, will be needed for a wider introduction of engineered nanomaterials in food related applications.

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# Delivery of Epigallocatechin-3-Gallate by Bovine Alpha-Lactalbumin Based on Their Non-covalent Interactions

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## Glossary

**Complexation** The combination of individual atom groups, ions or molecules to create one large ion or molecule such as the formation of complexes of proteins and small molecules (ligands).

**Fluorophore** A chemical compound with the ability to absorb light at a particular wavelength (excitation wavelength) and then emit it at a longer wavelength (emission wavelength).

**Fluorescence quenching** A process that decreases the intensity of the fluorescence emission of a given light-emitting substance. A variety of processes can result in quenching, such as excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching.

**Molecular docking** A computational method for bioinformatic modelling to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure, including investigations on ligand conformation, position and orientation within binding sites, and assessment of binding affinity.

**Non-covalent interactions** Different from covalent bonds with pairs of electrons shared by atoms, involve more dispersed variations of electromagnetic interactions between molecules or within a molecule, such as electrostatic interactions, van der Waals forces, hydrophobic interactions and hydrogen bonds.

## Nomenclature

$\text{kJ mol}^{-1}$  Binding energy

$K_a$  Binding constant

$K_{sv}$  Stern–Volmer quenching constant

$n$  Number of binding sites

$fa$  Fraction of fluorophore accessible to solvent

$K_Q$  Quenching constant

## Introduction

Dietary polyphenols exert many health promoting activities. However, their bioactive role in the organism is limited by their poor bioavailability. Epigallocatechin-3-gallate (EGCG) is the well-known representative of the bioactive diet polyphenols of poor bioavailability.

Food macromolecule-based nanoparticles have been fabricated using reassembled proteins, crosslinked polysaccharides, protein–polysaccharide conjugates (complexes), as well as emulsified lipids via safe procedures that could be applied to food processing (Hu et al., 2017; Shpigelman et al., 2012).

Milk proteins are natural vehicles for bioactives. Many of their structural and physicochemical properties facilitate their functionality in delivery systems. These properties include binding to ions and small molecules, excellent surface and self-assembly properties; superb gelation properties; pH-responsive gel swelling behavior (useful for programmable release); interactions with other macromolecules to form complexes and conjugates with synergistic combinations of properties; various shielding capabilities, essential for protecting sensitive payload; biocompatibility and biodegradability, enabling the control of bioactive bioaccessibility and promotion of its bioavailability (Livney, 2010).

## $\alpha$ -Lactalbumin

$\alpha$ -Lactalbumin (ALA) is one of the most important milk proteins, making up 20%–25% of the whey proteins and about 3.4% of total milk proteins. It has important biological functions in mammary secretory cells. ALA is a single chain protein with molecular mass of 14.2 kDa and structure homologous to the lysozyme family. It is a two-domain protein, with a larger  $\alpha$ -domain comprising three major  $\alpha$ -helices (residues 5–11, 23–24 and 86–98) and two short  $3_{10}$  helices (residues 18–20 and 115–118), and a smaller  $\beta$ -domain with a three-stranded antiparallel  $\beta$ -sheet (residues 41–44, 47–50 and 55–56), one short  $3_{10}$  helix (residues 77–80) and loops (Chrysina et al., 2000). There is a high-affinity  $\text{Ca}^{2+}$ -binding site in the loop connecting the two domains (Fig. 1).

ALA in its apo-form has the ability to interact with hydrophobic substances such as retinol (Livney, 2010), vitamin D3 (Delavari et al., 2015), hydrophobic peptides, model lipid membranes, and fatty acids (Barbana et al., 2006; Kehoe and Brodtkorb, 2014). The bioactive complex between ALA and oleic acid is known as BAMLET/HAMLET (Bovine/Human ALA made lethal to tumors) and has been shown to be cytotoxic to a range of cancer cell lines (Mok et al., 2007). Binding of the anthocyanins in the extract from grapes by whey protein isolate presumably involves ALA as a tight binder to malvidin 3-glucoside (Stănciuc et al., 2017). ALA based nanoparticles or nanotubes could find applications in foods and pharmaceuticals for delivery of bioactive substances (Kamau et al., 2010).

## $\beta$ -lactoglobulin

Bovine  $\beta$ -lactoglobulin (BLG), the major whey protein of cow's milk, comprises about 50% of total whey proteins and 10% of whole cow's milk proteins (Le Maux et al., 2014). BLG belongs to lipocalin protein family, which members have a widely diverse series of functions, including ligand binding function. It is assumed that BLG's ability to bind and transport lipophilic compounds is a physiological reason for its high abundance in milk (Kontopidis et al., 2004). BLG has at least two ligand binding sites, which in previous studies have been shown to simultaneously bind different ligands, such as retinol, fatty acids, cholesterol and phenol compounds, such as catechins and phenolic acids (Teng et al., 2015; Kanakis et al., 2011; Jia et al., 2017). Because of its compact structure, BLG is resistant to proteolysis, especially by pepsin. Therefore, ALA is more digestible than BLG in the gastric phase of protein digestion (Fu et al., 2002).

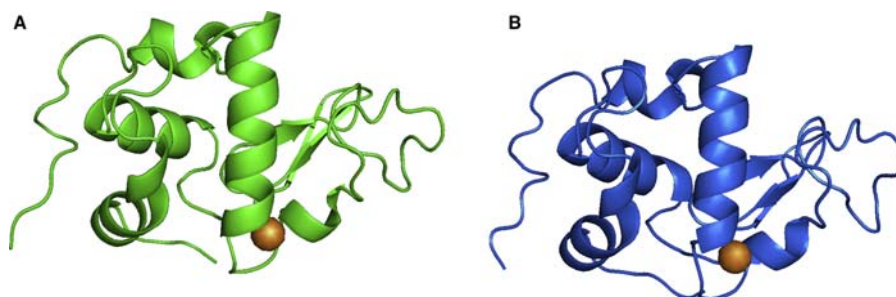
## EGCG

Epigallocatechin-3-gallate is the major and the most biologically active catechin in green tea (Kim et al., 2014), and many of the biological properties of the tea have been attributed to this compound. EGCG can be easily oxidized in aqueous environments, especially at neutral and basic pH (Shpigelman et al., 2012), and EGCG bioavailability and stability can be improved by complexation with food proteins, i.e. heat-treated BLG nanoparticles (Li et al., 2012). Among tea catechins, EGCG has the highest binding affinity to BLG due to galloyl functional group with multiple peptide binding sites, able to form hydrophobic interactions and hydrogen bonds with the proteins (Kanakis et al., 2011). EGCG had stronger binding affinity toward BLG than chlorogenic acid and ferulic acid (Jia et al., 2017).

## Bioavailability

The human gastrointestinal digestive tract is the major place where the food grade macromolecule nanoparticles exert their effects on improving the bioavailability of dietary polyphenols, via enhancing their solubility, preventing their degradation in the intestinal environment, elevating the permeation in the small intestine, and even increasing their contents in the bloodstream.

The stability, structural features and behaviors of nanocarriers in the gastrointestinal tract environment and the effects of nanoencapsulation on the metabolism of polyphenols warrant more focused attention (Hu et al., 2017; Shpigelman et al., 2012).



**Figure 1** Model of native ALA (PDB ID 1F6S) and recombinant ALA (PDB ID 1HFZ) with denoted position of  $\text{Ca}^{2+}$  ions (orange).

Polyphenols are classified into three categories based on their solubility: (1) high solubility but poor cell membrane permeability; (2) low solubility and poor cell membrane permeability; and (3) low solubility but high cell membrane permeability. EGCG belongs to category 1. Therefore, in order to improve bioavailability of EGCG, an attempt should be made to improve membrane permeability (Hu et al., 2017).

Polyphenols from foods and in combinations may have very different bioaccessibilities. Addition of bovine milk, soy milk, rice milk, ascorbic acid, or citrus juice(s) could increase the bioaccessibility of green tea catechins, partially due to the stabilization and protection of polyphenols from auto-oxidation at an alkaline pH (Green et al., 2007). EGCG quickly degrades in the intestinal juice (neutral or alkaline environment), which is mainly attributed to the auto-oxidation of EGCG, forming derived homodimers (Yoshino et al., 1999). Moreover, polyphenols bound with proteins can also be available for the absorption in the small intestine.

Food proteins are abundant and renewable raw materials for developing nanocarriers to deliver drug or nutraceuticals, which is mainly attributed to their exceptional binding capacity of various drugs or nutraceuticals. In addition, food proteins are biodegradable and nonantigenic and have high nutritional value. Protein nanoparticles can be easily prepared and such a production can be scaled up for commercial manufacturing.

### Binding Characteristics of ALA as a Delivery Vehicle for EGCG and Other Polyphenols

Milk proteins can act as delivery vehicles for bioactive compounds, including polyphenols, through noncovalent protein-polyphenol interactions. There are numerous reports dealing with interactions of phenolic compounds with whole milk proteins, whey proteins, as well as individual milk proteins (caseins, bovine serum albumin and BLG) (Ozdal et al., 2013), whilst only several studies described the interactions of phenolics with ALA.

Interactions of ALA with phenolics were investigated including phenolic acids commonly found in food (Zhang et al., 2014), procyanidins (Prigent et al., 2009), genistein and kaempferol (Mohammadi and Moeeni, 2015a), resveratrol and curcumin (Hemar et al., 2011; Mohammadi and Moeeni, 2015b). EGCG binds covalently to ALA (Wang et al., 2014b) and also forms stable noncovalent complexes (Al-Hanish et al., 2016).

Table 1 compares the binding parameters obtained for ALA-EGCG binding and the parameters obtained for binding of other polyphenols to ALA using the method of fluorophore quenching. Mathematical modelling is often applied to estimate binding affinity, and a recent study on the non-covalent interactions of EGCG and ALA estimated the binding affinity using four mathematical models (Stern–Volmer, Lehrer, Double logarithm, Langmoir Isotherm) (Al-Hanish et al., 2016).

Two different ALA crystal structures, native and recombinant, were used to probe the binding site for EGCG. Results of the docking analyses, based on the predicted binding energies and the nature of the putative interactions, indicated the existence of one high-affinity EGCG binding site situated in the cleft between  $\alpha$ -helical and  $\beta$ -sheet-rich domains (Fig. 2) (Al-Hanish et al., 2016). For both studied ALA crystal structures, docking of EGCG to this binding pocket produced a structure with the highest binding energy (Fig. 2A and B). Binding energies estimated by molecular docking simulation were 28.4 kJ mol<sup>-1</sup> for native ALA and 30.1 kJ mol<sup>-1</sup> for recombinant ALA. Bonding of EGCG to this high-affinity binding site is dominated mainly by hydrogen bonding and hydrophobic interactions (Al-Hanish et al., 2016).

Only two molecular docking studies on the binding of polyphenol compounds to ALA are reported, and both (Mohammadi and Moeeni, 2015a) have demonstrated that resveratrol, curcumin, genistein and kaempferol would bind to different regions of ALA. EGCG-binding site is close to, but not the same as, the resveratrol-binding site. This implies that there is no specific polyphenol-binding site in ALA.

When comparing the EGCG binding site on ALA with the EGCG binding site on structurally similar protein, lysozyme (Ghosh et al., 2008), one may find that these binding sites are in the same region. Aligned structures of ALA (PDB 1F6S) with EGCG bound to high-affinity binding site and lysozyme (6LYZ) are presented in Fig. 3. It can be seen that EGCG binds to ALA in the same region as to the lysozyme, e.g. at the entrance of cleft between  $\alpha$ -helical and  $\beta$ -sheet-rich domains in the hydrophobic pocket (Fig. 3), where EGCG interacts with tyrosine and tryptophane residues of lysosyme (Ghosh et al., 2008).

*In silico* analysis suggests that ALA contains at least one putative high-affinity EGCG-binding site, which is positioned in close vicinity to, and interacts with, the dominant fluorophore Trp104 in protein. The high-affinity site is situated at the entrance of the cleft between two ALA domains in the hydrophobic pocket and includes aromatic cluster II residues. It seems that EGCG is spatially too voluminous to penetrate deeper in the cleft of ALA molecule, resulting in a lower affinity in comparison to other less voluminous phenolics (i.e. phenolic acids).

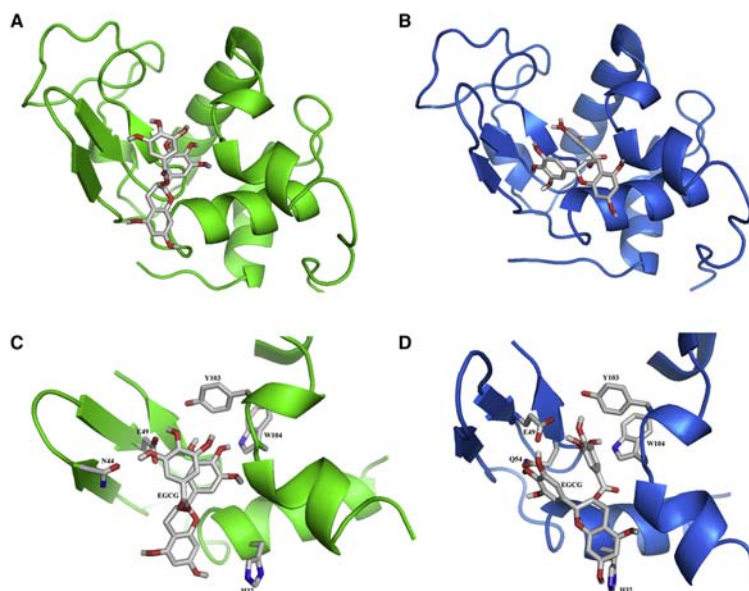
ALA is a good vehicle for delivering EGCG owing to its binding characteristics for other dietary proteins (as summarized in Table 2). The affinity of ALA-EGCG binding is comparable to the binding affinity of EGCG to BLG, Ara h 2 (2S albumin of peanut), trypsin, lysozyme, human serum albumin (HSA), lipase, ovalbumin, alpha- and beta-caseins, catalase, lipase, or alpha-chymotrypsin (Table 2). Interestingly, ALA is more digestible than BLG in the gastric phase of protein digestion (Fu et al., 2002). Therefore, for an EGCG-ALA and EGCG-BLG system, EGCG may be gradually released predominantly from ALA during the gastric digestion, and then progressively released from BLG during the intestinal phase.

It is known that EGCG can influence the fluidity of cell membrane (Kim et al., 2014). Among the important biological activities of EGCG, the inhibition of protein uptake by antigen-presenting cells, such as peripheral blood monocytes (Ognjenovic et al., 2014) and immature dendritic cells (Yoneyama et al., 2008) deserves particular attention. Hagiwara and Matsushita (2014)

**Table 1** Comparison of binding parameters obtained for ALA/EGCG complex, determined by the fluorescence quenching method and application of four different mathematical models, to binding parameters obtained for binding of ALA to phenolic compounds

Equation	References	Polyphenol	Binding parameter	Reference value	ALA/EGCG experimental value
Stern–Volmer	Mohammadi and Moeeni, 2015a,b Mohammadi and Moeeni, 2015a	Resveratrol	$K_{SV} (M^{-1})$	$4.19 \times 10^5$	$2.4 \times 10^4$
		Curcumin	$K_{SV} (M^{-1})$	$3.86 \times 10^5$	
		Genistein	$K_{SV} (M^{-1})$	$7.95 \times 10^4$	
		Kaempferol	$K_{SV} (M^{-1})$	$4.94 \times 10^4$	
Lehrer			$K_D (M^{-1})$		$3.5 \times 10^4$
			$fa$		
Double logarithm	Hemar et al., 2011	Resveratrol	$K_a (M^{-1})$	$8.31 \times 10^4$	$1.7 \times 10^4$
			$n$	1.08	
	Mohammadi and Moeeni, 2015a,b	Resveratrol	$K_a (M^{-1})$	$6.32 \times 10^6$	0.97
			$n$	1.16	
	Zhang et al., 2014	Curcumin	$K_a (M^{-1})$	$1.47 \times 10^6$	
			$n$	1.20	
		Caffeic acid	$K_a (M^{-1})$	$4.30 \times 10^6$	
			$n$	1.47	
		Chlorogenic acid	$K_a (M^{-1})$	$3.22 \times 10^6$	
			$n$	1.46	
		Coumalic acid	$K_a (M^{-1})$	$6.83 \times 10^6$	
			$n$	1.38	
		Ferulic acid	$K_a (M^{-1})$	$9.62 \times 10^5$	
			$n$	1.54	
		Genistein	$K_a (M^{-1})$	$5.92 \times 10^5$	
			$n$	1.23	
	Mohammadi and Moeeni, 2015a	Kaempferol	$K_a (M^{-1})$	$1.44 \times 10^6$	
			$n$	1.29	
Langmoir isotherm			$K_a (M^{-1})$		$1.8 \times 10^4$
			$K_d (M)$		

Taken from Al-Hanish, A., Stanic-Vucinic, D., Mihailovic, J., Prodic, I., Minic, S., Stojadinovic, M., Radibratovic, M., Milcic, M., Cirkovic Velickovic, T., 2016. Noncovalent interactions of bovine  $\alpha$ -lactalbumin with green tea polyphenol, epigallocatechin-3-gallate. Food Hydrocoll 61, 241–250 with permission from Elsevier.



**Figure 2** The molecular docking analysis reveals the putative EGCG-binding site in ALA (A) The high-affinity site for binding EGCG to native ALA (PDB ID 1F6S) (B) The high-affinity site for binding EGCG to recombinant ALA (PDB ID 1HFZ) (C) The close-up view of high affinity site for binding EGCG to native ALA (D) The close-up view of high-affinity site for binding EGCG to recombinant ALA. Color codes: native ALA – green, recombinant ALA – marine blue, carbon – white, oxygen – red, hydrogen – grey, nitrogen – blue. Taken from Al-Hanish, A., Stanic-Vucinic, D., Mihailovic, J., Prodic, I., Minic, S., Stojadinovic, M., Radibratovic, M., Milcic, M., Cirkovic Velickovic, T., 2016. Noncovalent interactions of bovine  $\alpha$ -lactalbumin with green tea polyphenol, epigallocatechin-3-gallate. Food Hydrocoll 61, 241–250 with permission from Elsevier.







reported that the mechanism of endocytosis inhibition could be due to the interference of EGCG with the interaction between small GTPase Rab5 and caveolin-1. To exert such a function, EGCG should be delivered to Rab5 in a biologically active form.

In atherosclerosis, damaged endothelial cells generate more reactive oxygen species (ROS), such as hydrogen peroxide, and inflammatory mediators, which recruit monocytes to endothelium. Antioxidant-loaded monocytes target endothelial cells, and the antioxidants scavenge more than 90% of intracellular ROS generated by cytokines or exogenous ROS (Lee, 2014). Thus, monocytes could contribute to retardation of early-stage atherosclerosis via target-specific delivery of protein-bound natural antioxidants, such as EGCG, to endothelium. Two previous studies examined the uptake of EGCG by monocytes in a complex with dietary proteins, and in both cases, the impact of endocytosis inhibition by EGCG was observed. Therefore, both ALA and ovalbumin complexed with EGCG can efficiently deliver the biologically active polyphenol to monocytes (Ognjenovic et al., 2014; Al-Hanish et al., 2016).

## Conclusions

ALA can serve as a suitable delivery system of biologically active EGCG and possibly other nutraceuticals, due to the low cost and availability of ALA. Further studies are needed to develop EGCG-encapsulating ALA-based or BLG/ALA-based nanoparticles or nanotubes, in order to improve EGCG stability and protect its activity during a prolonged storage. Not only the affinity binding of EGCG to ALA and BLG, but also to other whey proteins, can be utilized for efficient food fortification with EGCG. As a result, in such fortified foods, whey proteins can continuously release EGCG during the digestion process resulting in improved uptake and conveyed health benefits of this powerful polyphenol. As ALA is more digestible than BLG by pepsin, during gastric digestion EGCG will be gradually released predominantly from ALA, and then during intestinal phase, progressively released from BLG.

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# Food Structure, Rheology, and Texture

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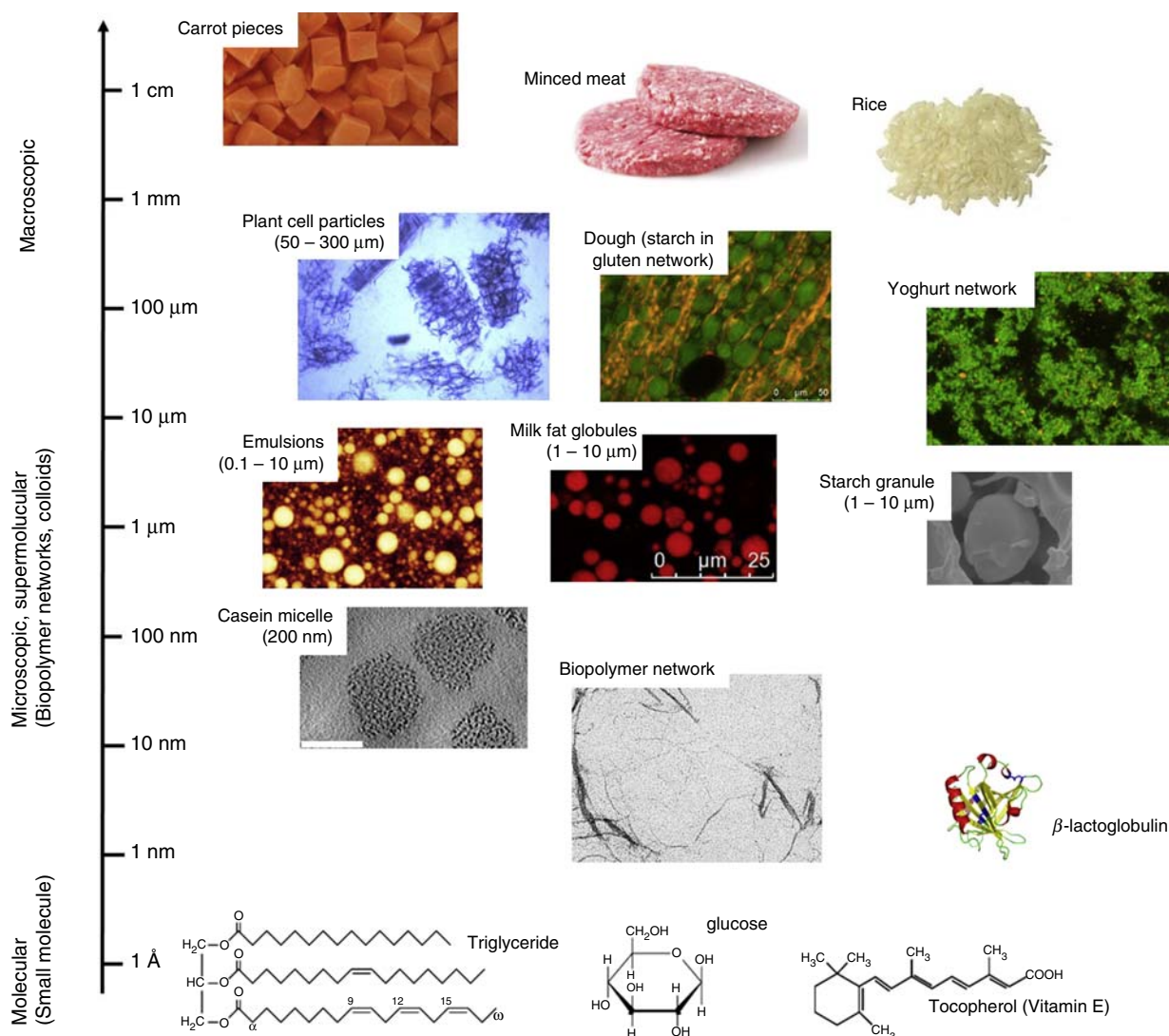
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## Introduction – Food Structure

Food structure is the organization of food constituents at multiple spatial scales and their interactions. In simple terms, different types of food structures are formed when food ingredients are mixed together to make a food product. Many food properties relevant to process engineering and product quality are related to structure. Insights into food structure and how it changes during processing operations are essential for producing high-quality food.

Structures that are relevant to food properties can be considered at different length scales (**Figure 1**):

1. At the smallest scale is the molecular structure: chemical structures that make up the constituents such as water, minerals, vitamins, flavor components, fatty acids, lipids and protein monomers, etc.
2. The next level up is the microscopic level: aggregation of molecules and their assembly into components, colloids and networks which are typically measured between nanometers and micrometers. A range of classic supermolecular structures in food are casein micelles, milk fat globules, starch granules, biopolymer network/gels such as the gluten structure in dough, pectin gel, etc.



**Figure 1** The length scale of structures and examples of representative molecules, food components, networks, and food structures. Microscopic images are kindly provided by Drs S.K. Øiseth, T.H. McCann and A. Leis, CSIRO (Werribee and Geelong, Australia).

3. The macroscopic level of structure considers those features that are perceived by human senses, such as texture and taste. They are often associated with specific types of foods by consumers, for example, tomato paste or fruit purees, recombined meat such as burgers and sausages, porous structures such as cakes and biscuits, and different types of grains, etc.

The processability, texture, flavor, and shelf-life qualities of food are controlled not just by chemical composition, but also by how the various ingredients are distributed and interact at the nano and microscopic length scales. Food structures vary enormously from relatively homogenous liquids to complex, multiphase colloidal systems containing fats, proteins, polysaccharides, salts, and water in the form of fibers, droplets, crystals, networks or even air bubbles. The size, shape and distribution of these structures greatly influence product stability as well as sensory properties and bioavailability of nutrients for absorption when food is consumed and digested (Turgeon and Rioux, 2011).

The important role of food structures in the area of nutrition is more and more recognized: how digestion of food macronutrients such as starch, fat and protein occurs depending on the matrices of food structure and thus accessibility of digestive enzymes in the gut. The same is true of micronutrient accessibility – both the chemical and physical states affect whether the body is able to derive the potential benefit of minerals, phytochemicals and vitamins, etc. (Parada and Aguilera, 2007).

## Food Colloids

Colloids are a widely encountered structural component present in many different food systems. A colloid can be classified as a dispersion of one material state in another, in which the dispersed component typically has dimensions across the micron length scale. The material states that comprise colloids are usually called the dispersed phase and the continuous phase, and each of these can be comprised of solid, liquid, or gas. Usually, the two phases are immiscible with each other, and thus colloidal systems are thermodynamically unstable. Instead, colloids can be kinetically stabilized, either through manipulation of the dispersed phase (such as reducing particle size) or through the continuous phase (increasing viscosity, or imparting a yield stress). In this way, the physical stability (or structural lifetime) of a colloid can be controlled and that for foods can range from a few seconds to in excess of a year, depending on the product in question (Dickinson, 2015).

## Emulsions

Oil and water, when combined, are well known for their immiscibility, yet both materials are frequently encountered together in many food systems. The particular colloidal state used to achieve metastability of mixture of oil and water is the emulsion, in which one phase is dispersed in the other in the form of small droplets. For food products, particularly soft solid foods, the oil-in-water state is more commonly encountered, providing a structural, material, and sensory contribution to products such as homogenized milk, yogurt, mayonnaise, ice cream, and cheese (with butter and margarine as examples of water-in-oil type emulsions). Emulsion stabilization is usually achieved through a combination of processing, in the form of homogenization, and surface stabilization of droplets through formulation design. High-pressure homogenizers and colloid mills are most frequently used in food manufacturing to produce emulsions. Both processes exert high shear forces on mixtures of oil and water, and the resulting flow properties under these shear conditions are sufficient to disperse the oil phase into small oil droplets (typically ranging from  $\sim 0.2$  to  $20\ \mu\text{m}$  in foods). Ingredient stabilization, in which surface active components in the formulation (such as proteins – notably dairy proteins, and polar lipids such as monoglycerides and lecithins) are able to adsorb at the newly formed interface, can be used to not only provide stabilization against coalescence and phase separation, but also to promote specific interactions between droplets. Emulsion structuring through controlled flocculation or partial coalescence contributes to the stability and material properties and sensory properties of many foods, such as cream cheese, which comprise a network structure consisting of protein and fat droplets, and whipped cream for which the foam structure is stabilized by a scaffold of agglomerated fat droplets.

## Foams

Foams are another example of a colloidal state commonly found in foods, such as cakes, breads, whipped cream, confectionary and beverages. Foams are dispersion of air in a liquid continuous phase (which is usually water). Formation and stabilization of foams is similar to that of emulsions, requiring the generation of air bubbles as the dispersed phase (through incorporation of air under high shear, but also potentially through sparging or pressure drop) and their stabilization through adsorption of an appropriate surface active material at the air–water interface (again, proteins and polar lipids can be effective in this respect). Loss of stability occurs through phase separation (drainage), coalescence and disproportionation (in which air diffuses from small bubbles into large bubbles).

Bubble size is typically larger than that observed for emulsions (ranging from  $\sim 50\ \mu\text{m}$  to several millimeters depending on product), and due to the large density difference between air and water, long-term stabilization can be challenging. For some products, such as beer foam, bubble lifetime only needs to be maintained over a matter of few minutes (although even during this short time, the foam provides both a sensory contribution and visual cue to the product). However, for other products such as ice cream and bread, where the foam phase also provides an essential contribution to the sensory properties of the food, foam stability needs

to be maintained for considerably longer. Generally, to avoid separation and destabilization, immobilization of bubbles can be achieved through modification of the continuous phase rheological properties (e.g., increasing viscosity, creating a yield stress or completely solidifying the continuous phase).

## Gels

The formation of a gel state usually occurs as a consequence of the formation of bonds between interacting molecules or colloidal particles within a solvent medium. This can lead to the establishment of a percolating structure that is essentially able to immobilize the solvent phase, transitioning a material from a sol state to a gel state. Gels are often considered elastic materials, and while this is certainly true for a number of examples (such as boiled egg white), it is important to note that depending on the type and extent of interactions, significant diversity in gel material/textural properties can be generated. Accordingly, structures can be produced with material properties varying from soft, readily yielding materials (such as yogurt), through to firm, elastic materials (jelly) and hard, fracturable materials (such as aged cheddar cheese). Gels can also be formed from a variety of food ingredients, based on the broad premise that these materials are able to form bonds, either with each other, or with other ingredients. Specific gelling materials are considered as follows.

**Protein gels** – Proteins with globular molecular structures (such as whey and soy proteins) can form gels as a consequence of protein denaturation (through a variety of mechanisms, such as temperature, pressure, and solvent conditions) usually followed by formation of covalent bonds between neighboring molecules via disulfide exchange. Gel strength can be influenced by protein concentration, ionic and pH conditions and the interactions with other food ingredients.

Proteins can also form noncovalent gels as a consequence of other forms of interactions. Two common examples include casein micelles in milk (which can form particle gels as a consequence of either acidification or rennet treatment) and gelatine, which can form thermoreversible gels as a consequence of hydrogen bond formation.

**Polysaccharide gels** – A number of polysaccharides are able to undergo gelation. This can be achieved through varying mechanisms of interaction including hydrogen bonding (such as for agar), electrostatic cross-linking (low methoxylated pectin, sodium alginate in combination with calcium ions), and hydrophobic interactions (hydroxymethyl cellulose). Gel properties can be influenced as a consequence of polysaccharide concentration, the number and availability of junction sites, and by solvent conditions. Combinations of polysaccharides can generate some interesting synergistic behaviors, allowing expansion of the range of material/textural properties achievable, and similarly interactions with other ingredients (e.g., the interaction between k-carrageenan and milk) can also create specific pathways for gelation.

**Emulsion-based gels** – The interfacial layer of emulsion droplets can allow droplet–droplet or droplet–continuous phase interactions to take place. Where droplets are able to stick together (again, a number of mechanisms may allow interaction to occur), network structures can be formed that percolate the system, causing gelation to take place. Gel material properties are dependent on the type and strength of interaction, the amount of fat present, and the influence of the continuous solvent phase (and any other materials present within it). Cream cheese provides an example of such a structure.

## Suspensions

A suspension is a colloidal state in which a solid phase is dispersed in a liquid. This particular state can be found in a number of food products such as cake batters, molten chocolate, salad dressings, and chocolate milks. For suspensions, the dispersed phase is generally of higher density than the surrounding continuous phase and (where the continuous phase remains liquid) will tend to sediment over time. To ensure that a solid dispersed phase remains uniformly distributed within a food often requires some form of kinetic trapping, usually as a consequence of altering continuous phase rheology such that the solid particles become locked into the structure.

In this way, sugar particles dispersed in the molten fat phase of chocolate are fixed in place on solidification of the chocolate on cooling. For a product such as chocolate milk, solidification of the continuous phase to immobilize cocoa particles would not be appropriate for the product. However, the implementation of a very weak yield stress (through the addition of the polysaccharide carrageenan) can be sufficient to inhibit sedimentation (while still allowing the liquid to properly flow during pouring and consumption). A similar approach is taken with some salad dressings, where xanthan (another polysaccharide) can be used to impart a weak yield stress to arrest the sedimentation of added herbs.

## Food Rheology

Food rheology is the study of the consistency and flow of food under specified applied forces, to understand the underlying physicochemical principles of ‘structuring’ by food materials and their interaction.

The degree of fluidity, consistency and other mechanical properties are important in determining how food ingredients can be mixed and processed during food manufacturing, what kind of product texture can be achieved, and how stable it will remain. The application of rheology to study food is primarily for the following three purposes (Fischer and Windhab, 2011). First, food engineers, who try to develop rheology–process relationships of the food, use rheological data for process or product optimization. Typical flow processes in food processing include mixing/stirring, dispersing, extrusion, coating, injection molding, and spraying.



Rheological measurements are also used in analytical to semiempirical modeling as well as in numerical flow process simulations. Second, material scientists or physicists focus on rheology–structure relationships of soft materials. Model food systems are often used to establish the material properties and develop science for new formulations and food properties. Last, food technologists aim at comparative characterization of food products to establish rheology–property relationships. Typical ‘properties’ of interest correlated with rheology are (1) sensory/perception characteristics (e.g., texture), (2) stability, (3) convenience aspects (e.g., portioning, scooping, dosing, filling), and (4) nutritive characteristics (e.g., release kinetics, satiety).

Rheological characterization involves quantification of the functional relationships between deformation, stresses, and the resulting rheological properties such as viscosity, elasticity or viscoelasticity, flow behavior, and recovery (Fischer et al., 2009). In principle, the experiments are usually carried out by either imposing a small force (*stress*) and measuring the deformation of the sample (*strain*), or a fixed amount of movement (*strain*) and measuring the *stress* developed in the sample. The small deformation measurements enable materials to be probed over supermolecular distances (e.g., nanometer to micrometer range) to establish the relationships between levels of structures and structural organization. On the other hand, measurements made under conditions of large deformation can provide information complementary to that obtained in the small deformation range, particularly time-dependent and nonlinear viscoelastic behavior at large strains and stresses. Measurements obtained at a large deformation regime are more relevant to the use of food materials in practice and the eating experience such as cutting, spreading, or chewing.

## Food Texture

Food texture plays an important role in whether the consumers like the food product or not (Foegeding et al., 2011). Texture is one of the attributes used by consumers to assess the food quality. Food texture is part of our sense when we feel the food in our mouth. It is can be described in the terms such as ‘hard,’ ‘soft,’ ‘liquid,’ ‘solid,’ ‘rough,’ ‘smooth,’ ‘creamy,’ ‘crumbly,’ ‘crispy,’ ‘lumpy,’ ‘gritty,’ etc. Such textural terms are directly related to the density, viscosity, surface tension, and other physical properties of a particular food product (Pascua et al., 2013).

Ultimately, the textural characteristics of a food are measured by sensory assessment tests. Since texture is inextricably linked to food structures at micro- and macrolevels and are strongly influenced by the interactions of food biopolymers such as proteins, polysaccharides, and lipids, instrumental methods designed to measure rheological and/or mechanical properties may be used to establish parameters that relate to relevant sensory textural characteristics (Chen and Opara, 2013).

In addition to the direct contribution to consumer acceptance, texture also has a vital secondary role on modulating flavor release and perception. If flavor components in a food are to be sensed, they must be released from the food matrix to reach the appropriate taste receptors. This release of flavor is closely associated with the way in which the food structure breaks down in the mouth relating to both the initial texture of the food and the change in texture throughout mastication (Stieger and Van de Velde, 2013).

## Food Breakdown

The structure of any given food product is essentially a template of the properties of that food system, representing its material behavior, physical stability, appearance, sensory performance, and other attributes related to the overall quality of that particular food. For manufactured foods, this is primarily considered for product structures essentially in the final processed and packaged form; in other words, a snapshot of the structure as it sits on the supermarket shelf. However, if one wishes to develop correlations between food structure and aspects such as technical functionality (e.g., the spreadability of butter), sensory, and even behavior during digestion, then it becomes essential to determine the structural changes taking place for any given food in response to the environmental conditions that are applied to that food during use, consumption, or digestion.

Thus, the sensory properties of any food can be established as a consequence of the in-mouth failure of that material in response to the conditions of shear generated by the teeth and movement within the oral cavity, by the interaction and dilution with saliva (including the presence of salivary enzymes), by the temperature in mouth, and of course the residence time needed to render the food into an altered structural state appropriate for swallowing (Chen, 2015). The investigation of structural dynamics in the mouth is termed oral processing and is a rapidly growing branch of the Food Structure discipline. This is facilitated by the development of increasingly sophisticated models able to replicate the oral cavity, particularly regarding the shear forces present during mastication, and which allow the changes in food structure during mechanical simulation of the eating process. Additionally, *in situ* analysis of structure changes during eating can be achieved through techniques such as articulography, which allows the breakdown of foods during eating to be directly related to the temporal development of sensory attributes. This approach can be applied to virtually any food type.

Similarly, the study of food material breakdown during gastrointestinal transit is increasingly being used to demonstrate the role of food structure in the digestion and uptake of micro- and macronutrients (Norton et al., 2014). This has been a rapidly developing field of research, aided by the development of sophisticated static and dynamic *in vitro* models that can increasingly be considered representative of digestive physiology (particularly with regard to pH, biochemical, and mechanical conditions) within the stomach and small intestine. Such models are able to determine how the digestion key nutrients, such as proteins and lipids, are influenced by the response of food materials to the digestive environment. An understanding of this relationship can allow food structures to



be developed with the ability to impart specific digestive properties, providing appropriate validation using human studies has been carried out (Bornhorst and Singh, 2014).

## Conclusion

The quality of a food product, in terms of sensory, shelf life, appearance, or nutritional value, is governed by the structure of that food. An understanding of how ingredients are assembled through processing, and how structural elements are across diverse length scales, impacts the material and technical properties of foods and can provide a considerably more iterative means of manipulating product properties. Thus, the intrinsic functionality of raw materials, ingredients, and additives can be tailored to specifically deliver a requisite structural contribution to any given food product.

More recently this approach has been extended to relate the structural disassembly of foods. Knowledge of how foods break down in the mouth through to the human digestive system provides new insights as to how the dynamics of product microstructure influence both food perception, digestion and nutrient absorption. Such knowledge will ultimately allow for the design and development of products able to meet specific requirements in terms of their properties, thus enhancing product value, whether it is in terms of cost, quality, or nutritional benefit.

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# Applications of Microrheology to Food Systems

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## The Deformation & Flow of Food

Rheology is the study of how materials flow and deform in response to mechanical stress (Barnes, 1994). It is an important tool for food scientists for two reasons (Fischer and Windhab, 2011). Firstly, flow properties define food structure during preparation. Most processed foods are edible structures that form when ingredients like proteins, polysaccharides, and lipids respond to mechanical and thermal stress (Mezzenga and Fischer, 2013; Van der Sman, 2012). Secondly, how a food is perceived and digested is affected by its flow behaviour (Witt and Stokes, 2015; Scholten, 2017). The focus of this review is the rheology techniques being developed to probe flow behaviour on the multifarious length- and time-scales that are relevant to food on its journey from production to end-use.

Traditional rheometers were developed to study materials that have a well-defined response to stress. A homogeneous liquid like brine, for example, flows steadily when poured out of a container. The flow is characterised by measuring the liquid viscosity on the macroscopic scale. Soft foods like mayonnaise, peanut butter and yoghurt on the other hand flow only once a critical stress had been applied. Otherwise they deform in a finite way, like elastic solids. Small angle oscillatory rheology (SAOS) characterises this behaviour on the macroscopic scale by quantifying the elastic storage modulus ( $G'$ ), which is a measure of the energy stored during a strain cycle, and the viscous loss modulus ( $G''$ ), which is a measure of the energy dissipated when the material flows during a strain cycle. Typically the stress (or strain) is applied sinusoidally and studies are carried out as a function of frequency in order to arrive at a viscoelastic spectra. Changes in the  $G'$  and  $G''$  of materials as a function of the frequency at which they are strained reflects the contribution of different relaxation mechanisms over different time windows, and as such informs on internal dynamics. Recent advances in rheology have been motivated by the complex flow behaviour of many soft materials which can depend on time, the size of the sample being probed, and the history of applied stresses (Chen et al., 2010; Liu et al., 2016).

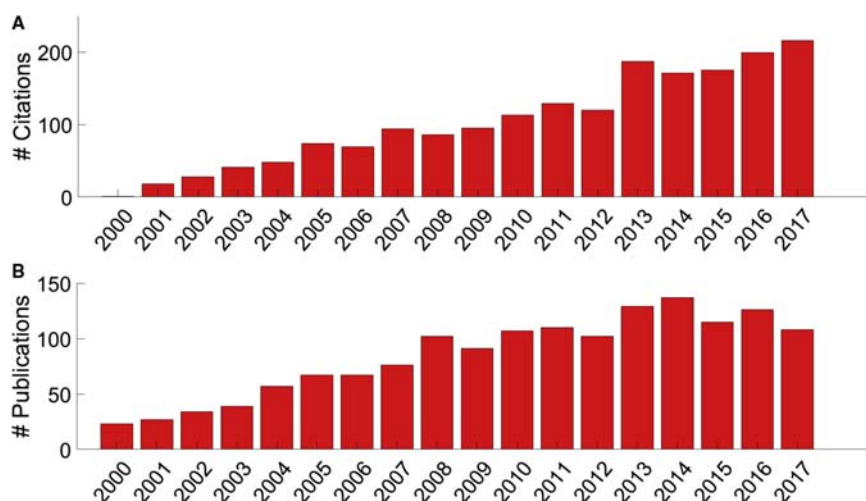
Many foods are inhomogeneous solutions that drip and splatter when poured, rather than flowing steadily and indeed show complex, time-dependent responses to mechanical stress down to the micrometre scale. Their flow behaviour is complicated by the presence of nanometre and micrometre-sized structures formed by proteins, polysaccharides, and lipids. The microstructures are often meta-stable, which means that how the food responds to stress changes as it ages. The challenge then is to understand how local variations in microstructure and dynamics contribute to the complex interplay between food structure, function and rheology.

## Microrheology

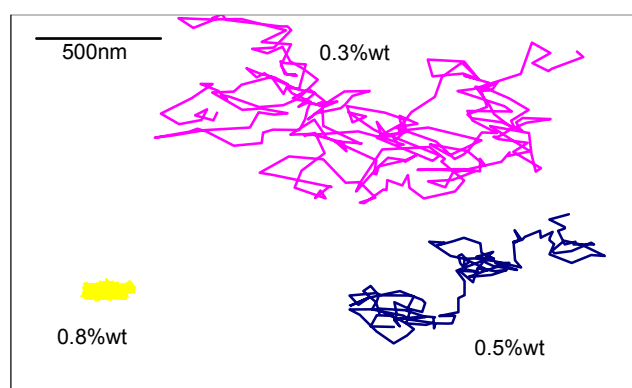
Microrheology is the collective name for a relatively new set of techniques that seek to probe the mechanical properties of soft materials over increasing ranges of time and distance, spanning the molecular to the macroscopic. They have steadily become more visible in food science literature (Fig. 1).

In essence microrheology seeks to replace the traditional, rather invasive, application of stress to the entirety of bulk samples, and the concomitant *sample-averaged* measurements of induced deformation or flow, with what might be thought of as a rheological microscopy. Stresses are imposed locally through microscopic probe particles that are internalised within the samples (such tracer particles can be added at a low phase volume during the preparation of samples if no appropriate native constituents can be identified), and the resultant movement of the probes obtained for example by microscopy in multiple particle tracking (MPT) captures the strain response, potentially allowing a localised measurement of the viscoelastic properties to be performed. The first advantages of microrheology are thus clear: i) applied stresses are considerably less likely to cause measurement-induced modifications to the structure being interrogated, ii) only small sample volumes are required, and iii) there exists the potential to obtain local mechanical properties. Even qualitatively visualising the movement of such micron-sized probe particles in a sample simply with a light microscope conveys the feeling of how 'gloopy' or 'jelly-like' different regions of samples are, holding clues to understanding creep, fracture and transport in food matrices. Fig. 2 shows the trajectories of the centre of mass of three different tracer particles, each one taken from a different concentration of a model food gel, an ionotropic pectin system formed with calcium. The progression from relatively free diffusion in the more viscous, low concentration system, through interesting "cage-jumping" behaviour at 0.5%, to firmly constrained motion as the concentration is increased further seems immediately intuitive.

As described, the stresses in microrheological experiments are not imposed onto the bulk of the sample through the surface of a rheometer plate or other geometry, they are applied by the probe particles being moved within their local environment. The application of these forces can be carried out either by using the modulation of external fields with which the particles interact, such as optical tweezers (OT) or magnetic tweezers, or simply by harnessing the thermal fluctuations of the particle. The former techniques



**Figure 1** (a) The number of publications since 2000 with “food” and microrheology” listed as keywords and (b) the number of times these publications have been cited. Data from Web of Science, December 2017.

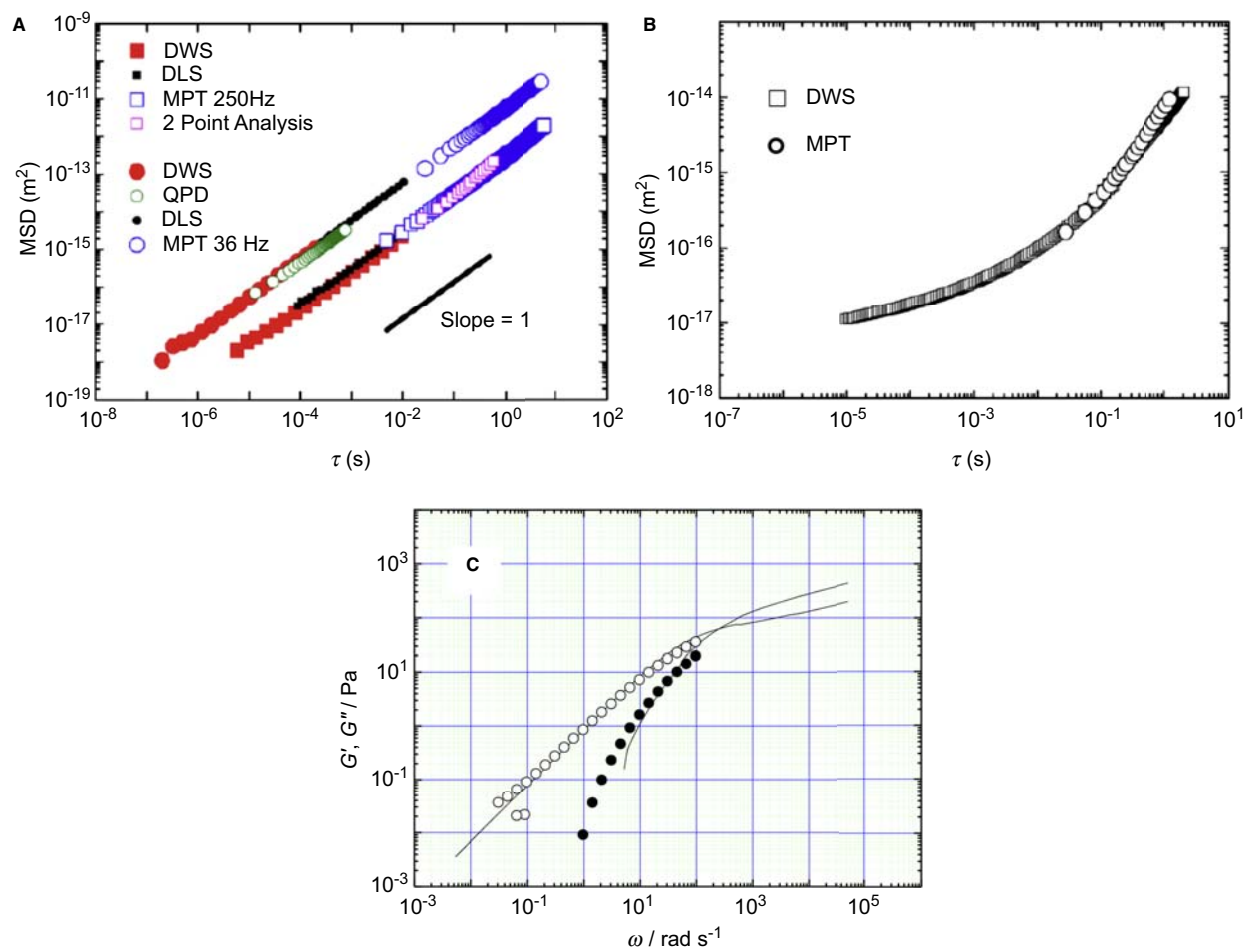


**Figure 2** The centre-of-mass movement of 500 nm latex particles undergoing thermal motion in pectin gels of three different concentrations as shown.

are called “active microrheology” while the latter bear the name “passive”. Passive microrheology then essentially studies the Brownian motion of internalised tracer particles (Fig. 2), and discerns the local mechanical properties from the fluctuation-dissipation theorem of Einstein, in exactly the same way as particle sizing is carried out in techniques such as dynamic light scattering in solutions of known viscosity: here rather than the viscosity being known and the diffusion coefficient of particles revealing their size, their size is known and their diffusion behavior reveals the mechanical behaviour of the surroundings. Indeed, Einstein had eloquently mooted the idea of microrheology himself during his work on the interpretation of Brownian motion, describing microscopic particles as moving like “a buoy in a pudding”.

The main task of quantitative microrheology then is to quantify small movements of tracer particles. In passive microrheology how the mean squared displacement (MSD) of the particles changes with time is the central focus of the measurement. For purely viscous solutions such measurements reveal a linear relationship that can be used to extract a single diffusion coefficient and thus a viscosity (Fig. 3a). While for viscoelastic media how the MSD of the probe particles scales with time is more complex (Fig. 3b), with some extra work the data can be mapped to a traditional quantitative viscoelastic spectrum such as would be measured with SAOS (Fig. 3c) (Cicuta and Donald, 2007).

In active microrheology a materials response function is measured by either driving probe particles with external fields or driving the sample (with a piezo-electric stage for example) and observing the probe response. The response ( $G'$  and  $G''$ ) can be measured as a function of frequency in a similar fashion to SAOS simply by manipulating the different driving frequency (Furst, 2005). While this is somewhat more labour intensive than the passive measurements that essentially measure the response to the entire spectrum of thermal fluctuations, active microrheology does potentially allow one to probe larger forces and even non-linear behaviour.

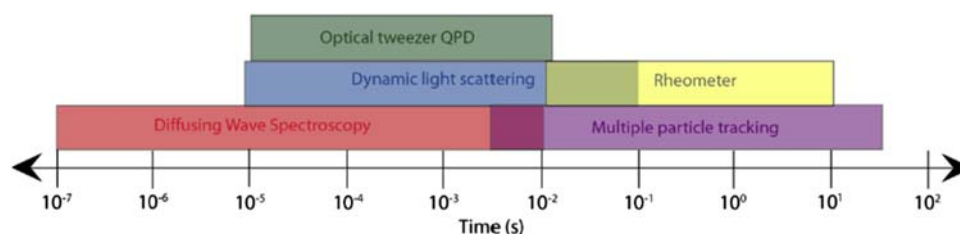


**Figure 3** (a) The mean-squared displacement (the central measurement) of tracer particles measured in viscous fluids with two different viscosities, measured by several techniques as described in the text; (b) the mean-squared displacement of tracer particles measured in a viscoelastic fluid (PEO) and (c) a viscoelastic spectra obtained from processing the data in (b) (Solid Lines) compared with results obtained by conventional rheological measurement.  $G'$  (Solid Circles) and  $G''$  (Open Circles).

In passive microrheology where no external field is required, simply looking at the thermal fluctuations over smaller and smaller timescales corresponds to measuring the mechanical properties at higher and higher frequencies. Another potential advantage of microrheology is thus revealed, iv) the viscoelastic spectrum of a material can be measured at frequencies several orders of magnitude higher than in a conventional rheometer, as long as the small displacements of the particles undertaken over very small times can be measured. While camera technology continues to improve and using a camera to film many particles at once in principle allows you to obtain information on heterogeneity, frame-rates of 100s of Hz are typically hard to surpass. However, the desire to observe probe particle motion over smaller and smaller time intervals is led, in-part, by the development of alternative methods for measuring the displacement of microscopic particles.

In one approach the position fluctuations of a single particle can be measured by illuminating it with a low power laser, capturing the light scattered onto a quadrant photodiode (QPD) and observing how the intensity of the light in each quadrant fluctuates. As the bead moves the amount of light captured by each quadrant of the photodetector varies, and these measurements can be calibrated to reveal quantitative displacements. However, freely diffusing beads will quickly leave the region of the probe laser so that obtaining a sufficiently averaged displacement is typically only possible by corraling the particle of interest using an optical trap (a focussed laser beam that interacts with the high refractive index particle and restricts it from moving away from the centre of the beam).

In another approach light scattering techniques such as diffusing wave spectroscopy (DWS), in which the ensemble-averaged motion of a set of tracer particles can be measured in steps smaller than a micron, correspond to rheological measurements at a MHz. In this case while sacrificing spatial resolution by monitoring fluctuations in the light scattered by the *ensemble* of particles, rather than observing the particles directly, temporal resolution is gained by using photomultiplier tubes rather than CCD (or faster CMOS) cameras to poll the intensity of scattered light.



**Figure 4** Timescales over which typical microrheology techniques (DLS, DWS, OT and MPT) operate compared with conventional rheology, revealing that a host of new information is available from these new experiments. This corresponds (left to right) to a frequency range from around 10 MHz to 0.05 Hz, and measurable storage moduli from around 10 kPa to 0.1 mPa.

Practically these timescales have an impact on the apparent frequency of the mechanical measurement and thereby on the sort of modulus values one can measure with a particular technique (Fig. 4). Techniques that can measure probe particle displacement at higher frequencies are capable of obtaining smaller displacements and thereby can typically probe materials of higher elasticity.

In summary, microrheology provides a set of new tools for food scientists to address the mechanical properties of food matrices, allowing deformation and flow to be studied over some eight orders of magnitude, from microseconds to hours. As the techniques continue to develop the hope is that more understanding of real structure-function relationships between desired properties such as processibility and breakdown, and different measurable mechanical properties, will be revealed.

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# Intrinsically Disordered Proteins: Polymers Without Structure but With Great Potential for Applications in Food Science

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## Glossary

**Structure-to-function paradigm** an expression used to define the mutually inclusive relation between molecular function and structure.

**Ingredients compartmentalization** internalization of one or more ingredients in food products, usually in a separated compartment embedded in the formulation or as a separated physical state (phase).

**Binding isotherm** a plot showing the fractional saturation of a quantity (e.g. complex formation between two molecules) as a function of ligand concentration. In order to be meaningful, the measurement has to be taken in conditions of constant temperature (isotherm) and pressure.

**Low-complexity protein sequence** a region of protein sequence space that is characterized by a small variety of amino acids. Low-complexity regions are particularly abundant in disordered proteins, in which particular residues are underrepresented (i.e. aromatic residues).

**Coacervation** process leading to the phase separation of molecular components within a solution, according to changes in concentration or micro-environmental conditions (pH, ionic strength, etc.).

**Cation- $\pi$  interactions** molecular interactions formed between an electron rich  $\pi$ -system and a cation. In proteins, the aromatic ring of phenylalanine or tyrosine residues and the sidechain of arginine usually compose the moieties taking part in cation- $\pi$  interactions.

**Molecular scaffolds** molecular components that are essential for the integrity of a particular cellular body and that provide interaction moieties for other molecular components within the cellular body (clients).

**Molecular clients** molecular components of cellular bodies that are not indispensable for the integrity of the body but that rely on molecular scaffolds (see previous definition) within the body for establishing interactions.

**Hydrotrope** molecule that can solubilize hydrophobic compounds into aqueous solutions and does not have the ability to self-aggregate.

## Introduction

### Intrinsic Disorder in Proteins and the Discovery That Function Can be Mediated by the Lack of Molecular Structure

One of the long-standing paradigms of biology states that the function of biomolecules is rigorously related to the three-dimensional structure that they adopt. DNA can be intended as a paradigmatic example of this: its double-helical structure ensures recognition of DNA-binding proteins as well as the preservation and propagation of genetic material from one generation to another. Proteins were thought not to be an exception to the structure-to-function paradigm.

In the early 1900's, Linus Pauling and Alfred Mirsky observed that protein denaturation generates functional loss (Mirsky and Pauling, 1936). Accordingly, renaturation (or refolding), which was accompanied by the restoration of function, undoubtedly indicated that structure and function would be mutually inclusive. Around that time, the denaturation-driven loss of function had been documented for a consistent number of molecules and the idea that structure and function would be intertwined was almost perceived to be as important as the genetic code (Kolata, 1986).

Considering such findings, the length of time that the community took to realize that some molecules effectively lack well-established secondary structure elements within their explored conformational ensembles, comes with no surprise. The past few years, however, have been characterized by an increased awareness that proteins without structure, now commonly referred to as *intrinsically disordered proteins* (IDPs), are very common and their recognition has ultimately expanded the universe of protein function as we know it. Interestingly, IDPs seem to have increased concomitantly with organismal complexity, for instance from prokaryotes to eukaryotes (Pancsa and Tompa, 2012; Schad et al., 2011), indicating that lack of structure could coincide with an expansion of function (Babu, 2016).

The increasing number of studies on IDPs has revealed exceptional properties of these molecules (Dyson, 2016; Wright and Dyson, 2015). Nevertheless, the uniqueness of such properties goes beyond the brilliance of their function inside cells and crosses disciplinary borders ranging from structural and cellular biology to food science and engineering.

To the question: "*what do intrinsically disordered proteins have to do with food science?*" the most appropriate answer would be *everything*. As food science constantly searches for new solutions to expand the repertoire of formulations for the creation of new products, IDPs provide the means for such solutions because of their chemical versatility that ultimately leads to a variety of behaviors which can be finely tuned by changing micro-environmental conditions.



In order to reply to the question above it is necessary to take a step back and consider the core interests of food science and the demands of the food industry. Based on the necessity to continuously create new products, to move towards the creation of super foods and functionalized ingredients, key methodological needs can be identified. Of these, three will be highlighted here as they are all of great importance: (i) the need to compartmentalize ingredients ultimately obtaining control over their timely release in desired quantities, (ii) the need to increase food security, especially in parts of the world severely affected by desertification due to climatic changes and (iii) the importance of designing and tuning the specificity and affinity of receptors for the development of more sensitive biosensors used for the detection of food pathogens.

### The Tendency of Intrinsically Disordered Proteins to Compartmentalize Makes Them Particularly Interesting for Food Science

One of the most fascinating discoveries of the last decade is that IDPs are majorly responsible, in cells, for the formation of molecular conglomerates composed of intracellular proteins and/or nucleic acids, which are generally referred to as membraneless organelles (MLOs) (Darling et al., 2017; Meng et al., 2015; Shorter, 2016). MLOs are functional entities formed upon the change in micro-environmental factors such as temperature, chemical modifications of their composing molecular components, pH and/or ionic strength (Banani et al., 2017; Molliex et al., 2015; Riback et al., 2017). Although the exact nature of the interactions and the conformational dynamics of the components forming MLOs is unknown, these bodies exhibit the physico-chemical properties of liquid–liquid mixtures, as they are able to drip, fuse and deform (Darling et al., 2017). Altogether, these characteristics are of great potential for both the pharmaceutical and the food industries, a more detailed understanding of the features that allow such behavior in cells needs to be elucidated: hence more research needs to be devoted to these interesting molecules.

Interestingly, the quantity and diversity of MLOs suggests that there is not a single mechanism behind the ability of these components to phase separate or disassemble in a micro-environmentally controlled manner. This ability is strongly related to the presence of low-complexity, intrinsically disordered regions (IDRs). Most IDRs tend to interact via coacervation, a process which is widely explored in the food industry (Yan and Zhang, 2014). The key to control coacervation is an extensive knowledge of the chemical moieties that, upon interaction, favor or disfavor the occurrence of molecular contacts. Due to their sequence variability, IDRs dispose of a large range of interacting moieties leading to phase separation and this can provide numerous possibilities for the industry to control coacervation with increased tunability. For example, IDPs containing hydrophobic residues tend to self-assemble, establishing homotypic interactions leading to simple coacervation (Reichheld et al., 2017). The lowest complexity peptides that have shown the ability to phase separate and at the same time have numerous traits of tunability are perhaps poly glutamine-containing stretches, which, even though glutamine has an extremely high solubility in its monomeric state, quickly modulate their phase behaviour according to their length (hence the overall concentration of glutamine), pH and/or ionic strength (Toretsky and Wright, 2014).

Highly polar random stretches interspaced with repeats containing hydrophobic residues such as phenylalanine, have also shown to phase separate and form hydrogels (Fu et al., 2007, 2010; Sun et al., 2013). An example of those is given by nucleoporins (Lemke, 2016; Peters, 2009; Rexach and Blobel, 1995), which in the nuclear pore complex of eukaryotic cells form hydrogels acting as a selective barrier regulating molecular transport between nucleus and cytoplasm (Rout et al., 2000; Schwartz, 2005). For those proteins it has been shown that the simple mutation of phenylalanine residues to tyrosine leads to the dissolution of the hydrogel (Frey et al., 2006). The phenylalanine residues are also responsible for specific interactions with nuclear transport receptors and mediate an ultrafast association between intrinsically disordered and structured partners, which is at the centre of nuclear transport (Milles et al., 2015).

On the other hand, proteins containing charged residues tend to assemble via complex coacervation in which oppositely charged groups interact in order to ultimately lower their net charge (Pak et al., 2016).

For these cases, it has been observed that charge–charge interactions coupled to cation- $\pi$  interactions between aromatic and positively charged residues, are of major importance for driving phase separation. One example of proteins that undergo phase separation through the formation of complex coacervates are Ddx4 proteins, which phase separate to form nuage organelles in mammals (Pak et al., 2016). In the case of Ddx4 proteins, interactions between FG- and RG-repeats numerously represented along the amino acidic sequence of several Ddx4 proteins, occur. Purely electrostatic interactions are also majorly responsible for the phase separating behavior of these proteins, which are a perfect example of how phase transition is, in this case, extremely sensitive to changes in ionic strength.

From a general and simplistic standpoint, the process of phase separating has been referred to as the interplay of two different components called scaffolds or clients (Salman et al., 2016; Harmon et al., 2017). While scaffolds are moieties that drive the process to exclude solvent and thus phase separate and are also called drivers of phase transition (Brangwynne et al., 2015), client molecules just show passive partitioning behaviour which leads to condensates. With respect to this, the maximum tunability of coacervation that has potential in food science would be driven by a scenario in which a coacervate and its responsiveness to the micro-environment is modulated by the activity of both the scaffolds and the clients composing it.

Recent studies show how the interplay between the conformational properties of scaffolds and clients can switch the equilibrium of assemblages. A classic example of this is given by the process of gelation that can be sometimes coupled with phase transition. Gels are prevalent in the food and pharmaceutical industries from the production of laxatives, gelling agents intended as gelatin substitutes (Armisen et al., 2000; Stanley, 2006), yogurts or desserts (Philips and Williams, 2009; Roopa and Bhattacharya, 2008) to the creation of immobilizing agents for enzymes (Philips and Williams, 2009). Chemically, gelation is considered as the formation of molecular networks that start interacting above a critical concentration defined as the percolation threshold (Broadbent and Hammersley, 1957). Nevertheless, gel formation can also follow phase separation if the gel point (the concentration at which the formation of molecular networks occurs) lies below the critical concentration

required to achieve phase separation. In an extremely interesting computational study, Harmon et al. pinned down the physical principle behind the ability of protein sequences to phase separate with or without the formation of a gel (Harmon et al., 2017). The study reported that the critical property of intrinsically disordered linkers in favoring phase transition followed by gelation is the effective solvation volume of the linkers spacing interacting structured SH3 domains. In the case linkers have effective solvation volume of approximately zero, gelation follows phase separation as the conformational dynamics of linkers is such that interacting moieties composing the polymeric network lie very close. In contrast, linkers with positive effective solvation volume will have a negative impact on phase separation or gelation. Very importantly, effective solvation models are directly related to sequence compositions and mostly due to the presence of charged residues along the linkers. This provides ground rules for the exploration of intrinsically disordered regions that, coupled with scaffolds having diverse binding valences, can modulate the concentration ranges at which polymer mixing shows either gelating, phase separating properties or both.

Another particularly interesting ability of organisms is to control the formation and dissolution of liquid-like super concentrated droplets using out of equilibrium processes. These processes are mainly mediated enzymatically by a series of chaperones or disaggregases to dynamically modulate composition and formation of protein coacervates (Hubstenberger et al., 2013; Kroschwald et al., 2015; Sweeny et al., 2015). A recently presented and very suggestive hypothesis, backed up by experimental evidence concerning the dissolution of FUS granules (Ratti and Buratti, 2016), suggested that the high concentrations of ATP normally present in cells (and falling in the mM concentration range) would be functional not only to promote enzymatic processes requiring energy (which would require concentrations of ATP estimated in the low  $\mu\text{M}$  range) but also for the dissolution of MLOs. Thus proposing ATP as an effective hydrotrope of protein coacervates (Patel et al., 2017). Controlling the formation and the dissolution of coacervates using small, biocompatible molecules by designing formulations featuring ingredients release upon the mixing of hydrotropes to coacervates, is of extreme interest for the food industry, especially in the need to compartmentalize and release functional ingredients in a timely manner.

An additional, extremely important level of regulation for potential food formulations using protein components is represented by the set of chemical modifications that amino acids can experience. *In vivo*, proteins continuously undergo post-translational modifications that *de facto* mutate the sequence composition of proteins through the addition or removal of chemical groups on the residues' side chains (Bürkle, 2001). IDPs, due to the lack of structure and to their extreme functional promiscuity, are particularly prone to post-translational modifications (Bah and Forman-Kay, 2016; Xie et al., 2007).

Overall, the possibility to translationally modify mixture components represents a great opportunity for tunability and therefore for the design and control of coacervates. All these examples demonstrate the great potential of IDPs in applied Science, and considering the fact that we are just beginning to understand the behavior of these fascinating molecules, more research exploring the potential of IDPs in food science will be key to go beyond their functional behavior in cells and support their utilization in different fields of applied research.

### **The Role of Intrinsically Disordered Proteins in Anhydrobiosis Provides Strategies to Enhance Food Security**

The rapid increase in the human population over the last few decades, combined with progressive environmental deterioration due to climate changes are posing questions on how to confront the subsequent lack of food security for large segments of the population (Misra, 2014). The consequences of climate change can also be imagined as an extensive increase in the desertification of several territories followed by extreme migrations (Campbell et al., 2016; Misra, 2014). One of the major aims of food science is to increase food security, especially in areas where desiccation threatens survival.

Importantly, the role of IDPs in anhydrobiosis (Wharton, 2015), which literally translates as "life without water", is becoming progressively evident. The knowledge that some organisms, adapt to extreme conditions and can survive desiccation isn't novel. Many creatures, spanning different phyla from plants to bacteria and fungi, rotifers, micro-animals and even nematodes, demonstrate the ability to "come back to life" after long periods (sometimes lasting several decades) of desiccation (McGill et al., 2015). Scientists have consistently tried to understand the molecular mediators and the mechanisms of such an ability. While in the past, the theory by which desiccation tolerance would be mediated by sugars (trehalose or sucrose) was dominant (Watanabe et al., 2002), further evidence posing IDPs as active mediators of desiccation tolerance is emerging (Boothby and Pielak, 2017; Liu et al., 2017): mostly after the realization that not all the organisms showing anhydrobiotic behavior possess the genes to synthesize or produce great enough quantities of trehalose or other sugars to prevent death upon desiccation.

It has been recently shown that tardigrades can over-express, in conditions of severe drought, a set of proteins actively involved in protecting cellular components from lack of water (Boothby, Tapia, Brozena, et al., 2017). These proteins, subdivided into three classes and defined as secreted abundant heat soluble (SAHS), cytosolic abundant heat soluble (CAHS) and membrane abundant heat soluble proteins (MAHS), share a major single feature: a large amount of structural disorder.

Besides abundant heat soluble proteins, a similar class of cellular components majorly responsible for desiccation tolerance is represented by the better characterized late embryogenesis abundant (LEA) proteins found mostly in plants (Dang et al., 2014; Gao and Lan, 2016; Rodriguez-Salazar et al., 2017; Villar et al., 2011) but also discovered across phyla (Hand et al., 2011). LEA proteins are small, mostly disordered, polypeptide chains showing a high content of hydrophilic residues (Hong-Bo et al., 2005).

Although the mediators of tolerance to dehydration have been identified, the mechanism by which these proteins arbitrate this activity is still unknown. It has been suggested that several mechanisms, which don't necessarily exclude one another, may take place and protect cellular components from the lack of surrounding water.

Firstly, it has been suggested that the ability of these molecules to form glass-like structures, a process called vitrification (Chavali et al., 2017; Uversky, 2015, 2016), may be related to their capacity to protect molecular components in the cell. The formation of glass-like structures has been observed for CAHS proteins and it has been directly related to the survival rate of organisms like yeast, which heterologously expresses CAHS; interestingly, the vitrification of CAHS proteins also leads to an increase in heat tolerance of approximately 10 °C (Boothby et al., 2017). Besides vitrification, a water replacement mechanism, in which IDPs would actively mediate a hydrogen-bonding network with cellular components has also been suggested (Boothby and Pielak, 2017).

Particularly fascinating is the mechanism suggesting that IDPs involved in desiccation tolerance may act as molecular shields. Molecular shields can protect cellular components by crowding their surroundings via non-specific interactions. This mechanism, if proven true, is particularly interesting to food science as it is transposable to other molecules, such as polysaccharides, that already have many applications in food preparations. With respect to this, an interesting study by Chakrabortee et al. investigated the ability of LEA proteins to act as molecular shields and protect crucial components of organismal metabolism. The study proved a divergent functional role of IDPs from molecular chaperones and suggested points of contact with neutral polysaccharides functioning as anti-aggregating molecules to ultimately preserve cellular components from desiccation (Chakrabortee et al., 2012).

Nevertheless, even though research has enhanced our understanding of IDPs in anhydrobiosis, the exact mechanism by which these proteins protect the structure of cellular components is still unknown. While the collected data points towards the fact that IDPs fully preserve function, the consequences of IDPs activity on the structure of the protected components are not always clear. Some evidence suggests that molecular shielding can indeed yield conformational changes leading to apparent functional loss and a suggestive hypothesis re-conciliating such conformational changes and preservation of function is the one from Tompa and Csermely (2004). According to this hypothesis the interaction between chaperone IDPs and targets would generate an initial partial unfolding of the target, which would then re-join the folding pathway and regain structure and function.

It is in the end plausible that the activity of disordered proteins in promoting tolerance to extreme lack of water would be mediated by a combination of the proposed mechanisms or by new mechanisms not yet identified. Nevertheless, just by looking at the collected evidence it is clear how food science can benefit from the study of these components for the creation of new foods (or even food crops, overlapping into plant biology) that would show an increased tolerance to harsh environments. This includes both the possibility to increase the resistance of crops to arid landscapes and the opportunity to reduce the need to refrigerate products in countries where refrigerating is problematic. This line of research would have a direct influence on how food science would impact food security in a scenario where desertification and food shortage will affect increasing portions of the planet's population.

### Using Intrinsic Structural Disorder to Design More Sensitive Biosensors to Increase Food Safety

A high priority of food science research is to enhance global food safety, especially considering the staggering numbers of foodborne diseases, which are estimated to be in the millions with thousands of deaths per year (World Health Organisation, 2017). Technology coupled with basic research therefore needs to step up and find ever more accurate ways to detect foodborne pathogens before they would cause harm. An enormous advancement in the field has been represented by the creation of biosensors that are able to detect elements of pathogens in food samples. The founding elements of biosensors are essentially two: a recognition element and a target analyte which is most commonly a molecule from the pathogen to be detected in the sample. Essentially, the analyte interacts with the recognition element (normally a receptor) and triggers the transduction of a signal which is electronically detected. It is of course necessary to have an interaction between the two molecules (the receptor and the analyte), but it is highly advantageous that the sensitivity of the receptor for the target would be such that the analyte could be detected in the smallest possible concentration. The concentration at which a receptor binds its ligand is a hardwired characteristic of the receptor linked to its structural and dynamical features. Nevertheless, decreasing the concentration threshold required for the detection of foodborne analytes would be a great advantage to enhance food safety further. From a molecular perspective, doing this means increasing the affinity of the receptor for the target, which in other words signifies reducing the binding constant that characterizes the interaction between the two molecules. Attempts to engineer molecules to rationally tune the binding properties of molecular receptors have been characterized by diverse strategies such as allowing a conformational switch of the receptor and pushing the binding-prone population to be maximized (Ricci et al., 2012), combining multiple receptors in tandem (Vallée-Bélisle et al., 2012) using a compound (depletant) that would sequester the ligand without producing an output until ligand concentration would rise enough to stimulate a second, low affinity, receptor that produces a sharp response (Kang et al., 2012; Ricci et al., 2011), or mutating parts of the receptor's structure that would not be directly involved in binding (distal mutations) but that would somehow increase the affinity between the two molecules (Porchetta et al., 2012).

In contrast, part of the research has been devoted to favor cooperative allosteric receptor activation, which means increasing the receptor's affinity by transducing a signal across its structure (Dueber et al., 2007; Liu and Lu, 2006). Usually such a signal is generated by the binding of an analyte that allows the coupling of a second analyte. The two analytes binding the receptor can, at the same time, have a regulatory role and be a substrate of the enzyme (in this case giving rise to homotropic allostery, which mostly occurs when the regulatory element and the substrate are the same molecule) or act either as a regulatory element or enzyme substrate (heterotropic allostery).

Since the point of this review is not to describe allostery in detail, but to show how intrinsic disorder can contribute to food safety through the development of more sensitive biosensors, I will herein limit the explanation of receptor binding to the minimum necessary, to then focus on the use of IDPs in serving the purpose of increasing receptor's affinity.

Receptor occupancy can be described by the fraction of receptor bound as a function of target concentration:

$$\text{Receptor occupancy} = \frac{[\text{target}]}{[\text{target}] + K_D} \quad (1)$$

where  $K_D$  corresponds to the concentration of ligand at which the target is half occupied.

The concentration of analyte required to increase the occupancy of a receptor from 10% to 90% is defined as *dynamic range* of a receptor and essentially provides a measure of the receptor's sensitivity.

$$\text{Dynamic range} = \frac{\text{Receptor occupancy}_{90\%}}{\text{Receptor occupancy}_{10\%}} \quad (2)$$

It would therefore be ideal to decrease the minimum concentration of analyte required to generate an increase in receptor occupancy from 10% to 90%.

While in heterotropic allostery the binding of the second analyte (different from the first) generates a shift in the analyte binding isotherm but it doesn't affect its shape, in homotropic allostery binding of the second analyte can be favored or disfavored by the binding of the first analyte, giving rise to negative or positive cooperativity, respectively (Simon et al., 2014). In this case, the isotherm is described by the Hill equation (Hill, 1910) as follows:

$$\text{Receptor occupancy} = \frac{[\text{target}]^{n_H}}{[\text{target}]^{n_H} + K_{1/2}^{n_H}} \quad (3)$$

and the dynamic range is dominated by the Hill coefficient  $n_H$ . To reduce the concentration at which the receptor's occupancy increases to 90%, strategies to increment the Hill coefficient need to be designed. A Hill coefficient of 1 suggests lack of cooperativity, whereas higher values would indicate cooperative binding and the narrowing of the concentration window required to detect a signal and identify foodborne pathogens.

Intriguingly, intrinsic disorder has been indicated as a strategy to increase cooperativity or to transform non-cooperative into cooperative receptors (Simon et al., 2014). The creation of chimeric proteins composed of two non-cooperative DNA beacons, alternated by non-pairing (disordered) DNA stretches has shown to increase the Hill coefficient and the affinity for the second binding event. The first binding is indeed disfavored by the entropic cost of folding the intrinsically disordered stretch engineered on the receptor. Once the first analyte is bound to the receptor, the gain in structure (due to the disorder-to-order transition) favours the binding of the second analyte through the formation of a binding pocket, thus increasing cooperativity (or transforming a non-cooperative receptor into a cooperative one (Simon et al., 2014)) and ultimately improving the dynamic range.

Interestingly, the enhancement of dynamic range has been partially related to conformational properties of the disordered linker such as its persistence length. Although this approach has successfully been proven only on DNA constructs, the possibility to expand its applications and potential using intrinsically disordered peptides rather than nucleotides can be of enormous advantage. The intrinsic advantage of using DNA is given by its predictive base-pairing which can yield wanted behavior due to limited conformational freedom. Proteins don't carry this advantage as their conformational flexibility is much greater than that of DNA, but at the same time they would come with a much greater potential for applications of this kind. The examples of IDPs folding upon binding, which can be a mechanism useful to favor the occurrence of cooperativity, are numerous and IDPs offer a wide range of post-translational modifications that can play a regulatory role in the attempt to engineer and control cooperative behavior. In the future, it is certain that further investigations of intrinsic disorder used to improve the dynamic range of receptors useful for biosensing will certainly lead to an improvement in the quality of tools available to detect food pathogens.

## Conclusions

In the past decade intrinsic disorder has emerged as an evolutionary advantage for organisms, especially in the framework of increased system complexity. Nowadays, it is documented that the key and lock mechanism describing molecular interactions (Fischer, 1894), their specificity and affinity, is only part of the story and the need to obtain further information about the biophysics of molecules without a well-defined structure is a must to utilize these entities in the numerous fields of applied Science (Csermely et al., 2010). The extreme functional versatility of disordered molecules is of particular appeal to food science, with many areas that can benefit: from ingredient encapsulation to enhancement of food security and safety. Nevertheless, the paradigm shift that intrinsic disorder has brought to the world of Biology is such that new approaches, dealing with the greater molecular flexibility need to be explored. A key aspect of such need is interdisciplinarity (Uversky, 2002, 2013). As intrinsically disordered molecules fall into a new category of functionally viable entities, biology has had to combine the expertise from multiple disciplines to study how intrinsic disorder impacts cellular processes (Uversky and Dunker, 2013). Similarly, the embracement of interdisciplinarity, leading to the combination of multiple and diverse sets of expertise from a wide range of disciplines will empower the food science community with the additional tools necessary to improve human nutrition and drive to market the next generation of food products.



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## Structured Lipid Functionality and Application

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### Introduction

Due to the over consumption of fats, obesity has become the major cardiovascular risk factor according to epidemiologic studies (Nakamura et al., 1994), resulting in an increased risk of diabetes, atherosclerosis, hyperlipidemia, hypertension and cardiovascular disease. Oils and fats in their natural form are usually not desirable due to their physical properties, nutritional value and chemical composition however, lipids can be modified to obtain desired attributes and specific function. Structured lipids (SL) are lipids that are modified chemically or enzymatically to change the fatty acid composition and/or their positional distribution in glycerol molecules (Lee and Akoh, 1998). It involves modifying the chemical structure of the lipid by addition or rearrangement of fatty acids on the glycerol backbone (Iwasaki and Yamane, 2000). SL consist of the most common types of food lipid triacylglycerols (TAG) and others including monoacylglycerols (MAG), diacylglycerols and phospholipids. SL provide specific fatty acids to improve functionality and nutritional properties that could be used to target specific diseases and metabolic conditions (Kim and Akoh, 2015). Therefore, it is important to modify lipids by introducing beneficial fats such as long-chain polyunsaturated fatty acids (LC-PUFAs), fatty acids with 18–20 carbons or more, to replace saturated fatty acids.

Other ways of lipid modification include hydrogenation that converts lipids from liquid or semi-solid forms into solid by partial or full saturation of double bonds to produce fat with high oxidative stability. During the process, hydrogen atoms are added onto double bonds of unsaturated fatty acids in order to obtain physical attributes and functionality to provide cooking stability, creaming ability, steep melting properties and desired appearances (Kadhun and Shamma, 2017; O'Brien, 2008). Due to the production of *trans* fatty acids from partial hydrogenation, industries are replacing hydrogenation with interesterification that produces zero *trans* fat. *Trans* fats have been reported to be associated with increased risk of coronary heart disease hence, regulation on *trans* fats have been implemented and industries are finding alternative methods to avoid their production. Research has observed that consumption of *trans* fats raises the level of low-density lipoprotein cholesterol levels, reduces the level of high-density lipoprotein cholesterol, and increases the ratio of total cholesterol to high-density lipoprotein cholesterol (Katan et al., 1995; Mensink and Katan, 1990; Mozaffarian et al., 2006). *Trans* fat also increases the levels of triglycerides in the blood as compared with the intake of other fats, which further increases the risk of cardiovascular heart disease (Mensink et al., 2003).

### Interesterification

Interesterification is a process which acyl groups exchange positions either within the same glycerol backbone or with other glyceride molecules among triglycerides and phospholipids. A simple description of the reaction would be a random or direct attack on the fatty acids attached to the glycerol backbone resulting in the breakage of the bond between the fatty acids and glycerol backbone. After which, the fatty acid released is mixed with the free fatty acids (FFA) and the empty glycerol position is replaced randomly by another FFA (O'Brien, 2008). The desired properties of lipid can be manipulated by modifying the chemical composition of fats and oils through interesterification. Thus, interesterification has found a wide range of applications in the edible oils industry in order to obtain the desired nutritional properties, plasticity, melting profile of fats and the compatibility of TAGs.

#### Chemical Interesterification

In the process of random chemical interesterification, fat is heated to approximately 100–140 °C for a short period of time, in the presence of an inorganic catalyst such as sodium methoxide. The fatty acids are released from the glycerol backbone and re-esterified by randomization or directed fatty acid interchange within and among the TAGs. The process continues until it reaches thermodynamic equilibrium and this depends on the temperature, time and other conditions of the reaction (Gunstone, 2006). This method is the most common industrially implemented method as it is cheaper and requires a shorter reaction time than enzymatic interesterification. However, the reaction lacks specificity and offers limited positional distribution of fatty acids (Kadhun and Shamma, 2017). Side products that are hard to eliminate may be produced and oxidation of lipids can increase due to the application of high heat.

#### Enzymatic Interesterification

This process utilizes an enzyme such as lipase to catalyse the rearrangement of fatty acids and one of its most important features is the positional specificity that results in higher efficiency and mild conditions with few or no by-product. Specific lipases can hydrolyse sn-1 and sn-3 position, incorporating fatty acids without affecting the fatty acid in sn-2 position allowing improved nutritional

properties in SL. Enzymatic interesterification occurs under mild conditions that reduces the loss of temperature sensitive LC-PUFAs that are susceptible to oxidation, reduces the use of energy and solvents allowing easier recovery of the final product. Lipases are used for enzymatic synthesis of SL that are used as biocatalysts in the hydrolysis and esterification of TAG. Enzymes are used to increase the control over the nature of the product due to the specificity of many different lipases (Farfán et al., 2015). However, industrial application has been slow due to the high cost of enzymes (Kim and Akoh, 2015).

### Acidolysis

The acidolysis reaction consists of the exchange of acyl groups between an acid and an ester. The benefits of utilizing acidolysis is the simpler prediction of reaction product composition, however, it requires thermostable lipases in a solvent-free system, lower fatty acid incorporation, purification and recovery of end products, and requires oil saponification (Şahin-Yeşilçubuk and Akoh, 2017). Acidolysis is usually utilized to enrich oils by increasing the content of fatty acids such as LC-PUFAs in SL.

### Functionality of Structured Lipid

The synthesis of SL utilises a variety of fatty acids to increase melting points of lipid such as medium-chain fatty acids (MCFAs), a fatty acid with 6–12 carbon atoms, which possesses beneficial nutritional and metabolic attributes for nutraceutical and pharmaceutical application. The physical and chemical properties of lipids that can be modified include the solid fat content, slip melting point, melting and crystallization profiles and oxidative stability. Therefore, SL are referred to as nutraceuticals and functional foods that could be modified to satisfy consumer demand (Kim and Akoh, 2015). The nutritional functionality of SL can also be manipulated by utilizing a wide variety of fatty acids that could be used to provide health benefits. Short and MCFAs are used as substrates for SL due to their benefits of increased absorption, digestibility and metabolism (Huang and Akoh, 1994).

Studies have shown the positional distribution of fatty acids on the glycerol backbone influences physical behaviour and metabolism of lipids (Hu et al., 2017). SL with essential LC-PUFAs located at the sn-2 position and MCFAs located at sn-1, 3 positions of the glycerol backbone otherwise called medium-long-, medium -(MLM) chain TAG have increasing interest due to their nutritional properties as SL are able to provide essential LC-PUFAs in a form that provides the hydrolysis and absorption advantages of MCFAs (Jandacek et al., 1987). TAG in the sn-1, 3 position is preferentially hydrolysed by lingual, gastric and mainly pancreatic lipases resulting in FFA, thus producing sn-2 MAGs that can be absorbed efficiently (Bradley, 1998; Derya et al., 2015; Karupaiah and Sundram, 2007). MCFAs act as fast energy via oxidation of the more hydrophilic MCFAs and have lower tendencies to be deposited in the adipose tissue because they are not readily re-esterified into TAGs and also due to their direct transport via the portal vein to the liver rather than through the lymphatic system (Kim and Akoh, 2015). Hence, digestion of incorporated MCFAs at the sn-1, 3 positions are hydrolysed and rapidly absorbed as FFA while LC-PUFAs esterified at the sn-2 position provide increased protection against oxidation of fatty acids and increase absorption (Derya et al., 2015; Kim and Akoh, 2015). LC-PUFAs are the main forms of edible oils and provide essential fatty acids. However, they are metabolised slowly and tend to deposit in human adipose tissue compared to MCFAs which are more easily and readily digested as they isomerise rapidly and target FFA and glycerol (Christophe, 1998; Kim and Akoh, 2015). Beermann et al. (2003) studied the effects of dietary intake of fat containing 72% MCFAs and 22% n-3 LC-PUFAs on the metabolism of fat in healthy people. It was found that a significant reduction of total plasma TAG was possibly caused by increased  $\beta$ -oxidation with a synergistic effect from MCFAs and LC-PUFAs. Therefore, essential LC-FAs at the sn-2 position with MCFAs at the sn-1, 3 position is utilized most efficiently as they are hydrolysed readily with pancreatic lipase and readily absorbed into mucosal cells and through the intestinal wall (Karupaiah and Sundram, 2007; Kim and Akoh, 2015; Xu, 2000).

### Application of Structured Lipid

Cocoa butter is the main component of chocolate but has a natural limited availability resulting in a high cost. Therefore, alternative replacements for cocoa butter are being explored by modifying lipids. Cocoa butter equivalent is an example of a SL, which has a similar TAG composition to cocoa butter but is produced from low-cost vegetable oil instead.

Human milk fat substitutes are SL with a nutritional application. Resembling human milk fat in terms of the fatty acid composition and distribution, they are produced for use as infant formulas, enteral and parenteral nutrition. Human milk is the main source of nutrients and energy for infants and due to the limited availability of human milk, infant formulas are produced to mimic human milk fatty acid composition where high proportions of palmitic acid are esterified at the sn-2 position to provide fat with improved absorption capability in infants (Kim and Akoh, 2015). Current research on human milk fat substitutes have shifted its focus towards the enrichment of LC-PUFAs such as arachidonic acid, stearidonic acid, eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) as they are converted more efficiently into EPA as compared to the conversion of  $\alpha$ -linolenic acid to EPA in the human body (Bryś et al., 2014; Li et al., 2014; Nagachinta and Akoh, 2012; Pande, Sabir, Baeshen and Akoh, 2013a, 2013b; Simões et al., 2014; Tecelão et al., 2010; Teichert and Akoh, 2011; Turan et al., 2012; Vázquez and Akoh, 2012; Yüksel and Şahin Yeşilçubuk, 2012; Zou et al., 2014).

Shortening and margarine are produced by lipase-catalysed interesterification to produce SL with low-trans or trans-free plastic fats. This method is used to replace the partial hydrogenation process of producing margarine and shortening that results in the

production of *trans*-fats. Low-calorie fats and oils are SL produced through the synthesis of short-chain fatty acids as their caloric values are significantly lower than typical longer chain fatty acid however, to-date there has been no enzymatic production of low-calorie SL commercially. There have been a wide variety of fatty acids used as substrate to produce SL containing health-beneficial fatty acid to obtain a particular functional or nutritional purpose. Interest surrounding conjugated linoleic acid has increased due to its anti-carcinogenic activities, reduced atherosclerosis risk and reduction of in the level of body fat (Brown et al., 2001; Degen et al., 2012; Kritchevsky et al., 2004). Another important substrate with potential health benefits is omega-3 LC-PUFA especially DHA and EPA that could reduce risk of cardiovascular disease, inflammation, cancer, hypertension, immune response, diabetes and renal disorders (Mori and Beilin, 2004). DHA has potential health benefits for brain health and the development of the brain in infants, thus it is used in infant formula (Lien et al., 2018).

## Conclusion

Currently, most research has been undertaken on MLM TAG and other areas including human milk fat substitutes, cocoa butter equivalents and low-*trans* or *trans*-free plastic fats. Studies on structured phospholipids are limited but have shown potential as an efficient carrier of beneficial fatty acids due to the increased absorption of phospholipids in the body (Küllenberg et al., 2012). Research is on-going on various substrates to produce SL but fatty acid composition is not the only factor that contributes to the nutrition properties of fats. The distribution of fatty acids on the glycerol backbone is important as well. Research into nutraceutical applications of SL has been thorough with the introduction of functional MCFA and essential LC-PUFA from different oils and fats. However, the main issue in developing industrial scale production of SL is the high cost of enzymes thus, development of low cost and robust enzymes via genetic modification and directed evolution could open up more opportunities in the application of SL (Song et al., 2005).

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# Application of Electrospinning as Bioactive Delivery System

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## Introduction

Consumer awareness surrounding the relation between food and health has enhanced the interest to research functional foods, which can exert positive health effects beyond the basic nutrition requirements (Menrad, 2003; Sensory, 2014; Siro et al., 2008). However, the development of functional foods can be challenging, as the bioactive compounds, which are responsible for the beneficial health effects, can be susceptible to degradation during storage. Additionally, the low bioavailability of some of these bioactive compounds has been of concern, posing additional obstacles in the research and development of functional food (Aceituno-Nedina et al., 2013; McClements, 2015; Zhao and Tang, 2016). Consequently, finding a proper delivery system that can improve both the stability and bioavailability of bioactive compounds is a major concern in the field of functional foods. Various bioactive delivery methods, such as coacervation (Calderon-Oliver et al., 2017), spray-drying microencapsulation (Zhao and Tang, 2016), liposome entrapment (Istemic et al., 2016) and many others have recently been studied. Most of these methods involves exposure to heat and/or usage of organic solvents in the processing steps, which might lead to the loss of some bioactives and also raise a toxicity concern associated with presence of residual organic solvents and toxic waste disposal (Ghorani and Tucker, 2015; Lopez-Rubio and Lagaron, 2012). Henceforth, extensive research has been conducted on various novel methods that do not utilize high temperature and possibly less or no organic solvents, including electrospinning, which is the main focus of this paper.

## Fundamentals of Electrospinning

Electrospinning has recently gained significant interest in the fields of bioactive encapsulation and functional food development as it is relatively simple, versatile, low cost, non-thermal, and does not use organic solvents (Ghorani and Tucker, 2015; Kayaci and Uyar, 2012; Lopez-Rubio et al., 2012; Neo et al., 2013; Nguyen et al., 2011). Fig. 1 shows an illustration of the electrospinning system consisting of four major components: a metallic needle or capillary, a high voltage power supply (1–30 kV), a grounded conductive collector and a syringe pump that contains the electrospun solution (Zhang and You, 2014; Bhushani and Anandharamakrishnan, 2014).

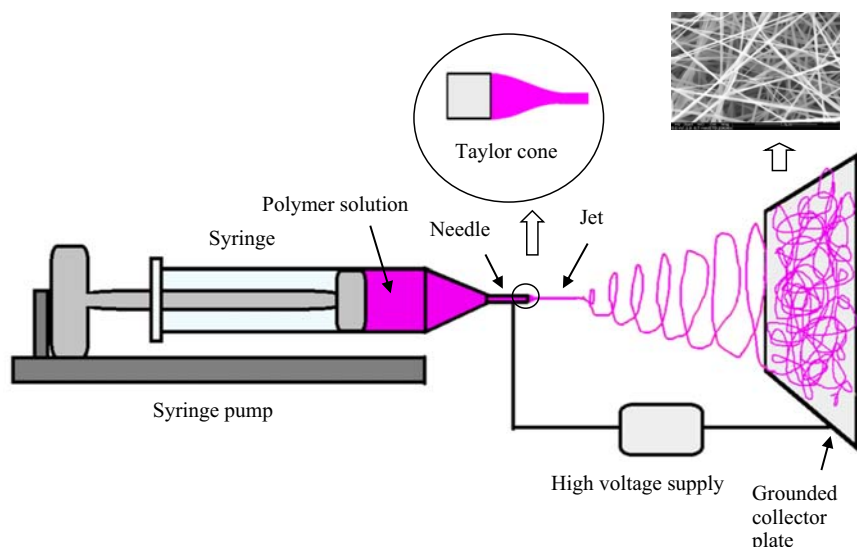
At the beginning of the electrospinning process, the polymer is extruded by the syringe pump into a droplet at the needle tip. Two major electrostatic forces, including the Coulombic force of the external electric field, which is applied between the needle tip and the collector, and the internal electrostatic repulsion of similar charges, distort the droplet's hemispherical surface at the needle tip into a conical shape called the Taylor cone. With the increase of electric field, more electric charge accumulates on the suspended droplet until a critical point is reached, where the electrostatic force of the polymer droplets counteracts the surface tension. A charged polymer jet is then ejected from the tip of the Taylor cone. This polymer jet is driven toward the grounded collector, it is whipped or bent by unevenly distributed charges. As a consequence, the jet is elongated and the solvent rapidly evaporates. Then, the thin and solid fiber with random orientation is deposited on the collector as a non-woven mat which has high surface area to volume ratio (Aytac et al., 2014; Neo et al., 2013; Nieuwland et al., 2013; Bhushani and Anandharamakrishnan, 2014). The formation of the Taylor cone is essential in the electrospinning process because if the cone formation does not occur optimally, nano-droplets might be produced instead of nanofibers or worse, the solution might drip without being converted into nanoparticles (Nieuwland et al., 2013). The diameter of electrospun nanofibers can range from 10 to 1000 nm (Kriegel et al., 2008) with the average diameter ranging from 50 to 500 nm (Nurwaha et al., 2013). Despite the simple installation, the electrospinning process is affected by many factors such as flow rate, viscosity of solution, concentration of polymer, applied voltage, humidity and temperature (Drosou et al., 2017).

Nieuwland et al. (2013) stated that both the formation of the Taylor cone and the efficiency of the overall electrospinning process are highly dependent on the characteristics of the solution and polymer used. The solution to be electrospun should have the optimum conductivity, viscosity, and surface tension as all three parameters directly affect the fiber formation and the overall process (Aceituno-Nedina et al., 2013; Doshi and Reneker, 1995; Zeng et al., 2003; Nieuwland et al., 2013). In addition, the polymer used should be highly soluble and able to entangle and overlap with each other. Therefore, since the types of polymer used highly affect the efficiency of the electrospinning process, the following section will discuss food-grade polymers that have been successfully used for the delivery of bioactive compounds in recent years.

## Food-Grade Polymers Used in Electrospinning

Proteins and polysaccharides are common polymers for electrospun nanofibers (Schiffman and Schauer, 2008). As the carriers of bioactive compounds, the selected polymers should be edible, natural and soluble in non-toxic solvents (Ghorani and Tucker, 2015). Over recent years, many food-grade polymers have been used for electrospinning, namely amaranth protein (Aceituno-Nedina et al.,





**Figure 1** Illustration of the electrospinning system.

2013), cellulose acetate (Wongsasulak et al., 2010), gelatin (Nieuwland et al., 2013), egg albumen (Wongsasulak et al., 2010), whey protein (Aceituno-Nedina et al., 2013; Lopez-Rubio et al., 2012; Nieuwland et al., 2013), and pullulan (Lopez-Rubio et al., 2012; Stijnman et al., 2011). Yet, very limited amounts of them have been used to encapsulate bioactives. On the contrary, some bioactives have also been encapsulated with non-food grade polymers like poly(vinyl alcohol) (PVA) (Kayaci et al., 2014) and poly(lactic acid) (PLA) (Vega-Lugo and Lim, 2009).

### Proteins

Proteins are suitable materials for electrospinning because they are bio-compatible and can enhance the nutritional value of food. However, their complex tertiary and secondary structures cause difficulties in electrospinning, therefore, they need to be present in random coil conformations to be spinnable. For instance, soy proteins must be denatured to unfold their globular structures in order to be successfully electrospun (Shanker et al., 2013). Common proteins applied in electrospinning are zein, whey protein, soy protein isolate, collagen and gelatin (Ghorani and Tucker, 2015).

Zein is the most common material used for electrospinning and has been successfully used to encapsulate compounds including fish oil (Moomand and Lim, 2014), beta-carotene (Fernandez et al., 2009) and gallic acid (Neo et al., 2013). Zein is a hydrophobic corn-based storage protein (prolamin) with high thermal stability and oxygen-barrier properties (Neo et al., 2013). Their nanofibers are lustrous and flexible but have poor mechanical properties, however, this can be significantly improved by crosslinking (Yao et al., 2007). Whey protein is another commonly used polymer for electrospinning. The electrospun nanofibers of whey proteins can be treated with heat to improve thermal stability and decrease water solubility due to crosslinking (Sullivan et al., 2014). The nanofibers formed from Collagen are characterized by high porosity with a wide range of pore size distribution, a high surface area to volume ratio and outstanding mechanical strength (Rho et al., 2006). Gelatin gives similar properties, since it is a collagen derivative (Shanker et al., 2013).

### Polysaccharides

Polysaccharides are often considered as a suitable wall material for encapsulation due to their versatility (ability to react with other substances via various functional groups), temperature stability and large particle size (Fathi et al., 2014). The following polysaccharides have been applied for electrospinning: alginate, cellulose, chitin (and chitosan), hyaluronic acid, inulin, pullulan, dextrans and guar-gum (Lee et al., 2009; Ghorani and Tucker, 2015). Additional treatments or a co-solvent are frequently required before polysaccharides can be successfully electrospun. For instance, glycerol is required as a co-solvent to facilitate the formation of electrospun nanofibers from aqueous alginate (Nie et al., 2008), derivatization of cellulose is required to increase solubility (thus electrospinnability) (Wang et al., 2007; Frenot et al., 2007; Zhao et al., 2004), and solubilisation of pure chitosan using 90% acetic acid is required before electrospinning (De Vrieze et al., 2007; Geng et al., 2005).

Cellulose is the most abundant natural biodegradable polysaccharide and is low cost. The nanofibers produced from cellulose have great thermal stability and mechanical properties, as well as having good stability in the human GI tract and are insoluble in most solvents (Rezaei et al., 2015). Another polysaccharide, chitosan has good bio-functionality (e.g. antibacterial, antioxidant and anti-hyperlipidemic properties) and is biocompatibility with living tissue (Arkoun et al., 2017; Goncalves et al., 2017). Electrospun chitosan nanofibers have high porosity and surface area-to-mass ratio (Arkoun et al., 2017). Sodium alginate is a polysaccharide



from algae with low toxicity, good biocompatibility with living tissue, low cost and non-immunogenicity (Wangkaya et al., 2017). However, sodium alginate will need to be combined with other polymers such as poly(ethylene oxide) or PVA to be electrospun. Electrospinning of food carbohydrate-based systems involving complexes with cyclodextrin (CD) have also been studied. This included electrospinning of vanillin and geradinol/CD inclusion complex in PVA to prolong shelf-life and temperature stability (Kayaci and Uyar, 2012a; Kayaci et al., 2014) as well as incorporated CD into zein nanofibres via electrospinning (Kayaci and Uyar, 2012b).

## Encapsulation of Bioactive Compounds via Electrospinning

Electrospinning has been applied to produce nanofibers capable of entrapping bioactive molecules in foods, such as antioxidants, vitamins, essential fatty acids, and living cells such as probiotics (Table 1).

### Technological Aspect

The main advantage of electrospinning may be related to its ability to produce small sized particles in a one-step process, thus it is relatively cheap compared with other methods (Bhushani and Anandharamakrishnan, 2014). The advantage of the small sized particles is that they may have better controlled release at specific body sites. It is also a non-thermal process (Bhushani and Anandharamakrishnan, 2014) that may be suitable for encapsulating heat-labile ingredients, including probiotic bacteria (Lopez-Rubio et al., 2012). This also demonstrates the versatility of electrospinning as an encapsulation method. In enzyme mobilization, the nanofibers produced via electrospinning have large surface area-to-volume ratio and high porosity to allow mass transfer with less limitation (Wang et al., 2009). Moreover, it is possible to obtain sheet or tubular fibril constructs from the same set up simply by using different collectors (Sill and von Recum, 2008). This latter feature demonstrates its adaptability to different processing requirements and ease of use.

However, electrospinning is a slow process and thus multi-jet systems are required to produce nanofibres on the industrial scale (Varesano et al., 2009). Varesano et al. (2009) further explained that increasing the number of jets may lead to technical difficulties

**Table 1** Bioactive components and the polymers applied in electrospinning

Bioactive component	Wall material/Polymer	Finding	References
Epigallocatechin gallate (EGCG)	Zein	Release of EGCG from fibre is dependent on relative humidity	Li et al., 2009
$\beta$ -carotene	Zein	Higher oxidative stability against UV irradiation	Fernandez et al., 2009
$\beta$ -carotene	Whey protein concentrate 10% and 20% glycerol solution	Higher oxidative stability against UV irradiation	Lopez-Rubio and Lagaron, 2012
Gallic acid	Zein	Thermal stability and retention of antioxidant activity	Neo et al., 2013
Anthocyanin-rich raspberry extract	Whey protein isolate	Enhanced antimicrobial activity and anthocyanin content	Wang et al., 2013.
Chitosan	Zein	Enhanced antimicrobial properties for active packaging and biomedical applications	Torres-Giner et al., 2009
Fish oil rich in omega-3 fatty acid	Zein	Enhanced oxidative stability of fish oil	Torres-Giner et al., 2010
Bifidobacterium	PVA	Increased cell viability at room temperature (20°C) and cold storage (4°C)	Lopez-Rubio et al., 2009
Bifidobacterium	Whey protein concentrate/pullulan	Both polymers could be used to encapsulate the cell; pullulan produced fibre with smaller diameter but WPI enhanced the cell viability at 75% humidity, better than pullulan.	Lopez-Rubio et al., 2012
Lactobacillus acidophilus	Soybean solid waste + 8% PVA	Survival of 78.6% to 90% after 21 storage at 4°C.	Fung et al., 2011
Curcumin	Zein	Improved sustained release, free radical scavenging ability	Drosou et al., 2017
Quercetin and ferulic acid	Amaranth protein isolate and pullulan	Enhanced thermal stability, studied release characteristics and antioxidant protection ability during <i>in vitro</i> digestion	Drosou et al., 2017
R-(+)-limonene	Pullulan/ $\beta$ -CD	Increased storage stability, controlled release	Drosou et al., 2017

and less efficiency. Moreover, some materials may not be readily electrospun due to strong inter- and intra-molecular forces in conjunction with the complex three-dimensional structure of macromolecules and therefore, may require toxic solvents, which are undesirable in the food industry (Aceituno-Nedina et al., 2013; Mascheroni et al., 2013). These two areas require further research. A review by Persano et al. (2013) provides some insights into industrial scaling-up of electrospinning.

## Conclusion

Overall, electrospinning is suitable for a wide range of food systems (carbohydrate, protein and lipid) and is a useful method for encapsulation of food functional ingredients including flavour, antioxidants, enzymes and probiotic bacteria. Moreover, it is easy to use and adaptable to different process requirements, for example it is possible to obtain different fibril orientations from the same electrospinning set up simply by changing the collectors. However, some improvements are required concerning its application on the industrial scale. Further research on more food grade materials is required to optimize its use in the food industry, especially to determine suitable food-grade materials for use with different food functional ingredients.

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# Food Texture, Oral Processing and Satiation: Examining Their Relationship

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## The Texture of Food

The decisions a person makes regarding their food choice, food intake and behaviour are governed by a foods sensory properties (Sorensen et al., 2003), be it the aroma or colour of fruit to check for ripeness, potential toxicity or the a combination of sensorial properties to guide the perceived palatability and enjoyment.

Texture in particular, has a great influence on the acceptability of foods and also in determining quality (Çakör et al., 2012a). However, texture can be a challenging concept purely because it has different meanings to different people, often with different vocabularies used to describe similar sensations. Texture has been defined as “the sensory manifestation of the structure of the food and the manner in which this structure reacts to applied forces; the specific senses involved being vision, kinaesthetic and hearing” (Szczesniak, 2002). Kinaesthetics are described as “those factors of quality that the consumer evaluates with his sense of feel, especially mouthfeel” (Kramer and Twigg, 1959).

The analysis of a foods texture has traditionally meant the instrumental analysis of a foods microstructure and mesostructure or the sensory analysis of texture using a sensory panel. However, a combination of the material properties, oral processing and sensory evaluation of food are required to obtain a complete picture (Wilkinson et al., 2000). Material properties such as rheology and adhesion are commonly analysed in two groups comprising of ‘rheological properties’, which are prior to fracture and ‘fracture properties’, which are at the point of fracture, although these terms are not standard throughout the literature as often the term ‘rheological properties’ is used as a general phrase (Foegeding, 2007).

## Oral Processing and Perceived Texture

In recent decades, it has been acknowledged that oral processing is a crucial component that must not be overlooked when analysing a food’s texture and can give real insight. Oral processing is generally broken down into three different phases: *preparation*, *chewing* and *pre-swallowing* (Guinard and Mazzucchelli, 1996; Wilkinson et al., 2000).

During the *preparation phase* the food is moved from the front of the mouth to prepare for chewing before the teeth close. The aim of the *chewing phase* is to reduce food into particles that are of a size suitable for swallowing. While in the *pre-swallowing phase*, tongue-palate movement moves the food to the back of the tongue without specific jaw movement and forms the bolus. The bolus is eventually swallowed and the process is repeated.

Structural properties, perceived as texture and changes to the food are sensed constantly from the initial bite to the final swallow and modification of the chewing pattern occurs frequently while reducing the food (Mioche, 2004).

Çakör et al. (2012b) described changes in texture as a result of the different stages of oral processing as:

- 1) *First chew*: fracturing of the food between the molars gives the sensation of firmness, fracturability and moisture release.
- 2) *Repetitive chewing*: food is broken down into smaller pieces and chewdown properties are dominant. Chewdown properties include; breakdown rate, particle size, particle distribution, adhesiveness and moisture release.
- 3) *Bolus formation and swallow*: bolus is formed, residual particles are swallowed and moisture is assessed and more lubrication in the form of saliva may be required for an effective swallow.

Considering the chewing variation between individuals during oral processing, many studies focusing on the chewing stage (or mastication cycle) observe masticatory performance (van der Bilt, 2002), which involves evaluation of chewing and particle size reduction and distribution (van der Bilt et al., 2006). During masticatory evaluation the human mouth is little more than a mechanical testing device with a feedback system to prevent damage to the teeth (Foegeding, 2007). The rhythmical sequence of mastication can be measured using methods such as electromyography (EMG) and jaw tracking, which monitor muscular activity and modifications of mandibular forces and movements as a response to the structure of the ingested food.

## Oral Processing, Satiation and Satiety

The amount of food consumed at a meal is controlled by a positive feedback system and negative feedback system. Generally, the orosensory effects in the mouth stimulate the positive feedback system while food entering the stomach and intestines promotes the negative feedback system of satiation signals (Smith et al., 1990; Yeomans, 1996, 2008). Satiation results in the termination of eating while satiety results in the halt of further eating, the decline in hunger and increased fullness post meal (Blundell et al.,

2010). Appetite control by increasing satiation onset or prolonging satiety is of great interest as the overconsumption of food during a meal is a factor in weight gain and obesity (Ebbeling et al., 2004; Louis-Sylvestre et al., 2003). The 'satiety cascade' (Fig. 1), which was initially proposed by Blundell et al. (1987) and modified by Mela (2006) shows how the composition of macronutrients, energy density, sensory attributes and food structure all add to satiation and satiety modulation (Blundell et al., 2010).

The nutritional components of food can have a bearing on satiation and satiety but will only briefly be covered here. General consensus is that consuming foods that are high in fibre promotes satiation and prolonged satiety because stomach distension is increased, which causes a delay in gastric emptying (Howarth et al., 2001). There is also evidence that protein promotes satiation and increases satiety (Lejeune et al., 2006), but as with fibre the background food matrix has an influence on these effects (Slavin, 2005). There has been a considerable amount of research also undertaken concerning peptides from the gastrointestinal tract, which are released as a result of food intake and have various physiological roles in food management (Blundell et al., 2010). It is believed that the intermediate postprandial state is typified by hormonal variations such as a decrease in the orexigenic peptide ghrelin and a simultaneous increase in anorexigenic peptides such as glucagon-like peptide (GLP)-1, cholecystokinin (CCK) and Peptide YY (PYY) (Chaudhri et al., 2006). These peptides have key roles in mediation of satiety, hunger and energy intake by acting on the brain and vagus nerve (Cummings and Overduin, 2007) and thus are often used as satiation biomarkers (de Graaf et al., 2004).

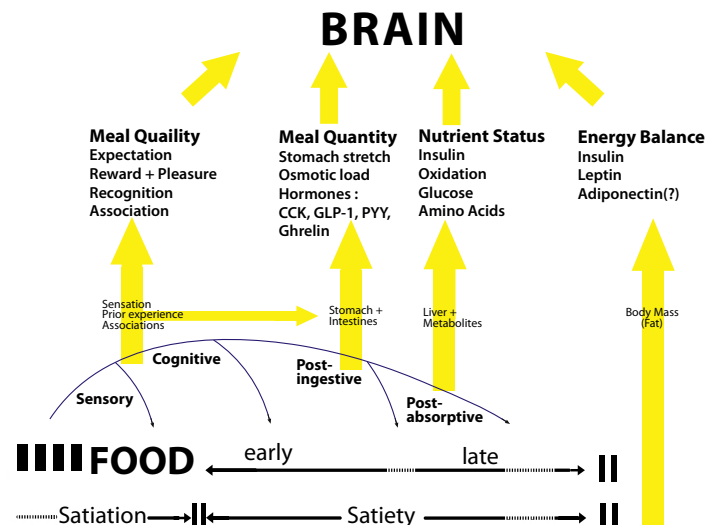
Current thought is that oral processing time (or oral transit time), the time that food has in contact with the oral cavity has some bearing on the relationship between food structure, sensory perception (texture) and satiation and satiety but how this arises is unclear (Campbell et al., 2017; James, 2018). During oral processing, a complex mechanism of interactions occur and being able to prove a link between any number of these is extremely difficult.

An increasing body of literature has shown that the bite-size, eating rate and food phase; liquid versus solid, can influence satiation and satiety and indeed all exhibit an impact on orosensory processing time (de Graaf, 2012). Studies have shown increased satiation and/or satiety by manipulating viscosities of dairy-based drinks (de Wijk et al., 2008; Zijlstra et al., 2008, 2009) with increased oral processing time decreasing the amount of viscous or semi-solid foods consumed. This has repeatedly been shown in the literature in a number of different foods (Bolhuis et al., 2011, 2014).

The difference in satiation response between a liquid, semi-solid or solid form of the same food could be attributed to effort required to bite, chew or swallow the food, with general consensus being the latter requires the most oral processing effort. This hypothesis has not been studied in great depth, although one such study showed that with bite effort eliminated there is no difference in the amount of the food consumed regardless of it being in a liquid or semi-solid state, in this case using chocolate dairy-based beverages of different viscosities (de Wijk et al., 2008). Contrary evidence has been found though, where solid foods with more work required during oral processing having a less satiating effect (Pentikäinen et al., 2017), highlighting the need for more research in the area.

There is also a school of thought that the longer the oral processing time, the greater the Cephalic Phase Response (CPR) (Smeets et al., 2010) such as the production of CCK and GLP-1 hormones as a part of the satiety cascade (Fig. 1).

Although there seems to be some compelling evidence on oral processing time and its effect on satiation and satiety, is it as simple as the effort required to chew a food or the time that it takes to swallow a food all that is required to be manipulated in order to trigger this response and limit food intake? There are many fundamental factors that dictate the oral processing pathway of a food and thus its oral processing time. Going back to the basis of this poses the question, does the structure and texture of a food have more of a role than currently thought and not just as a means of increasing oral processing time but in terms of activating the



**Figure 1** Satiety cascade modified from Blundell, et al. (2010) originally modified by Mela (2006).

perceived sensations one feels while eating and how these may affect the satiation or satiety response. Thus, the complexity of the texture of a food is becoming increasingly significant to this field of research.

### Textural Complexity and Satiation: Is There a Link?

The idea behind textural complexity is that most foods give the person eating them a range of different sensory sensations at once and thus the texture of a food can be difficult to describe using just one or two descriptors. Using sensory evaluation tools such as Temporal Dominance of Sensation (TDS) has allowed the understanding of the whole experience of oral processing by mapping changes in dominant sensation over time. It has also let us further understand the differences that individuals experience between one another when eating the same food. As often there is only consensus of the dominant textures at the beginning and end of oral processing amongst people, with the middle part quite different (Blundell et al., 1987).

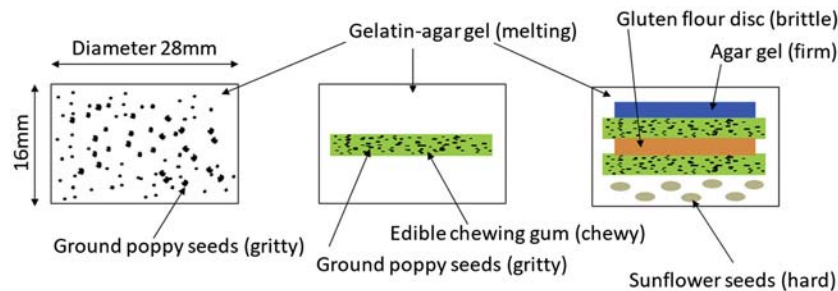
The proposed definition of “textural complexity” in its simplest form is “a succession of perceived textures from first bite to the point of swallow” (Larsen et al., 2016a). When studying textural complexity, it is important that oral processing time is not examined as the driving factor of satiation or satiety, rather it is the textural make up of the test food, be it a “real” whole food or a model food designed to create a specific texture profile.

A series of recent studies on textural complexity were undertaken using model gels with varying levels of textural complexity built into them by way of embedded inclusions (Larsen et al., 2016a, 2016b, 2016c; Tang et al. 2016, 2017) (Fig. 2).

These were made by a careful selection of ingredients to give varied and noticeable textures. A low complexity gel and high complexity gel were produced and tested via sensory panels and using TDS for observable texture attributes and for confirmation that the two samples were indeed of a low textural complexity and a high textural complexity. Throughout the R&D phase, chewing rate, chewing time and the number of chews required to swallow were equilibrated so that oral processing time was not a variable. Satiation was measured using a 2-course *ad libitum* meal after the consumption of the low or high complexity model gels as a preload in a single-blind randomized cross over study. Over two separate trials, the panelists who had consumed the high-complexity preload went on to consume significantly less of the *ad libitum* meal overall.

### Textural Complexity as a Future Tool in the Food Industry

Research on the effect of textural complexity on satiation and satiety is still in its infancy and there are clearly other factors that need to be researched to provide a better picture of the mechanism behind this potential link. However, if substantiated, textural complexity may be a promising tool to be used by food manufacturers to create new products with purpose built texture profiles in a bid to cut down food intake by triggering faster satiation responses. This is highly topical due to the escalating obesity epidemic. The World Health Organisation estimates that in 2016 more than 1.9 billion adults were overweight and 650 million adults in this group were obese, with a tripling of obesity prevalence between 1975 and 2016 (World-Health-Organisation, 2018). Even more concerning, is the rates of overweight and obesity in children under 5, estimated at 41 million (World-Health-Organisation, 2018). Overweight and obesity are caused by a number variables, sometimes due to medical reasons but often poor food choices in terms of nutritional quality or the quantity of food being consumed are driving factors along with sedentary lifestyles. Reducing food intake is one realistic way of combating the epidemic from the ground up and this is where a greater understanding of the satiation and satiety response could be used in a bid to improve overweight and obesity outcomes by manipulating peoples responses through the types of foods they eat.



**Figure 2** Gel-based model foods of low and high complexity (James, 2018).



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# Food Sensory Perception Influenced by Structure and/or Food–Saliva Interactions

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## Introduction

It has been long considered that the study of food, Food Science, ranges from raw material production and processing through to digestion and absorption inside the human body. For oral consumption of any type of food, ingestion is the very first step. The sensory experience and/or perception is one of the most important factors influencing consumers' preference of a food product and their intention of purchase. There is no record of when the earliest study of food texture took place, however, it is generally accepted that systematic study of food texture began in the second half of the last century and became an important discipline in the study of food science. Attributes related to food texture, including the elasticity, viscosity, adhesion and many others, for solid, semi-solid and liquid foods were objectively studied using empirical and fundamental techniques and instrumental measurements, then correlated with consumers' sensory perception (Sherman, 1969; Shama et al., 1973; Shama and Sherman, 1973; Christensen, 1979; Cutler et al., 1983). With the technological advances and the integrations of oral physiological studies, other aspects of food oral consumption such as food comminution and swallowing, food structural breakdown and mastication, etc., have become hot topics of food texture research in recent years (Agrawal et al., 1997; Dobraszczyk and Vincent, 1999; van Vliet, 2002; Lucas et al., 2004).

## The Complexity of Saliva

Sensory perception of consumed food does not only depend on the properties of the food, but also on how food is deformed and the distinct structural changes that happen in the mouth (Janssen et al., 2007). The incorporation of saliva drastically alters the physical, biochemical, rheological and tribological properties of food during oral processing, due to not only the diluting effect but more often the enzymatic breakdown of food components. The formation of a food bolus facilitates easy swallowing, but also changes flavor and aroma release (Engelen et al., 2003a,b; de Wijk et al., 2004).

Saliva is a bio-fluid secreted mainly from parotid glands, sublingual glands, submandibular glands and other major glands, which makes up to 90% of the total secretion, along with other small salivary glands. Saliva is generally described as 'tasteless and colorless', consisting of about 99% water, with the rest being electrolytes, proteins, enzymes, carbohydrates etc. 'Colorless' is an objective description; 'tasteless' on the other hand, is not. As a matter of fact, saliva must have a taste otherwise one won't be able to taste or detect real tasteless substances such as water. The reason that saliva is perceived as tasteless is because that saliva is continuously tasted by oneself all the time and functions as the base or the reference for one's sensory perception of any food. Saliva secretion is affected by factors such as medications, neurobiological activities, environmental changes including weather, temperature, humidity etc. Age, mood and diet also influence saliva secretion. Saliva secretion and composition is also affected by if and how secretion is stimulated (stimulated vs. non-stimulated; different stimulation methods). It is now generally accepted that when stimulation is triggered, either physical stimulations (mastication) or chemical/biological stimulations (smell or eat lemon for example), saliva secretion increases in total amount, as compared to resting saliva secretion.

Mejean et al. (2015) analyzed sensory perceptions of lipid, salt and sweet taste among French populations in relation to their saliva compositions and observed a close relationship between sensory perception and one's saliva flow rate and food composition, especially the concentration and variety of salivary enzymes. When using a basic stimulating tastant, Neyraud et al. (2009) demonstrated that, with citric acid stimulation, the saliva secretion was affected to the greatest extent, compared to other stimulations such as using sucrose solutions (sweet taste), NaCl solutions (salty taste), monosodium glutamate (umami taste), or MgSO<sub>4</sub> (bitter taste). When using real food as stimulation, Engelen et al. (2003a) had shown that, despite the stimulation approach, subjects' sensory evaluations for semi-solid foods were not affected by one's saliva flow rates. More interestingly, they designed a follow-up study (Engelen et al., 2003b) in which they added saliva or saliva-like fluid ( $\alpha$ -amylase solution and water) on the spoon below custard and then evaluated subjects' sensory perceptions of odor, flavor and after-feel sensations. They found that a number of textural attributes such as thickness, creaminess and fattiness after-feel were affected by the liquid addition. However, the distinctive attributes of the custard such as almond odor and vanilla flavor were not effected, this also might be attributed to the small volumes of liquid added (0.25 and 0.5 mL, respectively).

The physical properties of saliva have also attracted a lot of research interest. It is now well accepted that saliva secreted from different glands has distinct rheological properties; parotid saliva contains very little mucins and behaves like Newtonian fluid, while sublingual and submandibular saliva have a shear thinning non-Newtonian behavior (van der Reijden et al., 1993). When stimulated differently (chewing gum or citric acid mouth wash for instance), the rheological properties of acquired saliva varied (Stokes and Davies, 2007), let alone the differences in other biochemical and physiological properties (Engelen et al., 2006). The friction occurrence during food oral processing is an additional complexity (Mosca and Chen, 2017). The binding of alkaline proline-rich salivary proteins to polyphenols in food caused protein precipitation and thus increased friction,

a phenomenon commonly seen in consuming unripe fruit or high quality red wines (Brossard et al., 2016). Similar to fat sensation, de Wijk and Prinz (2005) observed that the fat content, which directly correlates with the roughness, creaminess and friction, affected the lubrication status in the mouth, hence a variation in the fat oral texture appreciation.

### Impact of Food Structure on Sensory Perception and Oral Processing

The structure and microstructure of food is clearly a crucial parameter affecting the sensory perception. Kim et al. (2015) found that when ingesting a mix of smaller particles with larger ones, the smaller particles have significantly fewer chances to be selected for mastication. During oral processing they observed gelatin-based gel particles of smaller sizes remained intact and had a relatively shorter oral processing duration. In addition to the food size influence, the structural complexity of the food also alters sensory perception. By using either emulsion-filled gels made of different mechanical properties (de Lavergne et al., 2016) or complex gels with 'built-in' layers (Larsen et al., 2016), researchers all found distinct sensory responses compared to normal and simply-structured food. With higher complexity (adding extra seeds into the food layers for instance), greater food sensory intensity ratings were obtained for common sensory attributes and an even a greater perception of a foods' textural complexity was attained. Interestingly, based on researchers' observations, the foods' complexity seemed to induce a different pathway of food oral breakdown thus a continuously changing bolus during mastication.

The structural change of food during oral processing is also effected by the saliva in mouth. Food gets instantly wetted and coated with saliva once ingested into the mouth (Mosca and Chen, 2017). During oral breakdown by mastication, food particles are either dissolved in saliva or wetted by saliva. During this process, saliva facilitates the diffusion of taste and aroma compounds and serves as an effective media for taste and aroma release. Simultaneously, with the help of tongue manipulation, saliva facilitates the clustering and aggregation of food particles to form a bolus suitable for swallowing. If less saliva is present in the mouth, or the food products are dry and/or porous in structure, longer oral processing time (with more saliva incorporation) is typically needed (Mioche et al., 2003; Gaviao et al., 2004; Drago et al., 2011; Tarrega et al., 2011).

### Impact of Oral Physiology on Sensory Perception and Food Oral Processing

There is no doubt that the sensory perception and eating experience of a food product has an indivisible relationship with saliva secretion, which is largely dominated by our neural system. Lucas et al. (2004) made it very clear that, despite the progresses made by food scientists and oral physiologists, more common ground could be discovered and achieved to further elevate our understanding of the mechanisms of food oral sensation and perception. A convenient example would be a study conducted by Keesman et al. (2016). Their carefully designed experiments showed that the antiquated jokes of quenching one's thirst by looking at plums or to fulfill hunger by drawing a pie in the sky have indeed scientific grounds. By simply imaging in the brain consuming an attractive or sour-taste food, a significant increase in the saliva secretion could be observed.

The physiological aspects of food oral consumption also include how food is manipulated in the mouth by the actions of the tongue and other jaw and oral muscles (Prinz, 1999; Prinz and Lucas, 2001; Schimmel et al., 2007; van der Bilt et al., 2010). When comparing the degree of oral movement complexity during food consumption, one study has shown that the normal oral behavior has the highest degree of oral movement complexity. Once tongue movement is restricted (such as forcing it to hold food still then swallow, or hold the food up against the hard palate then swallow, etc.), the intensity of one's sensory perception decreases (de Wijk et al., 2003). A recent investigation of the oral muscle and jaw activities demonstrated different food breakdown pathways for various types of food (Le Reverend et al., 2016). The difference in initial food properties, largely attributed to differences in the manufacturing process (baking or extrusion for example), would partially contribute to how a food is manipulated during mastication in the mouth. With this in mind, a possible 'family of certain foods' with different textural properties (during manufacture) might be of more interest to predict subjects' chewing behavior and the corresponding sensory perception.

The large oral variations of physiological and habitual behavior amongst individuals, together with the difficulties to obtain objective data from panelists during sensory evaluation, have inspired engineers to work on developing artificial or simulated mouth models with controlled parameters and conditions to mimic an eating process. From the mechanical mastication process (focusing on the clenching and grinding of the jaw during oral processing) (Takanobu et al., 1998) to the saliva-containing *in-vitro* simulation of food oral processing (Prinz et al., 2007), more features were included to reveal how different foods behave differently during oral processing. The devices can be either derived from a fermenter-like chemostat (with temperature and pressure control) equipped with an appropriate rotational apparatus and a set of pressing model teeth (Arvisenet et al., 2008; Poinot et al., 2009), or a made-from-scratch 'steel block' equipped with a simulated dental arch, with controlled shear and compression stress, regulated food comminution and bolus formation functions (Woda et al., 2010). The functionalities and reliability of these devices have been validated recently by Mishellany-Dutour et al. (2011), who compared properties of the bolus collected from these simulated devices with those prepared *in vivo*.

## Concluding Remarks

There is indeed much more research to be done to reveal the underpinning mechanisms of food oral processing and sensory perception. Saliva, as a complex and individual-dependent bio-fluid needs to be further studied from different perspectives, including its cross-interactions with solid, semi-solid and liquid foods and the corresponding sensory and aromatic perceptions (Ployon et al., 2017). It is of necessity to consider the multi-functions of saliva, such as its dilution effect, protein and enzymatic reactions with food. How the tongue behaves during oral processing, especially with different types of food still needs extensive investigation. It is now generally accepted that friction occurrence during food oral processing has a great significance on sensory perception, but the magnitude variation of the stress and friction between the tongue and the palate, teeth and other oral surfaces generated during food breakdown are still largely unclear. Furthermore, the triggering criteria and mechanisms of bolus swallowing remain largely a mystery. Research into this requires joint efforts from food scientists, oral physiologists, neural scientists and clinical doctors. With an elderly population in many countries around the world, research into their health concerns from a food science perspective has become a hot research topic. A recently developed concept of ‘oral comfort’ by Vandenberghe-Descamps et al. (2017) emphasizes the importance of appropriate chewing and easy/safe to swallow to the well-being of elderly individuals. Saliva-induced food moistening, softened texture and others are among the elements of their validated questionnaire for oral comfort assessment. All are aspects to keep in mind when looking into the future of research on food oral processing.

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# How Food Structure and Processing Affect the Bioavailability of Nutrients and Antioxidants

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## Glossary

**Anti-nutritional factors** Natural or synthetic compounds that interfere with the absorption of various nutrients (such as vitamins or minerals) in human body. For example, phytate and oxalate that can interfere with the absorption of minerals, such as calcium, iron, magnesium and zinc.

**Bioaccessibility** Fraction that is released from food matrix and is available for intestinal absorption

**Bioavailability** The fraction of ingested nutrient that is available for utilization in normal physiological functions and for storage.

**Bioactivity** The effect of a given agent, such as food, drugs or vaccine, in a living organism or on living tissue.

**Carotenoids** A family of lipid-soluble plant pigments providing red, yellow, green and orange colors to fruits and vegetables. The main carotenoids studied are lycopene,  $\beta$ -carotene, lutein, zeaxanthin and  $\alpha$ -carotene.

**Encapsulation** A process to entrap bioactive compounds within a carrier material as a useful tool to improve delivery of bioactive compounds or living cells into food. It is a technology where the bioactive components are completely enveloped, covered and protected by a physical barrier.

**Excipient foods** A food specifically designed to increase the bioavailability of bioactive compounds

**Nutraceuticals** Any product derived from natural food sources with extra health benefits in addition to the basic nutritional value in foods. They can be considered non-specific biological therapies used to promote general well-being. The term "nutraceutical" combines two words – "nutrient" (a nourishing food component) and "pharmaceutical" (a medical drug).

**Phytochemicals** Non-nutrient plant chemicals that have protective or disease preventive properties in human. There are more than thousand known phytochemicals.

## Introduction

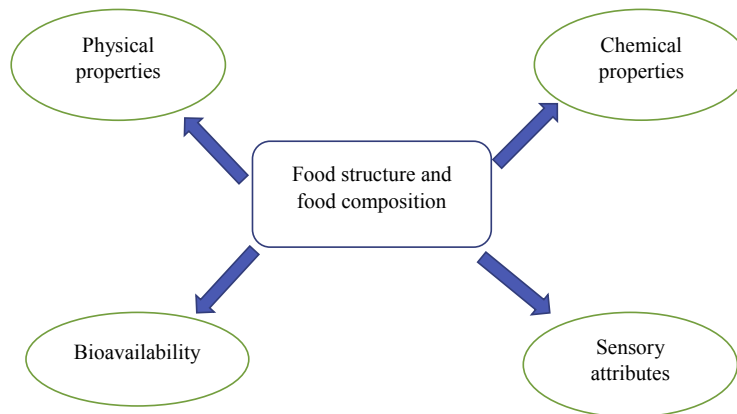
Nutrition is thought to play a major role in the primary and secondary prevention of non-communicable diseases (NCDs). Aside from pleasure, we eat to obtain nutrients to sustain our daily activity. Various epidemiological studies have suggested a relationship between the incidence of NCDs, such as diabetes, obesity and cardiovascular disease with the daily consumption of certain foods (Micha et al., 2017; Mueller and Appel, 2017). Modern consumers have become increasingly health conscious about the relationship between diet and health. Hence, the food industry has invested a great amount of time and money to develop foods with enhanced nutritional and health properties to keep up with the evolving preferences of consumers. Many new designed food products with attractive health properties, such as low sugar and fat, and greater bioavailability of phytochemicals are now available in the market (Sun-Waterhouse, 2011).

The natural food matrices (chemical structures) and man-made microstructures of manufactured foods have been shown to influence how nutrients from the ingested food becomes available for absorption in the human body. This happens because food matrices have significant effects on the nutritional quality (Jacobs and Tapsell, 2007; Parada and Aguilera, 2007). Moreover, the bioavailability of nutrients also depends on various factors, such as chemical state of the nutrient, interaction with other food components, presence of anti-nutritional factors, and their release from the food matrix. Although the nutrient content of natural and fortified foods can be obtained easily from international food composition databases, the understanding of the effects of processing and food structure on nutrients and phytochemicals, and hence, their bioavailability to exert physiological actions in the human body is limited (Cilla et al. (2017); Bohn et al. (2015); McClements and Xiao, 2014; Parada and Aguilera, 2007). In this chapter the effect of different food structures in relation to their physicochemical properties on nutrient bioavailability in the human body, is discussed. Examples of specifically developed food matrices to improve nutrition are also covered based on work from the last 10 years.

## Relationship Between Food Structure and Nutrition

The bioavailability of nutrients and phytochemicals are important determinants for the physiological health of the human body. The microstructure of food is a major factor governing the bioavailability of various nutrients and phytochemicals (Jacobs and Tapsell, 2007; Parada and Aguilera, 2007) (Fig. 1) Foods can consist of macromolecules, such as proteins and polysaccharides, plus



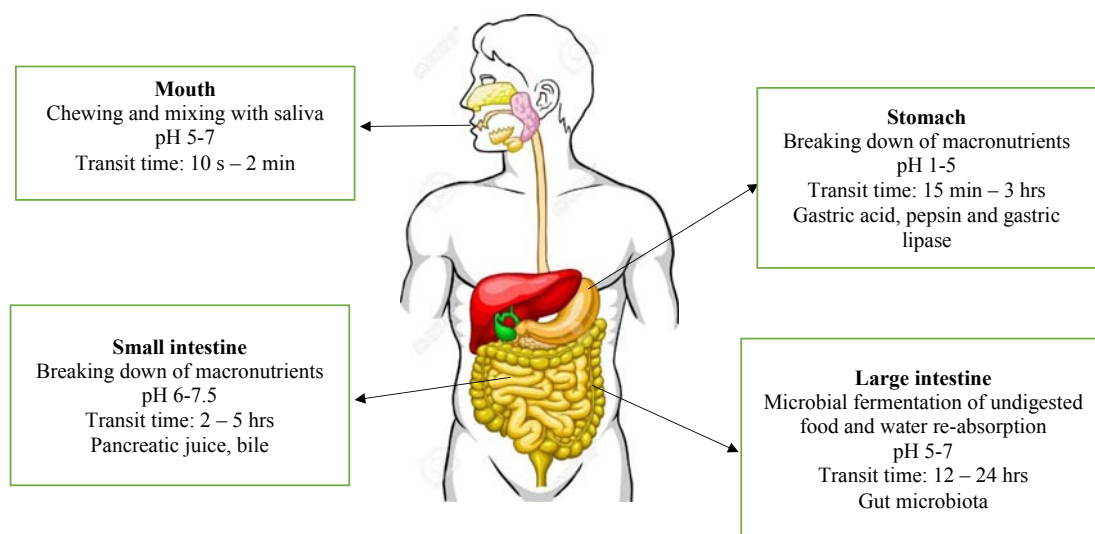


**Figure 1** The effect of different food structure and composition on human nutrition.

lipids, sugars, micronutrients, phytochemicals, and water. These components are arranged in different food matrices naturally or due to processing. Food processing, including fermentation, cooking, and grinding has been shown to improve bioavailability of nutrients and phytochemicals from plant foods since these processes disrupt the plant cell walls, releasing the nutrients stored within the plant organelles (Chang et al., 2013). The bioavailability of bioactive compounds is affected by digestion in the gastrointestinal tract, which includes transformation, transport and absorption at different sites (stomach, small intestine and large intestine) (Fig. 2). The following section discusses the molecular structure of some macronutrients in foods and their impact on the bioavailability of nutrients and phytochemicals.

## Proteins

Proteins exist as different molecular structures with differing chemical and physical properties (Phillips and Williams, 2011). Protein molecules are extremely sensitive to heat, pH and mechanical processes. Heating has been shown to affect the structure of milk  $\beta$ -lactoglobulin, increasing the rate of digestion by the enzymes, trypsin and chymotrypsin at 37 °C. However, the digestion rate was reduced after heating at 78 °C. While, high pressure treatment at 400–800 MPa enhanced the digestion of  $\beta$ -lactoglobulin by pepsin (Chicón et al., 2008; Zeece et al., 2008). It has been observed that protein from milk, which has undergone sterilization has greater resistance to GI digestion compared to unheated milk due to heat induced protein aggregation in milk with the formation of soluble and micelle-bound aggregates (Almaas et al., 2006; Dupont et al., 2010). Specifically, milk proteins, such as casein are susceptible to hydrolysis with or without heating, while  $\beta$ -lactoglobulin is susceptible to hydrolysis after heat treatment (Barbé et al., 2014). Heating at 100 °C can modify the primary structure of proteins by isomerization and destruction of amino acids (arginine, cysteine, isoleucine, lysine, threonine) (Bax et al., 2013).



**Figure 2** Influence of different specific conditions in the digestive tract which influences the digestibility of food and hence, the bioavailability of nutrients and bioactive compounds.

## Carotenoids

Carotenoids are important lipid-soluble antioxidants with numerous health-promoting activities. [Lemmens et al. \(2014\)](#) have published a comprehensive review on the bioaccessibility of carotenoids from fruits and vegetables influenced by food structure and processing in the presence of lipids. Generally, the bioavailability of carotenoids from cooked vegetables (50%) are much higher than raw vegetables (10%) ([Saini et al., 2015](#)). Similarly, processing may enhance the bioavailability of lycopene from the plant cells matrix. [Omoni and Aluko \(2005\)](#) demonstrated that the bioavailability of lycopene in humans is significantly higher in tomato paste compared to raw tomatoes. In contrast, ultrasound treatment reduced the bioaccessibility of lycopene from tomato pulp ([Anese et al., 2013](#)).

A study conducted by [Netzel et al. \(2011\)](#) demonstrated that the bioavailability of  $\beta$ -carotene in cooked and pureed carrots is significantly higher compared to uncooked carrot. Similarly, high pressure homogenization enhanced the bioaccessibility of  $\beta$ -carotene from carrot puree ([Knockaert et al., 2012](#)). However, high-pressure homogenization reduced the bioaccessibility of carotenoids (lycopene and  $\xi$ -carotene) in tomato pulp while the bioaccessibility of the xanthophylls (lutein, zeaxanthin) have increased ([Panozzo et al., 2013](#)). In another scenario, bioaccessibility of various carotenoids from orange juice increased significantly after industrial processing compared to hand-squeezed orange juice ([Stinco et al., 2012](#)). The bioaccessibility of free and esterified carotenoids from green and red pungent peppers was reduced after grilling and boiling. However, xanthophylls have higher bioaccessibility after grilling and boiling ([Victoria-Campos et al., 2013](#)).

## Phenolic Compounds

The daily routine of peeling fruits and vegetables resulted in significant loss of phenolic compounds because a large amount of these compounds are found in the skin rather than the pulp ([Parada and Aguilera, 2007](#)). It has been reported that flavonol contents of canned or frozen foods from onion, kale, apple and beans are significantly lower than the amounts found in fresh food ([Aherne and O'Brien, 2002](#)). However, processing of tomatoes contributed to higher flavonol bioavailability in tomatoes ([Stewart et al., 2000](#)). It was demonstrated that rutin from tomato puree had higher bioavailability even when the portion size was smaller ([Simonetti et al., 2005](#)). This shows that purees could be a good source of phenolic compounds, compared to natural foods.

[Sanz and Luyten \(2006\)](#) studied the matrix effect by determining the release of isoflavones from custard desserts made from starch or carboxymethylcellulose. They found higher amount of isoflavones were released from custards made from starch compared to custards made from carboxymethylcellulose. Presumably, because custards made from carboxymethylcellulose were more resistant to enzymatic digestion. [Nagah and Seal \(2005\)](#) demonstrated that cooking affected the antioxidant capacity of wholegrain foods by destroying the water-soluble antioxidant compounds, while the antioxidant compounds bound within the food matrix are not affected.

Cocoa is rich in phenolic compounds, as is its main product, chocolate. Processing, such as fermentation and drying reduced the total amount of flavanols in cocoa beans ([Parada and Aguilera, 2007](#)). [Serafini et al. \(2003\)](#) reported that the bioavailability of chocolate flavonoids was significantly lower in milk chocolate as compared to dark chocolate, suggesting that lipids and proteins from milk chocolate interacted with flavonoids when they are metabolized in the GI tract ([Serafini et al., 2003](#)). However, others suggested that the differences in the bioavailability of flavonoids from dark and milk chocolate was because of the food matrix interfering with the absorption kinetics instead of flavonoids-milk protein and lipid interactions ([Roura et al., 2007](#)). [Roura et al. \(2007\)](#) studied the possible interaction of milk on the bioavailability of (–)-epicatechin from cocoa powder in healthy humans. They found the presence of milk does not reduce the bioavailability of polyphenols and presumably their health effect in preventing various diseases ([Roura et al., 2007](#)). [Roura et al. \(2008\)](#) extended their studies to determine the effect of milk on the excretion of cocoa metabolites in urine. Results indicated that milk consumption does not significantly affect the total amount of metabolites excreted in urine. [Mullen et al. \(2009\)](#) demonstrated that milk didn't affect the plasma pharmacokinetics of an (epi)catechin-O-sulfate and had no effect on O-methyl-(epi)catechin-O-sulfate. However, the presence of milk significantly lowered the excretion of urinary flavan-3-ol metabolites. Similarly, [Urpi-Sarda et al. \(2010\)](#) discovered that milk partially affects the amount of urinary phenolic acids (protocatechuic, 4-hydroxybenzoic, 4-hydroxyhippuric, hippuric, caffeic and ferulic acids) derived from the colonic degradation of procyanidins and other compounds present in cocoa powder.

## Other Nutrients

Human studies conducted by [Verwei et al. \(2003\)](#) and [Verwei et al. \(2005\)](#) demonstrated that the bioavailability of folate (vitamin B<sub>9</sub>) was lowered with the presence of folate binding proteins [such as, pteroylmonoglutamic acid (PGA)] in fortified milk products. This shows that the food matrix can have significant effect on the bioavailability of folate after GI digestion. [Höller et al. \(2006\)](#) demonstrated that the type of food matrix influences the amount of biotin in food, capsules and premixes. Vitamin E is an important lipid-soluble vitamin with strong antioxidant capacity. It has been shown that the absorption of vitamin E is influenced by the amount of fat and the food matrix ([Reboul, 2017](#)). Recently, [Kim et al. \(2016\)](#) determined the effect of adding cooked whole egg to a raw mixed-vegetable salad on the absorption of vitamin E isomers,  $\alpha$ -tocopherol and  $\gamma$ -tocopherol using a randomized cross-over

trial. Results demonstrated that the consumption of cooked whole eggs helped to enhance the absorption of  $\alpha$ -tocopherol and  $\gamma$ -tocopherol from raw mixed-vegetable salad by healthy young men.

### Phytochemicals and Their Corresponding Antioxidant Activities

Phytochemicals, especially the phenolic compounds are non-nutrient bioactives, being the products of secondary metabolism in plants that have essential functions in plant physiology (Halliwell, 2012; Shahidi and Ambigaipalan, 2015). Polyphenols are the most well-known phytochemicals for their antioxidant capacities (Shahidi and Ambigaipalan, 2015). Nowadays, data from evidenced-based reviews supporting the notion that phenolic compounds from sources, such as fruits and vegetables have the potential to alter the risks of various diseases in humans (Chang et al., 2016a,b, 2018; Slavin and Lloyd, 2012). However, the findings from most of the studies on bioavailability of dietary polyphenols demonstrated that most of the polyphenols have low bioavailability (Halliwell, 2012, 2013; Quideau et al., 2011). Manach et al. (2005) demonstrated that absorption of phenolic compounds by the GI tract is between 1% and 5% of the amount taken from normal diet. The antioxidant polyphenols are poorly absorbed and extensively metabolized to non-antioxidant metabolites (Tomás-Barberán and Andrés-Lacueva, 2012). Even though some of the dietary polyphenols are poorly absorbed and rapidly metabolized, they are still the valuable bioactives in prevention of NCDs (Halliwell, 2013, 2013; Quideau et al., 2011). The low bioavailability of phenolic phytochemicals may be due in part to their low aqueous solubility and poor stability in the GI tract. In view of this, various ways have been developed to enhance the absorption, bioavailability and bioactivity of phytochemicals.

### Phytochemicals in Designed Food Matrices for Delivery

Macronutrients, such as lipids, carbohydrates and proteins have been used to enhance the bioavailability of bioactive compounds. McClements et al. (2015) have reviewed representative studies on the influence of excipient foods (carbohydrates, proteins, lipids, minerals and food additives) on the bioavailability of phytochemicals from various sources (Table 1). Martínez-Huélamo et al. (2015) demonstrated that the bioavailability of carotenoids from tomatoes increased when they were consumed with digestible lipids. The bioavailability of procyanidins from cocoa have also increased significantly when they are consumed with digestible lipids (Ortega et al., 2009). It has been reported that lipid-soluble bioactive compounds have lower bioaccessibility when they are consumed with non-digestible lipids, because the bioactive compounds were trapped in the non-digestible lipid droplets (Qian et al., 2012; Rao et al., 2013). The bioaccessibility of lipid-soluble bioactive compounds has been shown to be higher with small lipid droplet sizes, to be higher for liquid oils compared to solid fats, and to be higher for emulsifiers which enhances digestion in the GI tract (McClements et al., 2015). For example, curcumin can degrade in both neutral and alkaline pH environments in the GI tract (Heeger et al., 2014). Hence, the stability of curcumin could be enhanced by entrapping within the inner surface of large lipid droplets which will be digested slowly in the GI tract.

Carbohydrates, whether they are simple sugars or polysaccharides can affect the bioavailability of bioactive compounds through numerous mechanisms. For example, polysaccharides have been shown to affect the digestion and absorption process of lipids throughout the GI tract, by preventing the interaction of enzymes, such as lipase. Hence, the rate of lipid digestion would be affected (Espinal-Ruiz et al., 2014; Zhang et al., 2015). Mun et al. (2015a) found that lipids incorporated in starch-based hydrogels had higher bioavailability of  $\beta$ -carotene with higher lipid digestion rate. Possibly this happens because starch is able to prevent lipid molecules forming aggregates in the GI tract, increasing the accessibility of lipase.

Zhu (2017) has reviewed various methods of starch-based encapsulation systems to enhance the delivery of food ingredients. Vitaglione et al. (2013) encapsulated cocoa polyphenols in high amylose maize starch and tested the bioavailability of these encapsulated polyphenols in a randomized crossover trial. Encapsulation reduced the bioavailability of cocoa polyphenols as compared with that of polyphenols in a cocoa-nut cream. However, the encapsulated ingredient increased the delivery of flavanols into the gut of the human subjects (Vitaglione et al., 2013). In another study, Cohen et al. (2011) evaluated the bioavailability of genistein encapsulated in amylose inclusion complexes using simulated intestinal conditions in rats. Results showed that the genistein concentration was significantly higher in the plasma and urine of rats and was lower in the feces from the inclusion complexes compared to the control. This suggests that the amylose inclusion complexes increased the genistein bioavailability significantly (Cohen et al., 2011). This enhanced bioavailability of genistein could be due to the increased surface area of the polyphenols in the inclusion complex form as compared with the crystalline genistein (Cohen et al., 2011).

Proteins and peptides have been shown to demonstrate antioxidant capacity, and this may prevent the degradation of phytochemicals in the GI tract. Ribnicky et al. (2014) reported that blueberry anthocyanins bound to proteins had a better absorption rate. In contrast, the bioavailability of  $\beta$ -carotene was reduced in the presence of the milk protein lactoferrin. (Tokle et al., 2013). Some phytochemicals have been shown to enhance the bioavailability of other bioactive compounds. Bioactive compounds, such as quercetin, curcumin, piperine and some catechins interact with the transporters in the epithelium cell membranes, which could potentially affect the bioavailability of bioactive compounds (Martel et al., 2010). For example, the bioavailability of curcumin has been increased with the presence of piperine, where piperine inhibited the actions of digestive enzymes in the GI tract

**Table 1** Summary of various studies studying the effect of excipient foods on the bioavailability of various nutrients and bioactive compounds

<i>Excipient food component</i>	<i>Nutraceutical in co-ingested food source</i>	<i>Effects of excipient food</i>	<i>References</i>
<b>Lipids</b>			
Olive oil	$\beta$ -carotene and $\alpha$ -carotene in carrots	Adding olive oil to carrot during cooking enhanced the solubilization and the amounts of carotenoids	<a href="#">Hornero-Méndez and Mínguez-Mosquera (2007)</a>
Salad oil	Carotenoids in vegetables	Adding oil to vegetables enhanced solubilization and the amounts of carotenoids	<a href="#">Nagao et al. (2013)</a>
Phospholipids	$\beta$ -carotene in emulsions	Adding phosphatidylcholines to emulsions enhanced the bioaccessibility of carotenoids	<a href="#">Verrijssen et al. (2015a)</a>
<b>Carbohydrates</b>			
Sugars	Epigallocatechin-3-gallate in solution	Adding sugars to aqueous solutions prevented destruction of epigallocatechin-3-gallate	<a href="#">Shpigelman et al. (2013)</a>
Starch	$\beta$ -carotene in emulsions	Combining lipid droplets in starch-based hydrogels enhanced lipid digestibility and bioavailability of carotenoids	<a href="#">Mun et al. (2015b)</a>
Pectin	$\beta$ -carotene in emulsions	Adding pectin to emulsions affected lipid digestion and bioaccessibility of carotenoids	<a href="#">Verrijssen et al. (2015b)</a>
Indigestible polysaccharides	Phenolic compounds in chokeberry juice	Multicomponent food matrix reduced the phenolics content and antioxidant capacity of chokeberry juice	<a href="#">Stanisavljević et al. (2015)</a>
<b>Minerals</b>			
Milk minerals	Flavan-3-ols in green tea	The presence of milk minerals enhanced the bioaccessibility of flavan-3-ols significantly	<a href="#">Moser et al. (2014)</a>
<b>Food additives</b>			
EDTA	Iron in corn-masa tortillas	Adding EDTA enhanced the bioavailability of iron in corn-masa tortillas	<a href="#">Walter et al. (2003)</a>
Ethanol	Quercetin, resveratrol and anthocyanins	Ethanol increased cellular uptake of phenolic compounds and anthocyanins using GLUT2	<a href="#">Faria et al. (2009)</a>
Xylitol/citric acid and xylitol/vitamin C	Catechins in green tea	Xylitol/citric acid and xylitol/vitamin C enhanced the intestinal absorption of catechins	<a href="#">Shim et al. (2012)</a>

([Dudhatra et al., 2012](#)). Moreover, the high antioxidant capacity of quercetin has shown to inhibit the oxidative degradation of polyunsaturated compounds in the GI tract ([Lesser and Wolffram, 2006](#)).

Calcium plays an important role in the digestion of milk fat. In the digestive system, calcium enhances lipolysis, the release of fatty acids from triacylglycerols, but also limits the absorption of saturated fatty acids. Previous studies on *in vitro* digestion of cheese have shown that different structures of cheese lead to different behavior during digestion, demonstrating the potential role of the cheese matrix as a lipid-release modulator ([Ayala-Bribiesca et al., 2016](#); [Lamothe et al., 2012](#)). Additionally, Cheddar-type cheeses with different levels of calcium and different types of milk fats (with different contents of long-chain fatty acids) showed different lipid bioaccessibility profiles during an *in vitro* simulated GI digestion model ([Ayala-Bribiesca et al., 2017](#)).

## Microencapsulation

Various matrices can be used to enhance the stability of bioactive compounds during GI digestion in humans. Encapsulation or microencapsulation has been widely used in the pharmaceutical and food industries. The techniques used for microencapsulation include spray drying, spray chilling, extrusion, coacervation, liposomes, co-crystallization and freeze-drying. These techniques have shown to affect the survival of probiotics bacteria negatively, reducing their ability to exert health benefits *in vivo* ([Rajam et al., 2012](#)). Various alginate-based matrices have shown to be highly effective for microencapsulation of probiotics. For example, alginate-chitosan, alginate-gelatin, and alginate-pectin ([Chávarri et al., 2010](#); [Sandoval-Castilla et al., 2010](#)). Microencapsulation with alginate shown to enhance the viability of probiotic bacteria in freeze-dried yogurt stored for 6 months at 4 and 21 °C ([Capela et al., 2006](#)). Similarly, the survival of encapsulated probiotic bacteria in chitosan-coated alginate beads was found to be higher than

that of free cells after storing for a duration of 4 weeks at 4 °C (Krasaekoopt et al., 2006). Protein hydrogels have also been shown to be a useful carrier for the controlled release of bioactive compounds. Remondetto et al. (2004) observed different iron release profiles depending on the microstructure of the gels in which iron was trapped. This result was further supported by another study conducted by Moretti et al. (2006) where extrusion encapsulation of micronized dispersible ferric pyrophosphate in rice meal have resulted in low bioavailability of iron.

The use of dairy foods, such as yogurt, fermented milk drinks and cheeses have been used as carriers for probiotics is well established in the market (Tripathi and Giri, 2014). Dairy foods, specifically, the milk proteins are popular among consumers as they have useful physicochemical properties (Ranadheera et al., 2016). Recently, Prasanna and Charalampopoulos (2018) studied the survival of free and encapsulated bacterial cells *Bifidobacterium longum* subsp. *Infantis* CCUG 52486 in a variety of matrices, such as, sodium alginate, sodium alginate-cow milk, sodium alginate-goat milk and sodium alginate-casein hydrolysate. The bacterial cells encapsulated in sodium alginate-goat milk demonstrated the highest survival rate in cow milk and goat milk after storing for 28 days. The cells encapsulated in sodium alginate and sodium alginate-casein hydrolysate and the free cells had low survival rate under the simulated GI digestion and in all different storage conditions. Similarly, Chen et al. (2017) studied the effectiveness of xanthan-chitosan-xanthan encapsulation on the survival of *Bifidobacterium* BB01 in yogurt stored for 21 days at 4 °C and 25 °C, respectively. Results demonstrated that this encapsulation system improved the viability of the probiotics, which showed higher bioaccessibility in the GI tract. All these results demonstrated that probiotics encapsulated in modified matrices show improved survival during GI digestion.

## Nanoparticle Encapsulation

Nanoparticle delivery system has been widely used in the recent years in the field of pharmaceuticals and nutraceuticals. Nanoparticles have shown to enhance solubility of phenolic phytochemicals with low water solubility through hydrogen bonding and hydrophobic interactions (Li et al., 2015 and McClements (2015)). A study by Zou et al. (2012) demonstrated that the solubility of procyanidin in aqueous solution was increased by encapsulation with zein nanoparticles. Similarly, the water solubility of curcumin was enhanced by encapsulation with chitosan and protein nanoparticles (Kim et al., 2011). In addition, nanoparticles lower the risk of oxidative degradation of phenolic phytochemicals in the GI tract (Ishii et al., 2011). Moreover, nanoparticles have been demonstrated to be able to penetrate across the epithelial cells of the small intestine using paracellular or transcellular pathway. These pathways enhance the absorption of phenolic phytochemicals encapsulated in the GI tract (Li, 2015). However, the stability of nanoparticles in the GI tract are affected by various factors, such as pH, ions, digestive enzymes and mucus layer (Li and Gu, 2011). Hence, future studies are needed to formulate phytochemical encapsulated nanoparticles with higher stability in the GI tract.

## Conclusion

Food matrix (food microstructure) can play an important part in health and disease prevention by influencing the bioavailability of various nutrients and phytochemicals. Various food processing techniques can negatively or positively affect the nutrient contents and their bioavailability. The food matrix can be modified to protect a variety of nutrients and phytochemicals from being degraded during food processing, storage and during GI digestion. However, there is contradictory information on the effects of food matrix on the bioavailability of some nutrients. Hence, more sophisticated studies are needed to establish methods to determine the bioavailability of the genuine metabolites after extensive GI digestion. Additionally well-controlled analytical methods should be developed to determine the location and complexing of nutrients in a specific food matrix. More studies are also needed to determine the interactions between bioactive compounds and various macromolecules in the gut microbiome since the gut microbiota play an important role to influence the bioavailability of nutrients. Hence, the scientific community is expecting more interesting results from researchers in this field of study.

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# Locusts as a Source of Lipids and Proteins and Consumer Acceptance

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## Glossary

**Entomophagy** Consumption of edible insects

**Food Neophobia** Reluctance to eat new or novel foods

## Entomophagy

Entomophagy is the consumption of edible insects. Over 3000 ethnic groups in mainly African, Asian, and Latin American countries eat insects as part of their normal diet (van Huis et al., 2013). They can be eaten at various life stages including eggs, nymphs, and adults, depending on the species and processing method (Ramos-Elorduy, 2009). Even though over two billion people eat insects, it is still met with great resistance by many Western consumers. Most have grown up viewing all insects as 'bugs', initiating feelings of disgust or fear (Looy et al., 2014; Tan et al., 2015; Yen, 2009). However, there has been an increasing interest in edible insects' due to the nutritional and sustainable benefits. In 2013, United Nations' Agriculture and Food Organisation released a detailed report (van Huis et al., 2013) on the potential of insects as food and feed. The commercial production and distribution of insects and insect products such as cricket flours, protein bars, and chips has increased over recent years in Western societies. Schösler et al. (2012) indicated a trend towards more modern and gourmet insect products sold at specialist stores. Countries such as Canada, Netherlands, Belgium, and others are at the forefront of insect consumption, with a few insect products already on the market (Balzan et al., 2016; Schösler et al., 2012; Verbeke, 2015).

## Benefits

Edible insects have great potential to become a viable protein, fat, or meat alternative due to the numerous sustainability and nutritional benefits. With increases in population estimated to reach 9.7 billion by 2050, food demand will also increase by around 70%, compared to what it is today (FAO, 2009; van Huis et al., 2013). Within this statistic is the increase in meat consumption in both developed and undeveloped countries (Verbeke, 2015; Yen, 2009). Production of locust and other insect species is a very sustainable practice compared to livestock farming, as they require significantly less feed, land, and water. They can be reared vertically, utilising a fraction of the land space conventional meat sources do. As they are cold blooded, they can obtain water through the moisture in their food. When using crickets as an example, they only require 1.5 L of water to make one kg of protein, whereas beef requires an astonishing 3400 L (van Huis et al., 2013). Greenhouse gas emission is also a large concern for the agricultural sector. Insect's emissions are very low, as only cockroaches, termites, and scarab beetles emit methane gas (Oonincx et al., 2011). Insects such as locusts are commonly viewed as pests that ruin crops. In Australia, they are controlled by spraying harsh chemicals to reduce adult numbers (Yen, 2008). There is a potential to not only reduce pesticides into the environment, but to utilise these large outbreaks. Furthermore, insects are beneficial in terms of nutrition with large interest and research investigating the high protein content. They are also rich in unsaturated fatty acids and certain vitamins and minerals such as iron, zinc, calcium, copper, magnesium, manganese, and selenium (Rumpold and Schluter, 2013; Zielińska et al., 2015). With the rising number of health and environmentally conscious consumers worldwide, there is a great opportunity for production of insect products.

## Composition

Insects can be grouped into orders including: coleoptera (beetles), orthoptera (locusts, crickets, grasshoppers), lepidoptera (caterpillars), hymenoptera (wasps, bees, ants), hemiptera (bugs) and so on (Rumpold and Schluter, 2013; Zielińska et al., 2015). Due to the large number of edible species, many studies have presented broad array of results for species within these groups (Bukkens, 1997; Ramos-Elorduy et al., 1997; Rumpold and Schluter, 2013). Conversely, other studies have only investigated a small number of popular insects including, the yellow mealworm (*Tenebrio molitor*), black soldier fly (*Hermetia illucens*), and cricket (*Acheta domesticus*) (Shelomi, 2015). Literature on the composition of the locust species *Locusta migratoria* are limited. Furthermore, each of the 1900-edible species have a unique proximate composition, which differs largely due to their habitat, diet, metamorphic stage and extraction method. This creates variation within the literature for locusts and other insects (Kourimská and Adámková, 2016; Yi et al., 2013).

## Protein

When focusing on the orthopteran class (locusts, crickets, grasshoppers) in particular, protein contents can range from 48.69% to 77.01% (Ndiritu et al., 2017; Raksakantong et al., 2010; Ramos-Elorduy et al., 1997; Rumpold and Schluter, 2013). They also contain satisfactory levels of essential amino acids (Kouřimská and Adámková, 2016; Zhao et al., 2016). The high protein content of edible insects has been outlined in many studies, indicating their potential as a nutrient rich and sustainable protein alternative. However, the use of the nitrogen conversion factor 6.25 has been linked to overestimation of the protein in edible insects. The high amount of non-protein nitrogen (NPN), such as the polysaccharide chitin (found in the exoskeleton), produces a falsely high protein content. Overestimation of protein content in various insect species can be around 8.5% to 22%, with recommendations to subtract NPN from the total nitrogen content, or use a 4.76 conversion factor (Janssen et al., 2017; Jonas-Levi and Martinez, 2017; Zhao et al., 2016). Nonetheless, the high protein content in insects is comparable to other meat sources such as beef, chicken, or fish (Yi et al., 2013), indicating significant potential as a meat and protein alternative.

## Fat

Fat is the second most abundant nutrient in locusts and other edible insects. The average fat content (based on dry weight) for orthopteran species is around 13%, however can range between 3.24% and 53.05% (Kouřimská and Adámková, 2016; Rumpold and Schluter, 2013). Fat is an energy rich nutrient leading to similar insect energy values compared with other meat options (Paul et al., 2016). The fat content and composition of insects vary largely due to differences in species, diet, metamorphic stage, habitat, and extraction method (Dreassi et al., 2017; Ramos-Bueno et al., 2016; Zhao et al., 2016). Paul et al. (2017) found the larvae of the yellow mealworm (*T. molitor*) had a much higher fat content (32%) compared to orthopteran insects that were at the adult stage (10%–15%). The majority of the insect lipids are in the triglyceride form (80%–90%). However, phospholipids (20%), diglycerides, monoglycerides, and steryl esters are also present. Specific concentrations of each lipid class vary, depending on life stage, species, and location in the insect body (Grapes et al., 1989; Kouřimská and Adámková, 2016; Tzompa-Sosa et al., 2014).

## Carbohydrate and Ash

Generally, orthopteran species have reasonably low carbohydrate (CHO) and higher fibre contents. This has been linked to the presence of a hard exoskeleton and life stage of the insect (Mohamed, 2015a). The high amount of indigestible fibre called chitin, can lead to fibre contents of locusts ranging from 15.65% to 27% (Kouřimská and Adámková, 2016; Mohamed, 2015a). Lastly, mineral contents of insect species are diverse. Orthopteran species ash contents range from 0.34% to 9.10%, with an average 3.85% (Rumpold and Schluter, 2013). Some studies have identified high amounts of iron in locust species (Kouřimská and Adámková, 2016; Zielińska et al., 2015) whereas, one study only identified phosphorous in high amounts (Mohamed, 2015a). However, the bioavailability of these minerals still needs to be investigated further. Folic acid, riboflavin (B2) and B12 are also reasonably abundant, especially in cricket species (Rumpold and Schluter, 2013). Although the studies are diverse, the composition of edible insects including the high protein, fat, and energy contents are comparable to meat options. Consequently, insects have the potential to be a sustainable and nutrient rich protein, oil, or meat alternative in the future (Yi et al., 2016; Zielińska et al., 2015).

## Protein Extraction and Functionality

Extracting the protein from insects has been suggested in literature. It would not only improve the protein percentage of the extracted fractions, but also overcome consumer acceptance barriers (van Huis et al., 2013). Many studies have identified consumers are more willing to consume insects when they cannot visually see them in the food (Balzan et al., 2016; Schöslér et al., 2012; Tan et al., 2016). Adding extracted protein fractions means the whole insect can not be seen, and also provides added nutritional and potential functional benefits to food products. Limited research has investigated protein extraction and functionality from insects. Generally, three fractions are extracted following aqueous or alkaline extraction methods. The proteins are solubilised in certain pH conditions (usually pH 10–11) then removed after centrifugation, creating a pellet or insoluble fraction. The remaining soluble proteins are precipitated at their isoelectric point (pH 4–6) and removed again after centrifugation, leading to a residue (soluble) fraction, and the upper liquid layer called the supernatant (Bußler et al., 2016). After extraction, the protein contents of the three fractions (soluble, insoluble and supernatant) are higher than the initial protein content of the whole insect. This has been researched on insects such as *Acheta domestica* (cricket), black soldier flies, yellow mealworms and the Mexican soldier fly (Bußler et al., 2016; Del Valle et al., 1982; Ndiritu et al., 2017; Yi et al., 2013; Zhao et al., 2016). Studies have also investigated the difference in protein content and yields of each fraction, using different extraction parameters or methods. They found experimental parameters can significantly affect results such as extraction method, temperature, and pH (Tirgar et al., 2017; Yi et al., 2013; Zhao et al., 2016). The extracted protein could be used as a functional food in various food products. The foaming ability of such protein has been found to be insufficient (Bußler et al., 2016; Yi et al., 2013). However, sufficient gelling, water and oil holding capacity has been identified for soluble and insoluble cricket protein (Bußler et al., 2016; Ndiritu et al., 2017; Yi et al., 2013). The ability to hold water against gravity is an important application in certain food areas. Moreover, protein fractions are generally yellow and brown colored due to melanoïdins, enzymatic, or non-enzymatic browning (Purschke et al., 2017; Yi et al., 2013).

### Fatty Acid Composition

Fatty acid (FA) composition of insect oils is limited, especially for locust species. The most abundant FA's in most insects are polyunsaturated FA (PUFA), followed by saturated FA (SFA) and monounsaturated FA (MUFA) (Raksakantong et al., 2010; Rumpold and Schluter, 2013). However, again the exact FA composition varies depending on many factors. When investigating the FA composition of *L. migratoria* species in particular, Mohamed (2015b) and Ramos-Bueno et al. (2016) found higher amounts of SFA and MUFA compared to PUFA. The most common SFA were stearic and palmitic acid. Oleic acid was the most abundant MUFA, and the essential FA's linoleic and  $\alpha$ -linolenic (ALA), contribute to a reasonably high PUFA content of 17.8% and 32.3%, respectively. Overall, The PUFA/SFA ratio is higher in cricket species when compared to yellow mealworm's, with ratios of 1–3 and 0.68 respectively (Paul et al., 2017). The PUFA content in orthopteran species has been linked to the diet and enzymatic activity (Yang et al., 2006). Insects that consume grass or plants containing high PUFA also contain high  $\alpha$ -linolenic (omega 3) contents found in their FA composition (Raksakantong et al., 2010). Although commonly known for their high protein content, orthopteran species have huge potential as a sustainable and nutritionally rich lipid source. Omega 3 is an essential FA that humans cannot synthesis in their own bodies. It has known positive effects for heart disease and cholesterol levels (Kris-Etherton, 1999). Additionally, there has been a growing concern over the high amount of omega 6 consumed in human diets. The higher amount of omega 3 in certain insect species produces a desirably low omega 6/omega 3 ratio, indicating opportunities as a healthy oil option.

### Safety and Regulation

Locusts and edible insects are generally found to be safe to consume, with no additional hazards such as contamination and chemical concentrations, when compared to other animal products (Poma et al., 2017). Microbial counts of raw edible insects are reasonably high, with recommendations for processing steps to decrease risks (Stoops et al., 2016; Vandeweyer et al., 2017). Dried then powdered insects, such as cricket flour are popular in the insect market today. However, when the microbial counts of such products were investigated by Grabowski and Klein (2017), they did not pass Belgium and Netherland hygiene regulations. Even though no classical food pathogens were identified after processing, high numbers of microbes were still present. There is large variation in microbial counts, not only due to intrinsic properties of the products, but between species, due to difference in diet and habitat. Therefore, the safety of an insect product should be thoroughly investigated before production and distribution. People with crustacean allergies may also have reactions to insect's due to the high amount of chitin in the exoskeleton (van Huis et al., 2013).

Edible insects are classified as a novel food in European countries, with no specific regulations on breeding or marketing, except for Belgium and the Netherlands (Grabowski and Klein, 2017; Poma et al., 2017). The insects, *Zophobas morio* (super mealworm), *Achaeta domestica* (house crickets), and *T. molitor* (mealworm beetle) were reviewed by the Food Standard Australia New Zealand (FSANZ) Advisory Committee on Novel Foods (ACNF). Although they are not traditionally consumed, they are able to follow normal food regulations (non-novel), due to the lack of safety concerns. Labelling of the true nature of food is required for products containing these insects however. *L. migratoria* would have to be reviewed by the ACNF before classification (novel or non-novel food) and consequent regulations of locust products in New Zealand or Australia.

### Consumer Acceptance Barriers

There is great potential for insects, insect oils, and extracted protein fractions to be used as a food ingredient. With the health and sustainability benefits, consumers may be motivated to purchase insect products. However, in order to successfully introduce such a controversial and innovative product, consumers must first adopt it. Although entomophagy is not uncommon in some Asian, African, and Latin American countries, it is widely unaccepted in others. Most attitudes towards the idea are ones of disgust, fear, or curiosity (Yen, 2009). Over half of the Belgium participants in a study conducted by Caparros Megido et al. (2014) had negative feelings towards insects however, were willing to try due to the curiosity and novelty, as found in many other consumer studies (Tan et al., 2015; Yen, 2009). Culture is a significant barrier towards the actual adoption of entomophagy in many Western societies. With no innate aversion found, these cultures have developed behavioural norms of eating animals as a source of protein, and viewing insects as pests or disease transmitters (Tan et al., 2015; van Huis et al., 2013). This enculturation has created deep-seated ideas about what is and isn't acceptable to eat, consequently generating food neophobia towards edible insects (Caparros Megido et al., 2014).

Food neophobia is the avoidance of new or unknown foods. Rozin and Fallon (1980) claimed that foods are often rejected because they have unknown tastes, origins, or expected harmful consequences from consumption. This has been linked to what is known as the 'omnivores dilemma,' a survival mechanism in which we should be cautious of potentially new and harmful foods, but also need to explore novel options (Hartmann et al., 2015; Rozin and Fallon, 1980). Consumers are more willing to trial products that they have tried before, as they already possess known sensory expectations. If the food and its origin are unknown, they make inferences about the experience, which are often negative in terms of insects decreasing the willingness to trial them (Tan et al., 2015). Both Balzan et al. (2016) and Verbeke (2015) found that food neophobia was the biggest factor affecting willingness to eat or purchase insects. Even though around 60% to 70% of the participants knew about entomophagy, the unknown sensory expectations and consequences of consumption prevented most consumers' acceptance. Some research have found younger males are less neophobic than females (Schösler et al., 2012; Verbeke, 2015), whereas others have found no significant difference in age or gender



(Looy and Wood, 2006). Furthermore, some consumers are motivated by rational reasons such as the health and sustainability benefits, whereas others are motivated by the novel experience gained from eating insects (Hartmann et al., 2015; Tan et al., 2015). Although growing in interest, research on the barriers and motivating factors towards insect consumption is reasonably limited and often limited to European participants. With so many consumer acceptance barriers, just informing consumers of the nutritional and sustainability benefits of insects is not enough (Hartmann et al., 2015; Hartmann and Siegrist, 2016; Shelomi, 2015; Verbeke, 2015). Combined efforts to overcome the initial negative disgust emotions, promote the positive and decrease the negative taste expectations, and increasing the knowledge about preparation and the benefits of insects is hoped to encourage people to adopt or at least try insect products in the future. However, greater investigation into consumer acceptance, potential markets, and promotional strategies is needed for successful integration into Westerners' diets.

### Diffusion of Innovation

Literature commonly investigates consumer willingness to try insects, however actual adoption into their diet is a different thing. The novel vs normal issue is on-going. Even though consumers may be disgusted and fearful of insects, their curiosity and thrill-seeking nature may motivate them to taste an insect (Caparros Megido et al., 2014; Tan et al., 2015). However, actually incorporating insects into the normalised diet of Western consumers is a complex and still reasonably unknown issue. The diffusion of innovation theory described by Rogers (1987) outlines how new products such as insects are taken up by the population. Initially innovators and early adopter categories will be the first on board with the innovative product, and will communicate the benefits to the larger populations (early majority and late majority). Lastly, consumers called laggards who are categorised as traditionalists, will be the last to adopt the idea. The rate of insect adoption can be increased through improving the perceived advantages of insects, producing products consistent with the values of the target market, improving knowledge on how to prepare and source insects, and increasing taste experiences of insect products (Verneau et al., 2016). For example, association between edible insects and similar animals such as crustaceans, and also the dissociation with general "bugs," could help overcome some of the acceptance barriers (Caparros Megido et al., 2016). Changes in consumer behaviour can be difficult but has happened before. Crustaceans such as lobster used to be considered 'poor man's food', but are a delicacy in many parts of the world today. Moreover, in the past thirty years eating raw fish has become a normalised behaviour in Western culture with the introduction of sushi (van Huis et al., 2013). However, in order for insects to follow similar trends, further investigation into possible insect products, food production systems, and also changes in the legislative law need to occur.

### Insect Products

Limited research has investigated consumer expectation and preference of insect products. Some studies have found how insect preparation and presentation affects willingness to eat them. Incorporating insects into products so they can't be seen, and also using them in familiar food formats can increase acceptability (Balzan et al., 2016; Caparros Megido et al., 2014; Gmuer et al., 2016; Hartmann et al., 2015; Tan et al., 2016). Caparros Megido et al. (2014) found most of the Belgian consumers were more willing to try the products where the insects couldn't be seen, and were paired with familiar flavours such as chocolate. By adding insects to familiar foods and taking away their "disgusting" appearance, food neophobia to novel insect products can be decreased. Knowing this, effectively incorporating insects into attractive and familiar insect products is crucial for the adoption of insects in Western societies. However, even with the greater acceptance of insects when incorporated into food, there are still large discrepancies found between insect and non-insect containing snacks (Gmuer et al., 2016). Some have looked at insects as a meat substitute (Caparros Megido et al., 2016; Schösler et al., 2012) or researched acceptance of whole insects. Investigating consumer expectations and preferences for insect products could provide valuable information about potential product development ventures in the future.

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## Relevant Websites

- The food insect newsletter <http://www.foodinsectsnewsletter.org/>.
- Proti-Farm – The Protein Company <https://protifarm.com>.
- The rise of the incredible edible insect <https://www.popsoci.com/>.
- The resource on edible insects <http://www.entomophagy.com>.

## Edible Packaging

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### Glossary

**Brushing** Application of a coating with a brush on the food product surface.

**Coating** Solution that when applied directly on the surface of the food leads to the formation of a thin layer that covers the food surface.

**Dipping** Application of a coating by immersing a food product into a coating solution.

**Film** Thin layer that is prepared in a mold separately from the food product and is later applied to it.

**Hydrophilic–lipophilic balance** The balance of size and strength of the hydrophilic and lipophilic moieties of a molecule.

**Glass transition** Exhibited by amorphous polymers or the amorphous regions of partially crystalline polymers when a hard, brittle, glass-like state is transformed into a viscous or rubbery fluid with the increase of temperature.

**Melting** Characteristic of crystalline polymers and corresponds to the transition from the solid to the liquid state at a given pressure.

**Spraying** Application of a coating by spray to cover the food surface.

**Wrapping** Application of a film to cover a food product.

### Nomenclature

MW Molecular weight

GRAS Generally recognized as safe

RH Relative humidity

## Introduction

Packaging, in general, helps the delivery of goods to the consumers, being used to protect goods and inform the consumer. In the case of foods, this functionality is augmented once they are able to ensure preservation of perishable products, provide information to the consumers, and ensure convenience (Zepf, 2010). In the food industry, can, glass, paper, and plastic are the most-used packaging materials. In recent years, due to environmental concerns, the use of biomaterials has increased, in which due to their source and biodegradability, they decrease the use of petroleum-based materials and waste disposal, respectively.

Another type of packaging that is increasing in the food industry is edible packaging. Edible packaging is packaging based on food-grade materials that is applied to foods as coating or film and can be eaten. This packaging (film or coating) must maintain their edibility after processing and application, and thus ensure their edible status. Edible packaging is able to protect foods and bring convenience to the consumer, and thus is within the traditional packaging definition. To be considered edible, the used materials must be approved as food ingredients or additives, or be in the group of compounds that can be added and eaten with food products. Nowadays, the legislation is clear regarding to the ingredients and additives that can be eaten with foods. In the case of edible packaging, this legislation must be followed and studied case by case according to the food on which the edible packaging is intended to be used.

The performance of edible packaging depends on the materials used and their main characteristics, pertaining to the main properties of coatings and films, such as barrier, mechanical, and optical properties. The materials shall be selected according to the type of food (i.e., dairy, fruits, soluble coffee, and vegetables) and storage conditions [e.g., temperature and relative humidity (RH)].

Nowadays, several approaches have been evaluated aiming to reduce and control the poor properties of edible packaging materials. Moreover, the reinforcement of the matrix using nanomaterials and the addition of bioactive compounds with edible status has been used to increase the materials' functionality. This functionality could be related to the antimicrobial and antioxidant capacities of those compounds, for example, that help edible packaging increase the quality and safety of food products.

Thus, edible packaging should not be seen as a type of packaging that will replace conventional packaging materials, but as one that can improve the properties of conventional packaging, acting as an additional protection for foods.

## Materials

The materials used to produce edible packaging, both coatings and films, must meet two requirements; one is to be considered edible, and the other is the ability to form a continuous coating or film. In this group of materials are generally polysaccharides, proteins, and waxes that are able to form a continuous coating or film. They can be divided into different categories according to their production method and origin. These include: (1) materials from direct biomass or natural sources (e.g., marine, agricultural, and animal sources), typified by proteins, polysaccharides, waxes, and lipids; (2) materials produced by wild or genetically modified microorganisms, such as polysaccharides and some active compounds; (3) materials produced by classical chemical synthesis, including surfactants, plasticizers, and other active compounds that are normally used in edible packaging.

Among these materials, polysaccharides and proteins are the most used materials as edible packaging. They are widely applied in the food industry according to their source and intrinsic and external factors, which have the capacity to form coatings and/or films. In the food industry, they are also used as emulsifying, foaming, and gelling agents, which are added to foods to attribute new functionalities.

The final properties and behavior of films and coatings will depend on several intrinsic factors, which in the case of polysaccharides are: molecular structure and the presence of functional groups, molecular weight (MW), and charge. In proteins, the type, number, and sequence of amino acids determine their MW, conformation, electrical charge, hydrophobicity, and functionalities. **Table 1** presents the most used polysaccharides in the production of edible packaging, their source, and some of their main characteristics.

Waxes and lipids are materials also used for the production of edible packaging. Waxes have been applied for many years as a coating on fruits, mostly citrus fruits, but in recent years, they have also been tested in combination with other materials, such as polysaccharides and proteins, aiming at the formation of coatings or films with improved properties (i.e., hydrophobic nature). They can be divided into natural waxes and resins, acetoglycerides, fatty acids, and vegetable oils. Their constitution will dictate whether they are liquid or solid at room temperature and is its state at room temperature (related to the melting temperature) that will influence the way they will be used. Due to their hydrophobic nature, they are used to reduce the moisture sensitivity of other materials, such as polysaccharides and proteins, but can also be used directly in foods as a coating, and to decrease the moisture loss from food.

In most of the cases, the formation of films and coatings require the presence of a plasticizer. Normally, polysaccharide- and protein-based films without plasticizers present a brittle and stiff structure due to the extensive interactions between polymer molecules. The type of plasticizer used will depend on the main materials used for the production of films and coatings. In the case of hydrophilic materials, such as most polysaccharides and proteins, water is one of the most effective plasticizers and its presence in the matrix will influence the films' properties. Other plasticizers of low MW, such as polyols, can be incorporated into the matrix, interfering with molecular interactions and leading to increased film flexibility and processability. Others of the materials that can be added are surfactants. Surfactants are amphoteric substances that present simultaneous hydrophilicity and hydrophobicity behavior. They are normally classified according to the hydrophilic-lipophilic balance and are conventionally added to enhance the stability of films and coating produced by mixtures of polysaccharides or proteins and lipids or waxes. The use of surfactants allows the formation of an emulsion, oil in water or water in oil, increasing the stability of film or coating. Surfactants can also be incorporated to reduce the surface tension of the coatings and improve wettability.

## Production Methods

Edible packaging can be applied to foods as edible films or coatings, as commented previously. This choice will depend on the application and the main functionality that is sought. The coating is a solution that is applied directly to the food surface (by dipping, spraying, or brushing) and that after drying forms a thin film, which will perform the desired function. The films are a self-standing material applied on the food product (e.g., wrap). To be considered a film, the thickness must be less than 254  $\mu\text{m}$  (Robertson, 2012).

In the case of coatings, they are applied directly on the food surface and dried. The application method depends on the food product, but the most used methods are immersion and spraying.

The drying process can be performed at different temperatures, i.e., refrigerated and room temperature when they are left to dry for a few hours, and high temperature (40–70 °C), thus increasing the drying rate.

Films can be produced by two different processes, "wet" and "dry". The chosen process will depend on the material used and its application. The most common is the "wet process," in which the materials are solubilized or dispersed in a solvent forming a homogeneous mixture, which is then placed in a mold and, after solvent evaporation, the film is formed. Normally, an aqueous solution is used, but in some cases, a solvent such as ethanol and a change of pH are needed to improve solubilization. In this case, the solvents need to be approved for food applications (food grade); once past the evaporation, some residues can remain in the film matrix and be consumed in foods. The other process used is the "dry process," in which the thermal behavior of the materials is used to fluidize the materials (melting or glass-transition temperatures) without the use of solvents.

**Table 1** Examples of polysaccharides used for the production of edible packaging, source, and main characteristics.

<i>Polysaccharides</i>	<i>Source</i>	<i>Main characteristics</i>
Carboxymethyl cellulose	Cellulose from plant or produced by microorganism	<ul style="list-style-type: none"> <li>• Water soluble</li> <li>• Film- and coating-forming capacity in concentrations ranging from 0.5 to 3% (w/v), depending on the degree of substitution and MW</li> <li>• Negatively charged</li> </ul>
(Hydroxypropyl)methyl cellulose	Cellulose from plant or produced by microorganism	<ul style="list-style-type: none"> <li>• Water soluble</li> <li>• Film- and coating-forming capacity in concentrations ranging from 0.5 to 5% (w/v), depending on the degree of substitution and MW</li> <li>• Neutral</li> </ul>
Ethylcellulose	Cellulose from plant or produced by microorganism	<ul style="list-style-type: none"> <li>• Soluble in organic solvents</li> <li>• Film- and coating-forming capacity in concentrations ranging from 0.5 to 5% (w/v), depending on the degree of substitution and MW</li> <li>• Neutral</li> </ul>
Pectin	Plant	<ul style="list-style-type: none"> <li>• Water soluble (high temperature may be required for total solubilization)</li> <li>• Film- and coating-forming capacity in concentrations ranging from 0.5 to 2% (w/v), depending on the degree of esterification and MW</li> <li>• Negatively charged at pH &gt; 3.5 and sensitive to variations of pH</li> </ul>
Chitosan	Animal and microorganism	<ul style="list-style-type: none"> <li>• Soluble in acidic medium (normally soluble at pH &lt; 4.5)</li> <li>• Film- and coating-forming capacity in concentrations ranging from 0.5 to 3% (w/v), depending on the deacetylation degree and MW</li> <li>• Presents antimicrobial activity</li> <li>• Positively charged</li> </ul>
Galactomannans	Plant	<ul style="list-style-type: none"> <li>• Water soluble (high temperature may be required for total solubilization)</li> <li>• Film- and coating-forming capacity in concentrations ranging from 0.5 to 3% (w/v). This will depend on the mannose/galactose ratio and MW</li> <li>• Neutral</li> </ul>
Alginate	Algae or produced by microorganism	<ul style="list-style-type: none"> <li>• Water soluble</li> <li>• Film- and coating-forming capacity in concentrations ranging from 0.25 to 2% (w/v). This will depend on the mannuronic and guluronic acids ratio and MW</li> <li>• Ability to cross-link in the presence of multivalent counter ions</li> <li>• Negatively charged</li> </ul>
Carrageenan	Algae or produced by microorganism	<ul style="list-style-type: none"> <li>• Water soluble (high temperatures are normally required for total solubilization)</li> <li>• Film- and coating-forming capacity in concentrations ranging between 0.5 and 5% (w/v). This will depend on carrageenan types (e.g., lambda, kappa, and iota) and MW</li> <li>• Negatively charged</li> </ul>

## Characterization

Edible packaging can be characterized through different techniques, depending whether a coating or a film is considered. In the case of a coating, it should be guaranteed that it will spread on the food surface and form a film after drying, with adequate adhesion and durability. In this case, wettability is one of the most important properties. Wettability is determined by the balance between adhesive forces of the liquid on the solid and cohesive forces of the liquid, in which adhesive forces cause the liquid to spread over the solid surface, whereas cohesive forces cause it to shrink. The adhesive forces are determined by the measurement of the contact angle between the coating and the food surface, and the cohesive forces are determined by the liquid–vapor tension of the coating. In practical terms, the closer the wettability values are to zero, the better the surface will be coated.

Another characteristic that influences the coating performance is the surface properties of the food. Therefore, the surface properties of the food (e.g., surface tension) should also be determined. The surface tension of a surface is determined by the measurement of the contact angles between several standard liquids and the material's surface. Thus, it is possible to correlate the surface tension of the liquids with the obtained contact angle values and determine the so-called critical surface tension of the surface. In the case of coatings, another of the important aspects that should be determined is the viscosity, which will greatly influence the method chosen (i.e., dipping or spraying) for the coating application.

In films, barrier and mechanical properties are the most important properties. Barrier properties, the ease or difficulty of permeation through barrier materials by such environmental substances as water vapor, oxygen, and carbon dioxide, give information about the film's capacity to provide a barrier to gases and other volatile compounds between food and the external environment. A film with good barrier properties protects foods against moisture and aroma losses as well as oxygen and carbon dioxide permeation, thus increasing the shelf life and quality of foods. The mechanical properties also give useful information about film

performance, because films with low mechanical strength values will have limited applications. Normally, the mechanical properties of films are evaluated through the determination of tensile strength, elongation at break, and elastic modulus.

Because most materials are soluble in water, it is important to measure the film's resistance to water. This can be determined by measuring the solubility of the films in water, i.e., their integrity in aqueous environments. In some cases, a nonsoluble or low-solubility film is required if the stored foods need to be protected against water in an aqueous medium. However, in some applications, high solubility is required, such as bags or packets that need to be dissolved in water during use. Other of the properties studied in films are the optical properties. They are studied through the evaluation of opacity, transparency, and color, but also the gloss and the haze can be determined. The opacity and transparency indicate the amount of light passing through the film, being the high opacity and low transparency values related to a lower amount of light passing through the film. The color of the films can be determined by the International Commission on Illumination color system (CIE  $L^*a^*b^*$ ) using a colorimeter.

The chemical structure of films can be evaluated for a better understanding of their properties. Techniques such as Fourier transform infrared spectroscopy and X-ray diffraction can be used to evaluate the chemical structure and crystallinity of the materials. The surface morphology of films or cross section can be accessed by microscopy. Scanning electron microscopy and atomic force microscopy are good techniques for the observation of micro- and nanostructure of films whereas optical microscopy can be used as a direct imaging technique in transparent films, and polarized microscopy can be used on crystalline materials to observe how the crystals are dispersed. In addition, thermal analysis can give useful information about a film's chemical structure and thermal stability. Besides the determination of glass-transition temperature and/or melting temperature and enthalpy, it is also possible to evaluate the degradation of materials components that is followed by weight loss of the materials with increase of temperature.

One of the main issues of edible packaging is their sensitivity to external factors, such as temperature and RH. Most of the polysaccharides and proteins used are hydrophilic and the RH greatly influences the properties of the films. Consequently, during the determination of films' properties the RH and temperature should be controlled.

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# Active and Intelligent Packaging

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## Abbreviations

CO<sub>2</sub> Carbon dioxide  
ERH Equilibrium relative humidity  
AM Antimicrobial  
CPG Consumer packaged goods  
TTI Time-temperature indicators  
RFID Radiofrequency identification

## Introduction

Food packaging is a crucial element in consumption. It is potentially one of the most important forms of packaging in the consumer-packaged goods market, as it maintains product quality, prevents product loss, aids in transportation, allows safe storage and is a form of product advertising (Steenis et al., 2017).

## Traditional Packaging Systems

Traditional food packaging is an inert system, providing physical support and protection for a food product against external environments and stimuli (Robertson, 2012). This packaging must simply protect a product in the process of distribution, transport and storage.

In order to successfully incase and protect a product the packaging system will address the following key functions; containment, protection, convenience and communication (Robertson, 2012). The most basic function is containment, simply avoiding product loss and prevention of pollution during transport.

The primary function for food packaging is protection of the food product. Efficient packaging protection will often aid in the preservation and extension of shelf life, providing a physical barrier to microbial, physiochemical and physical damage.

The convenience of a packaging system simply refers to the ability for consumers to conveniently store and open packaging units.

Ease of use must be considered in design and construction. Effective communication within a packaging design will aid in brand identity while still providing all required information.

In addition to meeting the four key functions, food packaging is required to function in the physical, ambient and human environment (Robertson, 2012). This means that a food package must have the ability to withstand shocks, compression, gases, light, temperature, micro-organisms and dust, and still be usable by consumers with decreased dexterity, vision, strength or cognitive ability.

A poor design, that cannot perform in these environments will result in complaints and p rejection by consumers (Robertson, 2012).

## Smart Packaging

Smart food packaging is an extension of traditional packaging design. It fulfils the basic packaging functions (protection, communication, convenience and containment) and is functional in the physical, ambient and human environments.

Experts have forecasted that smart packaging is the future in food packaging solutions (Aday and Yener, 2015; Realini and Marcos, 2014; Vanderroost et al., 2014). It aims to reduce the safety hazards associated with traditional packaging through deliberate interaction with the environment and the food product (Restuccia et al., 2010). The new form of packaging offers properties and attributes that aid in product differentiation, emphasising unique qualities and ensuring product authenticity (Barska and Joanna, 2016). Smart packaging is understood to consist of two types of food packaging technologies; active and intelligent packaging.

## Active Packaging

Active packaging involves the desirable interaction of the product, packaging and environment through the addition of active agents to maintain product quality and extend product shelf life (Ahvenainen and Hurme, 1997; Barska and Joanna, 2016; Bastarrachea et al., 2011; Rooney, 2005). Active agents can be introduced to the food system either through a sachet containing active materials or

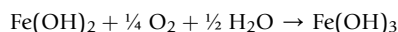
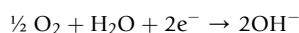
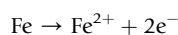
through the addition of active materials directly into the packaging film/material. Numerous active packaging technologies have been developed, with applications for specific food systems, allowing a substantial increase in shelf-life (Restuccia et al., 2010).

### Scavenging

A key limiting factor in a product's shelf life is high concentrations of oxygen ( $O_2$ ). Oxidation can cause off-flavours, unwanted odours, colour changes, nutrient destruction and facilitation of aerobic bacterium, moulds and insects (Ozdemir and Floros, 2004; Suppakul et al., 2003). The elimination of oxygen from packaging, especially the package headspace, has been a leading focus for packaging scientists (Suppakul et al., 2003).

Oxygen sensitive food products often utilise modified atmosphere packaging (MAP) or vacuum packaging, however permeation or complete exclusion of  $O_2$  from the package cannot be guaranteed by these techniques (Vermeiren et al., 1999). The implementation of an  $O_2$  scavenger to a package design will minimise the development of undesirable qualities in  $O_2$  sensitive products (Suppakul et al., 2003; Vermeiren et al., 1999). Current oxygen scavenging systems involve the oxidation of one or more of the following substances: iron powder, ascorbic acid, enzymes (glucose oxidase, ethanol oxidase), unsaturated fatty acids (oleic, linoleic, linolenic), rice extract and immobilized yeast on a solid substrate (Suppakul et al., 2003). These systems are often contained in a sachet.

Oxidation via iron powder is a very common method of oxygen scavenging:



The use of iron powder is common as it has the ability to scavenge oxygen in many food systems, including high intermediate or low moisture foods, and in food systems containing lipids (Vermeiren et al., 1999). Furthermore, they are effective in refrigerated, frozen or microwaveable items (Ozdemir and Floros, 2004).

Ethylene ( $C_2H_4$ ) is a growth-stimulating hormone that often has detrimental effects on the quality and shelf life of fruits and vegetables. It is often responsible for postharvest disorders (Ozdemir and Floros, 2004). This occurs due to the acceleration of respiration rates in climacteric fruits and vegetables, leading to maturity, senescence, softening and ripening (Vermeiren et al., 1999). A commonly used ethylene scavenging system utilises potassium permanganate imbedded in silica. Potassium permanganate cannot be integrated into the food packaging contact surface due to its toxicity, therefore the silica is kept in a sachet, with high permeability to ethylene. This inexpensive system will allow absorption of the ethylene by the silica, which will then be oxidised by the potassium permanganate into ethylene glycol (Ozdemir and Floros, 2004).

Product durability is often compromised by trapped (or product generated) moisture within packaging. Excess moisture can be generated by respiration of horticultural products, melting ice, temperature fluctuations with high equilibrium relative humidity (ERH) or drip of tissue fluid from cut meat and produce (Rooney, 1995). The trapped moisture can result in condensate or foggy film formation, microbial growth, decreased product durability and low consumer appeal (Barska and Joanna, 2016; Ozdemir and Floros, 2004). Excess moisture can be reduced either through liquid water absorption or humidity buffering.

The primary aim in liquid water control is to reduce the water activity ( $a_w$ ) thereby reducing the incidence of mould, yeast and spoilage bacterium growth (Vermeiren et al., 1999). Temperature cycling of high  $a_w$  foods has led to the use of plastics with an anti-fog additive, with the capability of lowering the interfacial tension between the condensate and the film. This contributes to the transparency of the film and allows customers to clearly view the packaged food items, while not affecting the level of liquid present within the packaging (Rooney, 1995).

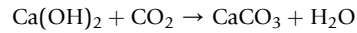
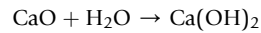
Drip absorbent sheets are often used in food packaging to remove excess moisture, especially in packaging where ice is expected to melt or under cut or whole meats. The liquid absorbing sheets consist of super-absorbent polymers located between 2 layers of micro-porous or nonwoven polymers, with polyacrylate salts and graft copolymers of starch usually the preferred polymer used (Rooney, 1995).

Humidity buffering involves the interception of moisture in the vapour phase by reducing the in-pack relative humidity and thereby the surface-water content of the food. It is achieved via one or more humectants between two layers of plastic film that is highly permeable to water vapour or by a moisture-absorbing sachet. It has been used commercially to wrap fish and chicken, reducing the ERH in the vicinity of the product.

Pouches containing Sodium Chloride (NaCl) have also been used in the US tomato market (Rooney, 1995). Desiccants have been successfully used in moisture control in a wide range of food items, silica gel, molecular sieves, calcium oxide (CaO) and natural clays (montmorillonite) are often provided in sachets (Rooney, 1995). Silica gel is the most widely used desiccant due to its non-toxic and non-corrosive nature (Ozdemir and Floros, 2004).

Carbon dioxide ( $CO_2$ ) formed after the roasting of coffee may cause coffee packaged in a can or aluminium foil pouch to burst. The  $CO_2$  released from freshly roasted coffee can be scavenged through an absorbing sachet composed of a porous envelope containing calcium oxide and a hydrating agent (silica gel), on which water is absorbed. The water will react with the calcium oxide,

producing calcium hydroxide, which will then react with the CO<sub>2</sub> to form calcium carbonate, as shown below (Ozdemir and Floros, 2004; Vermeiren et al., 1999).



Interactions between the product, product packaging and environment can result in undesirable smells. This degradation is due to volatile compounds, such as aldehydes, amines and sulfides accumulating inside the product packaging. These compounds can be selectively scavenged (Day, 2008). Odour proof packaging consisting of an odour impermeable plastic such as polyethylene terephthalate (PET) together with a port to allow for the passage of respiratory gases and a sachet consisting of charcoal and nickel to absorb odour has been used on Durian fruit (Biji et al., 2015). Further scavenging can be achieved via incorporation of acidic compounds such as citric acid in polymers, or the addition of films containing ferrous salt and organic acid (citric or ascorbic acid) capable of oxidizing volatile amines (Rooney, 1995).

### Emitters

Carbon dioxide (CO<sub>2</sub>) has been shown to suppress microbial activity. At high levels (60%–80%) it is shown to inhibit microbial growth on surfaces, aiding the increase in product shelf life (Suppakul et al., 2003; Vermeiren et al., 1999). In addition, increased CO<sub>2</sub> has been shown to delay the respiration rate of fruit and vegetables but may cause undesirable anaerobic glycolysis in fruits (Ozdemir and Floros, 2004). CO<sub>2</sub> permeability is 3 to 5 times higher than that of O<sub>2</sub> in most packaging films, resulting in the need for continuous CO<sub>2</sub> production to maintain the desired level within a packaging system (Ozdemir and Floros, 2004; Suppakul et al., 2003). Utilization of a dual function system, consisting of an oxygen scavenger and a carbon dioxide emitter is common place in increasing shelf life in fresh meat, poultry, fish and cheese (Suppakul et al., 2003; Vermeiren et al., 1999).

Spraying ethanol on food product surfaces, such as bread, cookies and other bakery products has been shown to be an effective method of extending the shelf life of these commodities through the suppression of mould growth (Ozdemir and Floros, 2004). A food grade ethanol absorbed or encapsulated in carrier material, such as an emitting sachet or film, can be used as a microbial growth inhibitor in food systems. The release of ethanol to the packaging headspace is regulated by the sachet material's permeability to water vapour. The ethanol in the carrier material is exchanged with the water absorbed by the carrier material.

In addition to ethanol, some sachets will contain trace levels of flavouring substances, such as vanilla, to mask the odour of alcohol within the packaging. It is costly to include ethanol embedded films into packaging; however, this is a more effective manner to ensure controlled release of the ethanol.

This cost can be worthwhile, as it eliminates the need for preservatives, such as sorbates and benzoates, for mould inhibition (Ozdemir and Floros, 2004).

### Antimicrobial Packaging

An antimicrobial (AM) packaging system aims to protect packaged food items against different microorganisms through chemical and physical factors (Barska and Joanna, 2016; Suppakul et al., 2003). Muscle based products provide an ideal growth media for a variety of micro-organisms, development of microorganisms will change the aroma, colour and texture of the food item, resulting in shelf life reduction and increased foodborne illness (Kerry, 2014; Kerry et al., 2006). The primary goal for AM packaging is to extend the lag phase and reduce the growth rate of microorganisms, extending shelf life and maintaining product safety and quality (Han, 2005). AM packaging can be classified into two types; migrating AM agents and non-migrating AM agents.

Due to the potential harm AM agents can pose, tight controls are placed on the method of AM release (Vermeiren et al., 1999). Significant research has been done in AM packaging. The most common AM packaging solutions utilise silver based materials, natamycin-based antifungal coating and allyl isothiocyanate a natural antibacterial and antifungal substance (Realini and Marcos, 2014). The primary implementation of AM packaging can be found in meat, bread, cheese, fruit and vegetable sectors (Vermeiren et al., 1999).

### Intelligent Packaging

Intelligent packaging provides information about the food item, environment within the packaging or the external product environment to the consumer. The information is provided via external product indicators (Aday and Yener, 2015; Robertson, 2012). Intelligent packaging can aid and contribute to improving "Hazard Analysis and Critical Control Points (HACCP) and "Quality Analysis and Critical Control Points" (QACCP) systems (Vanderroost et al., 2014). The primary areas targeted by intelligent packaging are; sensors, indicators and traceability technology.

### Sensors

Sensors are devices used to detect matter, then signalling for the detection of the particular matter sought (Kerry et al., 2006). Sensors will contain two main functional parts, a receptor and a transducer (Biji et al., 2015).

Chemical sensors utilise surface adsorption allowing the detection of the presence, activity, composition and concentration of particular chemicals or gases. The observed information is then converted into signals by a transducer, which can be active or passive depending on the external power requirement (Vanderroost et al., 2014). Nano based sensors can be used to detect pathogens,

chemical contaminants, spoilage, product tampering, trace ingredients and track the product through the processing chain (Liu et al., 2007). Sensors can make use of optical transducers such as a silicon based transducer, which do not require electrical power, allowing readings to be made from a distance using VU, visible and IR light (Yebo et al., 2012).

A biosensor can detect, record and transmit information regarding biological reactions (Yam et al., 2005). A bio-receptor will recognise the targeted analyte, such as an enzyme, microbe or nucleic acid. The transducer will convert the biochemical signals into a quantifiable response (Biji et al., 2015). An example of commercial application is the attachment of a pathogen-specific antibody to the membrane forming part of the barcode. Presence of contaminating bacteria will cause formation of localized dark bar on the barcode, rendering the barcode unreadable at the checkout (Yam et al., 2005).

Gas sensors can detect gaseous analytes within a package. There numerous forms of sensors including oxygen, carbon dioxide water vapour and ethanol sensors as well as metal oxide semiconductor field effect transistors, organic conducting polymers and piezoelectric crystal sensors (Kerry et al., 2006). Each sensor has a specific mechanism of action within a food packaging system.

Printed electronics are flexible printed sensors containing a receptor printed in top of a printed transducer. This technology allows the sensors to be light, bendable and more portable. It will also allow printing onto various substrates, allowing a tailored and unique sensor for the food package (Biji et al., 2015; Vanderroost et al., 2014).

An electronic nose consists of chemical or biosensors with specificity and statistical methods allowing recognition of simple and complex flavour or odour compounds (Vanderroost et al., 2014). The electronic nose mimics the mammalian olfactory system, allowing repeatable measurements that successfully identify and classify aromas. It can clearly distinguish volatile compounds produced during deterioration or ripening (Biji et al., 2015).

### **Indicators**

Indicators are substances that indicate the presence, absence or concentration of another substance, or the reaction between two or more substances through irreversible colour changes, by diffusion of dye along a straight path (Biji et al., 2015). Indicators are the most common type of intelligent packaging solution on the market today (Vanderroost et al., 2014).

Time-temperature indicators (TTI) display the measurable time-temperature-dependent changes related to the food to which they are attached. Commercially available TTI's include enzymatic, polymer based, solid state reaction and microbiological systems (Pennanen et al., 2015). TTI's monitor the complete time-temperature history of the food product, and can provide accurate predictions of the remaining shelf life (Pennanen et al., 2015).

Freshness indicators provide information regarding product quality and microbial growth or chemical changes within a food product. A reaction will occur between microbial growth metabolites and integrated indicators on the packaging. Each freshness indicator will be unique to the product, for example, an indicator created for seafood storage will react to volatile amines produced (Biji et al., 2015; Kerry et al., 2006).

Thermochromic ink is a dynamic ink that changes colour with exposure to different temperatures. The activation temperatures of the inks range from refrigerated temperatures up to high heating temperatures. The technology can be used to inform consumers of previous storage temperature as well as providing in home information regarding the product temperature (Vanderroost et al., 2014).

### **Traceability Technology**

The increased number of food safety incidents have resulted in a demand for traceability. Radiofrequency identification (RFID), an automatic identification technology, uses wireless sensors to allow tracking, recording and continued product information along the product lifecycle (Chrysochou et al., 2009). An RFID system can be classified as passive, not requiring a battery source, or active, requiring battery to power the system (Yam et al., 2005).

## **Consumer Perception Towards Smart Packaging**

Consumers are continuously demanding more from their food products. These demands drive innovation in the packaging industry, forcing manufacturers to develop novel solutions in order to remain competitive. However, the success of new packaging technologies relies on consumers' behavioural responses to the innovation (Chen et al., 2013). The success of these developments depends on the perceptions, responses and acceptance of consumers, rather than the effectiveness of the new technology (Siegrist, 2008).

The consumer trends that have a direct influence on consumer choice relating to packaging include; consumer lifestyle, changing retail practices, demand for convenience, purchase of high quality goods, preference for mild preservation, concerns regarding food safety, need for longer shelf life, environmental awareness and the desire for transparency and product traceability (Ahvenainen and Hurme, 1997; Grunert, 2005; Ozdemir and Floros, 2004; Realini and Marcos, 2014; Restuccia et al., 2010; Robertson, 2012; Yam, 2012; Zink, 1997). These consumer trends make it increasingly complex to define and understand consumer demand (Jensen, 2006; Yam, 2012). However, the complexity of these shifts result in increased market awareness, greater spatial and social mobility and innovation in seeking information related to goods and services (Barska and Joanna, 2016).

Food is a basic product, a necessary, often habitual purchase for consumers. Many consumers will not take a great deal of time in choosing the products they purchase, while others will ensure that their personal values are expressed through their purchasing decisions.

While some consumers are well informed about the technology utilised in packaging, the limited knowledge will not withhold consumers from forming opinions and making purchase decisions based on simple inferential cue utilization processes (Steenis et al., 2017).

Research has shown that risk perceptions and food safety concerns are the primary determinants of consumer resistance to novel food technology (Chen et al., 2013).

Providing consumers with information is of vital importance to the implementation of smart packaging. Without a basic knowledge into the technologies employed in smart packaging, consumers may begin to fear the science used to improve food packaging.

## Conclusion

Food packaging is a vital component in the success of emerging food products. Packaging is usually the first point of contact consumers have with a product.

Exciting developments are being made in the packaging industry. It is expected that continued technological advances will occur within the packaging industry, ensuring that food can remain safer for longer, without compromise on the quality.

With continued development, smart packaging will become more economically viable. Understanding consumer perceptions is key. Ensuring that consumers do not fear emerging technologies will be the central factor in whether smart packaging technologies are truly successful.

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# The Spaceflight Food System: A Case Study in Long Duration Preservation

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## Overview

NASA's food system is a unique case study for many of the preservation methods and chemistry principles that extend the shelf life of foods. Currently, foods must be safe, nutritious, and acceptable for a minimum of two years in room temperature storage for International Space Station (ISS) missions (Douglas et al., 2016). Since the food system is constrained by the limitations of microgravity and resources (no cooking, limited food transfer, no high-crumb producing foods, mass, volume, water, and crew time), the current food system is made up of fully processed, individually packaged portions in lightweight flexible laminates. The food system relies on processing and water reduction strategies to inactivate or control microorganisms and enzyme function. In addition to the unique NASA-produced food items, the current ISS food system uses several commercially available shelf stable foods. They are vacuum packed in individual serving sizes to reduce the introduction of oxygen and moisture. The result is a food system that ensures safety, stability, and ease of use in a microgravity environment (Table 1) (Douglas et al., 2016).

Although preservation strategies such as retort thermostabilization, freeze-drying, and irradiation have been adequate for all NASA missions to date, future Mars missions will require a five year shelf life (Douglas et al., 2016). The processing and storage of foods exposes them to light, heat, oxygen, and ingredients that may impact the chemical stability of the vitamins, volatiles, and macromolecules in the foods, ultimately impacting the nutritional and quality shelf life (Fennema, 1996). In this chapter, we will focus on the preservation methods currently used for spaceflight foods and potential alternatives that could be used for Mars missions. Since food quality will change during storage after it is preserved, it is also necessary to consider packaging, environment, and formulation. For example, high barrier packaging will reduce the introduction of oxygen and water into the food after processing, minimizing chemical reactions such as oxidation. Specifics about the packaging and storage is included in each of the subsections.

## Current Preservation Methods

### Freeze-Drying

Foods are freeze-dried by first freezing them and then holding them under vacuum at a pressure below the triple point, which enhances the removal of water by sublimation (Rahman and Perera, 1999). The resulting food structure is porous and more easily rehydrates than traditional air dried foods (Ratti, 2001; Rahman and Perera, 1999). Most water on the ISS is recycled (Carter et al., 2013), so freeze-dried foods reduce launch mass in addition to preserving the food for extended periods at room temperature.

**Table 1** The NASA food system currently being used on the International Space Station (ISS) uses several different preservation methods to ensure a safe, nutritious, and acceptable food system (Douglas et al., 2016)

Food/process	Preservation mechanism	Packaging	Food examples	Shelf life
Freeze-dried	Reduced water activity/inability of microorganisms to grow	Flexible laminate with needle interface for rehydration; overwrapped in foil laminate pouch	Asparagus, shrimp cocktail, mashed potatoes, strawberries	1.5–2.5 years
Retort thermostabilized	High temperature process results in commercial sterility, product is free of pathogens, spoilage microorganisms, and enzyme activity	Foil laminate pouch	Beef stew, candied yams, brown rice, peaches	2–3 years
Irradiated	Gamma ray treatment at 44 kGy results in commercial sterility, product is free of pathogens, spoilage microorganisms, and enzyme activity	Foil laminate pouch	Beef steak, Turkey	2–3 years
Low moisture or dried	Reduced water activity/Inability of microorganisms to grow	Flexible laminate, overwrapped in foil laminate pouch. Beverages in foil laminate pouch with needle interface for rehydration	Brownies, extended shelf life bread products, dried fruit, beverage powders	1.5–2.5 years
Fresh	No preservation mechanism, limited supply on resupply vehicles	Sealed in bags	Fruits, vegetables	1–2 weeks

The quality of a freeze-dried product and its porosity is related to the glass transition temperature ( $T_g$ ). The  $T_g$  is the temperature above which an amorphous solid changes from the glassy to the rubbery state (Bhandari and Howes, 1999). Above the  $T_g$ , some components may crystallize and the porous structure may collapse resulting in shrinkage (Ponginebbi et al., 2000; To and Flink, 1978). Crystallization and collapse expels moisture and volatiles, which results in inadequate rehydration, and loss of aroma (To and Flink, 1978; Ponginebbi et al., 2000). The collapse temperature is dependent on the molecular weight of the food components. For example, foods high in small molecular weight ingredients, such as simple sugars, will have a lower collapse temperature, which can be increased by the addition of higher molecular weight ingredients (Bhandari and Howes, 1999; Roos and Karel, 1991).

Freeze-drying enables daily access in spaceflight to fruits and vegetables that would not retain adequate quality if subjected to other production methods. Strawberries, for example, have a high water content and delicate structure that is more compatible with freeze-drying, as standard air-drying methods cause damage to the structure resulting in poor rehydration (Alvarez et al., 1995). The minimal loss in red color during freeze drying suggests that this process also preserves the nutraceutical content such as anthocyanins (Ratti, 2001).

Unfortunately, freeze-drying does not prevent degradation of labile vitamins. A third of the vitamin C degraded over three years of room temperature storage in freeze-dried strawberries packaged for spaceflight (Cooper et al., 2017). Instability of some vitamins or aspects of quality could be a factor of residual oxygen and moisture in the food package, oxygen and moisture ingress over time, or cooking prior to freeze-drying in some foods. Freeze-dried foods are very hygroscopic, and increases in moisture can reduce the collapse temperature (To and Flink, 1978). Spaceflight freeze-dried foods are gas flushed and vacuum packed in high barrier packaging that contains an aluminum foil layer to prevent moisture ingress. In the absence of cold storage on current missions, foods are stored at room temperature, which enables crystallization and collapse to occur in some foods over time. Several products become difficult to rehydrate or noticeably lose color and aroma within two years, which is a limitation for these foods on long duration missions.

### Retort Thermostabilization

This process, also known as the retort process, inactivates pathogens, spoilage microorganisms and enzyme activity in foods by exposure to steam or water overpressure that achieves elevated temperatures (Barbosa-Cánovas et al., 2014). Temperature and time exposures are calculated based on the product characteristics and the target pathogen lethality (Potter and Hotchkiss, 1998). Increases in the processing temperature and time may also increase the rate of the chemical reactions within the food. The final products are commercially sterile but the heat process does reduce the quality and nutritional content of the food (Chia et al., 1983; Branagan and Pruskin, 1993; Olivas et al., 2002; Rodriguez et al., 2003).

Spaceflight foods are packaged and processed in flexible laminate pouches that contain an aluminum foil layer, which provides an advantage over cans. The shape of the retort pouches decreases the distance to the cold point, allowing for shorter heating times that reduces nutrient losses and maintains quality better than the standard processing of cans (Potter and Hotchkiss, 1998). Thermostabilized spaceflight foods generally retain acceptable quality for at least two years and provide necessary variety to the spaceflight food system. The flexible pouches are also lower in mass and stowage volume, two very important resources in space travel (Perchonok and Bourland, 2002). However, several nutrients and quality factors degrade substantially in the foods, and current processing and storage parameters will not meet the requirements of future long duration space exploration missions (Catauro and Perchonok, 2012; Cooper et al., 2017).

Many foods are compatible with the retort process, but some items require alternative strategies. Spaceflight foods formulated with substantial egg content (63% w/w in Vegetable Omelet and 22.5% w/w in Broccoli Soufflé) are too rubbery and firm to be acceptable. At this time, NASA egg-based food items are freeze-dried (Catauro and Perchonok, 2012).

### Irradiation

Some products, such as larger slices of chicken or beef, become dry and texturally unacceptable if processed with the time and temperatures required in retort processing and will not produce an acceptable freeze-dried product. Irradiation is not typically used to process foods to commercial sterility, but NASA has special dispensation from the United States Food and Drug Administration (FDA) to prepare nine commercially sterile irradiated meat items that are considered to be more acceptable than comparable retorted items (FDA, 2011; WHO, 1999). The impact of irradiation on foods has been extensively reviewed and found to result in products with comparable safety, chemical, and nutritional properties to thermostabilized foods (WHO, 1999). Irradiation involves the use of gamma rays, x rays, or electrons, which at adequate energy levels inactivate spoilage and pathogenic microorganisms (Stewart, 2001). Oxidation products and chemical reactions can be minimized by irradiating foods at freezing temperatures or by packaging products in inert atmospheres (WHO, 1999) so space flight foods are deep frozen in metallized pouches for processing.

NASA has evaluated the nutritional content of some of the meat products following gamma irradiation and compared the results to database values. The results suggested that there may have been some degradation of vitamins A, B<sub>6</sub>, and B<sub>3</sub>, with the exception of B<sub>3</sub> in poultry (Douglas et al., 2016). Vitamin loss is comparable to retort processing (WHO, 1999).

Irradiation has been considered as a method to ensure microbial safety of bulk commodities that may potentially be launched from Earth for use on future long duration missions. Wilson (2005) found that surface irradiation of whole dry soybeans using

electron beam or gamma rays at 10 or 30 kGy provides microbial safety of the bulk commodity. However, oxidative quality changes to the beans resulted from the exposure, rendering them unacceptable for producing adequate soymilk and tofu. Some of the functional qualities altered by the radiation exposure included the soybean color, aroma, solid content, and the germinating capacity of the individual seeds (Wilson, 2005).

## Exploration NASA Food Systems

The nutritional and quality shelf life requirements for food increase from two to five years to support a mission to Mars. Evaluating the potential of strategies to extend the shelf life of the various types of spaceflight foods to five years is a lengthy process that would benefit from kinetic modeling advances. Kinetic data is dependent on formulation, packaging, processing, and storage conditions, so available kinetic data cannot be used to accurately predict losses under other conditions (Evans et al., 1981; Kamman et al., 1981; Lathrop and Leung, 1980; Rao et al., 1981). Ongoing research in modeling may eventually enable more accurate predictions to be made from limited data (Peleg and Normand, 2015; Peleg et al., 2016, 2017), which may expedite evaluations to identify strategies that extend shelf life.

Emerging processing technologies that may provide a higher quality commercially sterile product immediately post-production are some of the strategies that may be investigated (Park et al., 2014). High pressure processing (HPP) and microwave sterilization are expected to have the most potential (Jermann et al., 2015), and therefore are the focus of the rest of this chapter.

## Pressure Assisted Thermal Sterilization (PATs)

High Pressure Processing (HPP) is a pasteurization process that evenly applies up to 1000 MPa of pressure to inactivate vegetative cells and enzymes in foods, with associated temperature increases of only 2–3 °C/100 MPa (Patterson, 2005; Considine et al., 2008; Chefel, 1995). The high pressure can also influence the functionality and gelation of protein and starch ingredients, which has the potential to introduce novel attributes and textural variety to space foods (Knorr et al., 2006). Commercial sterility can be achieved through a variation on the HPP process called pressure assisted thermal sterilization (PATs), where high pressure is combined with a reduced sterilization temperature to inactivate spores (Wimalaratne and Farid, 2008).

Previous studies indicate that covalent bonds are not impacted by pressure, and that vitamins, flavors, and textures are less impacted in high-pressure processed products compared to thermal processes (Nguyen et al., 2010; Oey et al., 2006). Evaluations of PATs processed fruit at NASA indicated color and internal cellular structure were better maintained compared to retorted fruits (Cooper et al., 2015). However, temperature increases in combination with high pressure have been noted to result in degradation of natural pigments such as chlorophyll (Van Loey et al., 1998; Oey et al., 2008).

In 2015, the FDA approved a pressure enhanced sterilization process for low acid multiple components in an effort supported by several food industry partners, the U.S. Army Natick Soldier Research, Development and Engineering Center, and NASA (IFSH, 2015). Although this milestone indicates the potential of this technology to extend the shelf life of the space food system and provide higher quality products, the commercial application of PATs lags behind HPP and the technology is not yet available on a scale that will enable sufficient evaluation for shelf life or production of spaceflight foods (Tao et al., 2015).

## Microwave Assisted Thermal Sterilization (MATS)

Microwave sterilization is a high-temperature, short-time process that uses a frequency of 915 MHz, rather than the 2450 MHz used in conventional home microwaves (Tang, 2015; Barbosa-Cánovas et al., 2014). This shortened heating time, as opposed to the retort process, has the potential to preserve the nutrients, colors, texture and flavor of foods that are sterilized both within a package, and in a continuous flow liquid system with aseptic packaging (Steed et al., 2008; Tang, 2015). In a NASA study, MATS processing did provide better color and texture initially as compared to retort processing in two products (Sweet and Sour Pork and Carrot Coins) (Douglas et al., 2016). However, due to an inadequate oxygen barrier in the MATS package, there was significant loss in carotene pigmentation, chlorophyll stability, several vitamins, and fat stability in sauces, resulting in degradation that was similar or even more extensive than the retorted products (Douglas et al., 2016). Advancements in MATS-compatible packaging is promising (Zhang et al., 2015), but no packaging has yet been validated to sustain improved quality and nutrition over multiple years in complex, multicomponent foods like those in the space food system. Additionally, products do not respond similarly or evenly to MATS processing, and the FDA requires a complex data set, including the dielectric properties, cold spot determination, and microbiological validation for individual products prior to approval for production and consumption (Barbosa-Cánovas et al., 2014). However, if methods can be determined to sustain the initial quality improvements achieved with MATS packaging for multiple years of storage, this technology may yet have the potential to extend the shelf life of spaceflight foods.

## Conclusion

On a Mars mission, a large variety of foods will be required to deliver the required nutritional content, and provide acceptable variation in colors, textures, aromas and flavors to support crew health and well-being. The lack of a food system with a five year shelf life is an ongoing concern that must be resolved for a Mars mission, and will likely require the use of several preservation methods. Food chemistry and engineering advances that extend quality, nutrition, and acceptability while maintaining food safety and minimizing resource use will be a significant contribution to successful future space exploration. These same advances may also have terrestrial benefits. The pouch packaging is now commonly used for some commercial products due to higher quality and improved consumer convenience. Emerging preservation technologies have the potential to deliver higher quality shelf stable foods to consumers.

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## Relevant Websites

- NASA Human Research Program Inadequate Food System Risk <https://humanresearchroadmap.nasa.gov/Risks/risk.aspx?i=87>.
- Space Food Fact Sheets <https://www.nasa.gov/audience/formedia/presskits/spacefood/factsheets.html>.
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## Foods for the Military

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### Glossary

MRE Meals Ready-to-Eat individual field ration for the United States military

CRP Combat ration packs

### Purpose of Military Foods

Defence forces face unique logistical challenges in supplying food to maintain the health and performance of personnel on deployment. Military food may be the only source of nutrition while engaged in operations on land, in the air, or on-board vessels and meal times are also often the focus for social engagement and activities that promote cohesive teams. Military foods have to maintain the physical and cognitive performance of personnel by providing adequate levels of total energy, macronutrients (protein, fat, and carbohydrate), micronutrients (vitamins, minerals, trace elements) and fiber. Military forces must often operate far from supply bases and must be prepared to function in many different and potentially extreme environments. These can range from ultra-cold locations, to low pressure and cold at high altitude to hot dry desert or humid jungle conditions. Food supplies have to be adapted to remain usable in these conditions. The food format and the nutritional composition must also be varied to meet the specific needs of the personnel and their activities in those environments. Additionally the requirements for advanced preparedness and logistical flexibility for unscheduled or long range deployment means that military foods must also satisfy extreme shelf life requirements for preservation of eating and nutritional quality. Thus military food specifications can greatly exceed those of standard commercial food practice and often require purpose-developed products. Specific solutions in nutrition formulation, packaging and logistics have been designed to deliver the requirements of military foods and their related applications in emergency and remote station or recreational use.

### History of Military Foods

Military food technology arose from the necessity to adequately provision armies and navies as a prerequisite to successful field operations. Early combat feeding by requisitioning or plundering from local food resources had to be supplemented by portable food and water supplies, even in Roman times. Until the industrial revolution stimulated development of large scale food processing, armies and navies relied on traditional long-lasting foods such as dried or salted meats, preserved vegetables, grains that could be converted into bread products, oil, cheeses and wines to provide the nutritional and energy needs for sustenance of the forces. The first major advance in modern food technology was the invention of food sterilization in support of Napoleon Bonaparte's quest to conquer Europe. The original development was of preservation by boiling food in glass vessels. This was rapidly superseded from the 1820s by "canning" using tin-plated cylindrical steel canisters. Further refinements of canning and of dehydration techniques provided improved forms of stabilized foods by the time of the US "Civil War" in the second half of the Nineteenth Century. This was enabled by progression of a more basic understanding of food spoilage, microbiology and preservation science. Subsequently World War I drove the development of modern forms of combat feeding with long-shelf-life food items packaged and preserved in large hermetically-sealed metal containers. During World War II the first combat ration packs, comprised of purpose designed complete nutritional food supplies, came into existence to bridge the gap between the beginning of actual combat and the restoration of field kitchen based food supply (Forbes-Ewan et al., 2016). Post World War II developments in plastic technologies allowed production of flexible barrier packaging based on multi-laminates. These use layers of plastic polymers, aluminum foil, nylon, or paper bonded together where each layer either contributes to strength or provides a barrier to moisture or oxygen to achieve the necessary shelf life and ruggedness for military applications. Further military packaging innovation since the 1990's has resulted in the wide adoption of heat-resistant flexible retort pouches and shelf-stable ready-to-eat meals. These meals provide lighter versions of traditional canned foods reducing the bulk, weight of soldiers loads and waste volumes. Military needs for improved lightweight long shelf life meals also drove the large-scale implementation of freeze-drying technology from the 1960s. Both raw and cooked foods can be dried from the frozen state by low-pressure sublimation of the water directly to gas while retaining the shape, physical structure and nutrients of the food. The resulting ultra-lightweight freeze-dried meal, combined with packaging that excludes oxygen and water, results in food that can be maintained in good edible format for over 10 years under good storage conditions.

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\*Retired.



## Logistic Demands of Military Food

The military need for long-shelf-life and nutritionally complete foods has been a key driver of technological innovation in food technology. Armed forces have specifications for where food supplies may have to be stored for long periods in hot climates without refrigeration or periodic frozen exposure. For example, USA Ready-Meals (MRE's) have a minimum shelf life of 3 years at 27 °C (80°F) which is 2–3 times standard commercial practice of up to one year. The food must not only remain nutritionally complete but also be of adequate organoleptic standard. These specifications also make military foods, and the technologies behind them, useful in other applications such as lightweight rations for recreational hiking or long-stored reserve food supplies for emergency or catastrophic event planning. The developments in military food rations to meet specific use requirements of high-altitude pilots and for aircraft life raft survival supplies has also extended to providing the technologies for provisioning astronauts. In this context light-weight, long-shelf needs are also complicated by zero gravity feeding issues to avoid dispersion of crumbs and droplets through spacecraft and to facilitate storage and waste disposal. Ultimately, space colonization and planetary exploration will need new solutions beyond food preservation to introduce recycling of nutrients by growing food *in-situ* in constrained space and energy environments.

## Preservation Challenges of Military Food

The increased shelf-life specifications of up to three years at 27 °C (80°F) for military foods is in contrast with normal commercial shelf life purposes where food only has to meet “best-before” specification dates of typically no more than 12 months at ambient temperature. The additional costs of extra protection for standard commercial foods is not warranted and therefore foods for the military often have to be especially prepared and packaged.

Food storage solutions primarily rely on prevention of microbial and enzymatic degradation by applying heat sterilization or ultra-low water activity in combination with exclusion of oxygen and light. For wet foods such as meat-based casseroles, the specifications are easily met by retorting the food in sealed containers at 121 °C for up to 20 minutes to kill all bacterial cells and spores. The high barrier properties of metal containers also effectively prevent light, moisture or oxygen migration into the food. The major determinants of shelf life are therefore the degradation of flavor, texture and nutrients during the thermal processing step added to the slow progression of chemical reactions leading to off-flavours, degradation of texture and nutrient breakdown during storage. Thermally processed foods can have nutritive losses in excess of 40% for naturally occurring vitamin C, folic acid and pantothenic acid (Vit B5) (Cooper et al., 2011).

For dried foods, preservation relies on low water activity ( $a_w$ ). The water activity of a food is defined as the ratio of the vapor pressure of water in the food divided by the vapor pressure of pure water at the same temperature. While most fresh foods have a high  $a_w$  (i.e. > 0.98) the growth of microorganisms ceases below  $a_w$  0.7 and foods with  $a_w$  below 0.6 are stable and classified as a dehydrated foods (Labuza, 1980).

Dried military foods can also be made as intermediate moisture foods with generally less than 25% moisture. The water activity is modified using solutes such as salts or humectants such as glycerol or natural sugars and acids to maintain stability without becoming too hard or brittle to chew. Hurdle technologies, using a combination of preservation mechanisms such as acid pH, preservatives and antioxidants are sometimes used to ensure longer-term stability to meet military needs. Traditional examples are dried fruits ( $a_w$  0.72–0.8), jams ( $a_w$  0.82–0.94), honey ( $a_w$  0.75) and dried meats such as jerky ( $a_w$  0.85) that can be processed and packaged for achieving a long shelf life. Non-traditional items such as shelf stable ready-to-eat sandwiches and pizza were specially developed to diversify military MRE menus and are also used in the US Military First Strike Rations. Other shelf stable intermediate moisture foods produced for the military include pop-tarts, cereal bars, fruit roll-ups, brownies and filled wraps (Richardson, 2012).

As an alternative to standard drying approaches using hot or warm air foods can be freeze dried to extremely low moisture contents using combinations of vacuum and heat. Freeze-drying of food, also known as cryodesiccation, was developed initially primarily for military applications. Food pieces can be individually frozen to be dried as separate free-flow items such as dried peas or carrot pieces. Fully prepared meals can also be frozen as a block or in a tray and processed to dried form retaining the shape, color and nutritional content of the meals. During freeze-drying the free water content is sublimated off under vacuum to leave 1%–4% water remaining. The dried product is then packaged under vacuum or modified atmosphere containing only nitrogen to eliminate reactive oxygen. Commercially measured shelf life outcomes of up to 25 years have been achieved for meats and fruits preserved by this method.

Preservation by gamma radiation sterilization, although technically possible, is only used as a fail-safe additional mechanism for critical operations such as meat products for space flight.

## **Packaging and Oxidation Protection for Long Stored Foods**

Normal commercial applications of food packaging minimize the cost by using thinner walled, lower barrier packaging materials and therefore often do not meet military specifications for shelf life where extra protection is needed. Initial versions of flexible packaging technologies for preservation of military foods usually used aluminum foil based oxygen and moisture barrier films. These metallic barriers could achieve the levels of robustness and low permeability rates necessary for the extended shelf life. Increasingly more advanced polymers and multi-layer films are being developed that can achieve the performance needed for military foods while still using thinner and cheaper films. Ethylene vinyl-alcohol copolymers (EVOH) are an example of an oxygen barrier polymer used in multi-laminate films. EVOH can achieve oxygen permeabilities of less than  $1 \text{ cm}^3/\text{m}^2/\text{day}$  for a  $20 \text{ }\mu\text{m}$  thick film at 1 atm gas pressure. This is a similar level of exclusion to thin metallic foil laminates with the advantage that such plastic is also clear or semi-translucent for product visibility and is microwave permeable for heat processing or reheating of foods (Froio et al., 2012).

In practice, no films or containers are perfect barriers for oxygen and water. Oxidation is the predominant mechanism for nutritional spoilage and flavor defects of long stored foods. Even low levels of oxygen 100 times lower than air can still catalyze food changes. Vacuum packaging is used for densely packed products where the air can be almost completely removed without crushing the food materials to unacceptably compact or broken forms. Some foods such as rice or noodles, however, can contain sharp edges that may cause pinhole damage to the barrier integrity of the packages. In such cases, liners are needed to ensure the integrity of the barrier during packaging and subsequent shock or vibration along the logistics supply chain. In other food types where the packing density is low, the remaining spaces hold residual oxygen. In these instances, modified atmosphere packaging technologies can be used to flush from the food packages replacing it with nitrogen gas.

Remaining oxygen or oxygen slowly leaking through barrier films can be scavenged from foods to achieve the extended shelf life required for military applications. The oxygen can be chemically reacted using a sacrificial food grade reducing agent such as ascorbate although many food types such as meats and fruits contain high natural levels of reducing agents that help preserve product integrity. Oxygen absorbers sachets based on finely divided iron are commonly used to remove any residual oxygen to less than 100ppm through reaction to form iron oxide (Cichello, 2015).

## **Flavor Changes in Long Stored Foods**

Slow oxidation and rancidity changes creating unpalatable tastes are a frequent problem in meeting military food storage specifications. This is exacerbated by the need for military foods to contain a balance of unsaturated fatty acids and increasingly to provide sources of omega 3 essential fatty acids as well as lipid soluble vitamins such as Vitamin E that are easily oxidized. The development of new foods therefore has to consider, not only the exclusion of oxygen to the greatest extent possible but also the elimination of oxidation catalysts like iron that can cause the slow onset of rancidity in otherwise stable dried or intermediate moisture foods. Additionally synthetic lipid phase antioxidants or natural extracts such rosmarinic acid esters are being used to achieve the necessary extended shelf stability.

## **Food Satisfaction Issues With Shelf Stable Military Foods**

Military rations are usually formulated to meet national taste preferences to ensure high levels of acceptability and consumption. Variety and choice, however, are also key considerations for modern military foods, in addition to the conventional standards of palatability and nutritional completeness. While this is not critical for short-term ration consumption, it does apply to extended missions. In addition, changes in dietary preferences or necessities such as food allergies/intolerances and in the future long duration space travel create challenges for maintaining the acceptability of rations as desirable meals. New technologies are being developed to improve the preservation of fresh food characteristics such as textures and flavors that are altered by thermal or drying processing. High-pressure sterilization (HPS) of food uses molecular changes induced by pressures in the range of 400–600 MPa to completely inactivate microorganisms and enzymes at lower temperatures than possible with conventional thermal processing. This results in better retention of colors, textures and flavors to increase eating satisfaction. Similarly microwave assisted thermal sterilization technologies use the volumetric heating ability of microwaves to more rapidly achieve internal sterilization conditions for solid or semisolid food types that otherwise rely on slow thermal conductivity to achieve the required heat penetration. The more rapid processing correspondingly results in improved retention of food properties and nutritional content.

## **Meeting the Complete Nutrition Requirements of Military Foods**

As military foods can be the sole source of nutrition, they need to be formulated to deliver the complete nutritional needs and organoleptic preferences of personnel. Military specifications are different from national medical and health recommendations

to account for the anticipated special needs of members of the armed forces. The recommended nutrient composition of Combat Ration Packs (CRP) for use during combat operations is given in [Table 1](#). According to [NATO \(2010\)](#), combat operations missions involving sustained, dismounted light-infantry or Special Forces operations require approximately 60% more energy than needed by comparable civilians. The dietary macronutrient distribution favors high carbohydrate levels to meet the high-energy needs with dietary carbohydrate contributing 45%–65% of total dietary energy intake, dietary protein contributing 15%–25% and, the balance of the energy being derived from dietary fat intake.

For longer-term sustenance, military foods must also meet critical levels of micronutrient and vitamin content where body stores can be depleted over time. The most well known example was the development of citrus juice supplements, during the 18th Century to prevent scurvy on navy vessels undertaking extended voyages of more than 30 days. Roses Lime Juice was patented in 1867 as a formula to preserve the juice and its anti-scurvy properties with sugar. Ascorbic acid (Vitamin C) was only identified as the active ingredient in 1932 [Wikipedia, Scurvy](#).

The recommended maximum period of continuous use of military foods varies from 10 to 30+ days.

**Table 1** Recommended nutrient composition of individual combat rations for use during combat operations

<i>Nutrient</i>	<i>Unit</i>	<i>Value</i>
Energy	kcal	4900
	kJ	20,515
Carbohydrate	g	550–800
Protein	g	158–185
Total fat	g	110–190
Total Fiber	g	30
Vitamin A	μg	900
Thiamin	mg	1.2
Riboflavin	mg	2.5
Niacin	mg	16
Vitamin B <sub>6</sub>	mg	2.6
Vitamin B <sub>12</sub>	μg	2.4
Folate	μg	400
Pantothenic acid	mg	6
Biotin	μg	30
Vitamin C	mg	45
Vitamin D	μg	5
Vitamin E	mg	10
Vitamin K	μg	70
Choline	mg	550
Calcium	mg	1000
Phosphorus	mg	1000
Zinc	mg	15
Iron	mg	14
Magnesium	mg	410
Iodine	μg	150
Selenium	μg	70
Molybdenum	μg	45
Copper	mg	1.8
Chromium	μg	35
Manganese	mg	5.5
Fluoride	mg	4.
Sodium	mg	2300–12,000 <sup>a</sup>
Potassium	mg	3800

<sup>a</sup>Recommend that lower limit of sodium content be achieved by the main course of the ration primary with the additional sodium (up to 9000 mg) provided in supplemental snack items and seasoning packets to be consumed on individual basis.

[NATO \(2010\)](#).

## Military Foods for Specific Operational Needs

Individual ration packs (CRP) are designed to feed personnel for a 24-hr period. They typically contain two main meals, a number of light meals and snack (consumed either alone or in combination with other items), meal accompaniments, beverages and sundry items. Main meals are typically a casserole style with a fish, lamb, chicken, pork, beef and/or vegetable based in a flavored gravy/sauce. Light meals and snacks can include pouched tuna, noodles, soup, cereal bars, cheese, confectionery, dried fruit, bread, mashed potato and biscuits. Accompaniments include spreads, sweetened condensed milk, sauces and seasonings. Beverages include instant tea and coffee, protein-based and isotonic drink powders. Lightweight variants of ration packs are designed for easy of carriage and typically contain two dehydrated main meals with additional snacks to keep the weight to a minimum. Group rations are designed for feeding detachments such as three to five personnel. The ration is self-contained and does not require supplementation. They typically contain main meals, a number of light meals and snacks but may be supplemented with freshly prepared bread, rice, pasta or noodles.

The composition of rations are also formulated for special needs. Military food specifications differentiate between normal v combat operations (different energy expenditure levels from activity), hot v cold environments and high altitude environments. Soldiers also rarely consume all the food components issued in combat ration packs (CRP) and discarded foods increases the risk of depleted physical and cognitive performance. The development of 'modular' or personalized rations is being advanced to have the ability to tailor rations to meet preferences in foods as well as variations and changes in nutritional needs.

## Conclusions

Military foods require specialized technologies to enable them to meet the complete nutritional needs of personnel and logistics requirements for extended storage. The thermal degradation of nutrients, flavors and texture during sterilization and the slow oxidation promoting rancidity and maillard reactions during prolonged storage are countered by specialized technologies to deliver fit-for-purpose military foods. Further research on meeting new challenges such as making functional foods to deliver health and cognitive performance benefits or satisfying the needs of space exploration are ongoing.

## Examples of Ration Packs and Products



**Figure 1** Supplements example: US Defence Caffeine containing gum for energy and alertness.



**Figure 2** Military tube food: US Defence pilots in pressurised suits.



**Figure 3** Military energy bar: US Defence High energy density food for supplementary energy.



**Figure 4** Modular ration pack US Defence for High Altitude/Cold weather.



**Figure 5** Example CRP: US Defence.





**Figure 6** Example CRP: Spanish Forces Defence.



**Figure 7** Example CRP: Australian Defence.

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# Crop Plant Adaption to Climate Change and Extreme Environments

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## Introduction

There is now strong evidence that the world has moved into a phase of rapid global climatic change (Norris et al., 2016). This combined with an ever-increasing global population means that the need for sustainable food production and global food security will push current agriculture practices to their limits in the near future (Abdelrahman et al., 2017). To meet these challenges, we not only need to make more efficient use of diminishing fresh water reserves and other natural resources, but we also need to rapidly develop new crops and crop varieties to ensure future agricultural sustainability in a more resource limited and changeable environment.

Plant development is a highly coordinated process that involves complex plant-environment interactions that require the integration of external environmental factors and signaling networks internal to the plant. These signaling networks function together to control a genetic program that when expressed correctly results in a functional plant able to complete its lifecycle. As well as helping to regulate plant development, the environment in which a plant grows also provides the necessary resources to enable that plant to sustain itself. Environmental factors that can influence plant growth and development are generally divided into two groups, biotic and abiotic. Biotic factors are living organisms, including other plants, animals (e.g. insects) and microorganisms (e.g. bacteria, fungi and viruses) that can potentially positively or negatively interact with plants. Abiotic factors include temperature, carbon dioxide (CO<sub>2</sub>), water supply, nutrients and pollutants etc., all of which potentially can directly influence plant growth and development.

Both biotic and abiotic factors can be influenced by climate and it is by altering these factors that climate change will have impacts on plants. This means that crop plant productivity in the future and hence crop yields are highly dependent upon future climatic conditions. As a consequence, the production of most of the food produced for human consumption is also highly dependent upon the future climate.

There is some evidence to suggest that rising CO<sub>2</sub> levels could improve crop yields, by enhancing photosynthesis and reducing crop water use (Kimball, 2011) and that higher temperatures may lengthen growing seasons, in some parts of the world (Myneni et al., 1997), enabling more crop harvests. However, most of the evidence to date suggests that it is more likely that if climate change is not brought under control in the near future, many of our staple crops will be more difficult to grow where we have grown them in the past and that even in developed countries climate change could have impacts on food security (Lobell et al., 2011; Lake et al., 2012).

An important question is how do plants adapt to these changes and what can we do ensure global food security in the face of climate change?

## What Are the Main Climatic Factors Associated With Climate Change and How Can Plants Adapt to Them?

The three main climate related factors that are gaining the greatest attention with respect to crop plant productivity are: elevated CO<sub>2</sub> levels, temperature increases (both mean and the temperature range) and water availability. While it is the above three factors that will be addressed below, it is important to note that other climatic factors, such as wind and relative humidity could also directly influence crop plant productivity and that climate change could influence biotic factors, such as the incidence of plant disease and the availability of pollinators. By influencing these biotic factors climate change could have indirect impacts on crop plant productivity. Another very important point to note is that many of the atmospheric pollutants, which have increased as a result of global industrialization, also have the potential to negatively impact plant growth and development directly, and hence crop productivity. These include, sulfur dioxide and trioxide (SO<sub>2</sub> and SO<sub>3</sub>), nitrogen oxides, soot particles and many organic chemicals and heavy metals.

## A Future With Higher CO<sub>2</sub> Concentrations and Temperatures, and Reduced Water Availability

The composition of the air we breathe is not fixed and can vary in response to both natural e.g. volcanic activity and anthropogenic factors e.g. emissions and deforestation. Over the past decades there has been increasing concern regarding the release of greenhouse gases such as methane and nitrous oxide, and especially CO<sub>2</sub> into the atmosphere. Atmospheric CO<sub>2</sub> concentrations increased from a preindustrial level of approximately 280 ppm to 379 by 2005 and concentrations are predicted to rise to above well 400 ppm by 2050, even with the most conservative emissions pathways (IPCC, 2007).

This increase in CO<sub>2</sub> and other greenhouse gases is resulting in global warming. From the data gathered over several decades it is now clear that in many parts of the world increases in mean temperatures and the frequency of extreme temperature events, such as heat-waves, are already occurring as a result of climate change (IPCC, 2013). It is predicted that global temperatures will continue to increase, with a rise in mean temperatures of 2–3 °C by 2050 and as much as 4.5 °C by AD 2100, depending on the predictive model and mitigation scenario (Allen et al., 2009). Temperature increases are expected to be greatest at high-latitudes; with increases being more pronounced during winter than in summer, and ground-level air temperatures likely to warm more quickly over land

than over the oceans (IPCC, 2013). As temperature is one of the main factors influencing the rate of plant growth and development, these increases could negatively impact the productivity of many crop plants especially in the tropics and subtropics.

As temperatures rise and global weather patterns change, including the frequency and distribution of rainfall, water availability will become an increasingly important factor limiting crop productivity. Depletion and salinization of ground water reserves is already a major problem in many important crop production areas of the world and reduced rainfall and the potential for increased salinization of fresh water due to the depletion of major aquifers and increased sea levels will become an issue for agriculture in the future. Already there is extensive evidence that variations in the amount and regularity of rainfall affect the dominance of plants in natural communities, and growth and yields of crop plants (Jones et al., 2016). According to the FAO (2011), crop plant yields are doubled by efficient irrigation, compared to farming that relies on rainfall (rain-fed agriculture). Climate change induced disruption of rainfall patterns combined with a reduced ability to irrigate crop plants, due to the depletion of ground water reserves, could mean that in the future water availability will be the most critical driver of whether a crop harvest is successful, crops fail completely, or yields are greatly reduced.

### Adaptation of Plants to Elevated CO<sub>2</sub>

For plants, and many other living organisms, it is the oxygen and carbon dioxide levels in the atmosphere that are especially important. As with most other organisms oxygen is essential for the processes associated with normal growth and development of plants, as it is needed for aerobic respiration. While light energy is required to power photosynthesis in plants, CO<sub>2</sub> is the raw material used for the production of sugars and this can be thought of as the first link in the human food chain and in fact that of the whole earth. Hence changes in atmospheric CO<sub>2</sub> levels can have a direct impact on crop plants.

The impact of increased atmospheric CO<sub>2</sub> concentrations on plants is quite well understood as studies of plant specimens collected over the past 200 years, that have been maintained in herbaria, clearly show that a major response of plants to increasing atmospheric CO<sub>2</sub> concentrations is a decrease in the number of stomata (Woodward, 1987). This change in stomatal number is under genetic control (Franks et al., 2015) and varies widely within a species (Miskin and Rasmusson, 1970), suggesting that the ability to adapt to increased atmospheric CO<sub>2</sub> concentrations exists for many crop plants and that the potential exists for the stomatal number of crop plants to be genetically manipulated. This decrease in stomatal number in response to increased atmospheric CO<sub>2</sub> concentrations has the added bonus of reducing water losses, but does have potential consequences for crop productivity as reduced stomatal number can limit gas exchange and hence the uptake of CO<sub>2</sub> for photosynthesis.

With respect to productivity and potential yields increased CO<sub>2</sub> concentrations could have both direct and indirect effects on some of our major crop species. However, the influence of increased CO<sub>2</sub> concentrations on a particular crop will depend on the photosynthetic mechanism used by the crop in question. Terrestrial green plants can be divided into C<sub>3</sub>, C<sub>4</sub>, and crassulacean acid metabolism (CAM) plants, depending on the photosynthetic mechanism used. In C<sub>3</sub> plants their photosynthetic pathway involves a cyclic series of reactions, called the Calvin–Benson cycle, which involves the use and regeneration of ribulose 1,5-biophosphate (RuBP) as a means to fix CO<sub>2</sub>. As the first product of CO<sub>2</sub> photoassimilation is the three-carbon sugar 3-phosphoglyceric acid, the term C<sub>3</sub> is used for plants that use this photosynthetic pathway. The first step of carbon fixation in C<sub>3</sub> plants involves the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), which catalyzes the carboxylation of RuBP. In C<sub>3</sub> plants, rubisco is contained in mesophyll cells, which are in direct contact with the intercellular air spaces that are in turn connected to the atmosphere via stomata present in the epidermis. As the name suggests, in addition to carboxylase activity rubisco also has oxygenase activity and this oxygenase activity becomes more dominant under low CO<sub>2</sub> and high O<sub>2</sub> concentrations, and at high temperatures (Sage and Kubien, 2007). Under these conditions rubisco's oxygenase activity impairs photosynthetic efficiency as rubisco sometimes fixes O<sub>2</sub> to RuBP instead of CO<sub>2</sub>, resulting in the oxidation of RuBP and the production of 2-phosphoglycolate. This 2-carbon compound is toxic and undergoes a series of reactions in the peroxisomes and mitochondria that result in the release of CO<sub>2</sub> (photorespiration) and a loss of organic carbon and energy. C<sub>3</sub> plants represent approximately 95% of terrestrial plant biomass and generally grow well where water is plentiful and temperatures and light intensities are moderate (Still et al., 2003). Some important C<sub>3</sub> crop plants include, barley (*Hordeum vulgare*), oats (*Avena sativa*), rice (*Oryza sativa*), soybean (*Glycine max*), potato (*Solanum tuberosum*) and wheat (*Triticum* spp.).

In contrast to C<sub>3</sub> plants, C<sub>4</sub> plants use the C<sub>4</sub> carbon fixation pathway, also called the Hatch-Slack pathway, which first involves fixing CO<sub>2</sub> into the three-carbon molecule phosphoenolpyruvate (PEP) to produce the four-carbon molecule oxaloacetate. The enzyme involved in this process, phosphoenolpyruvate (PEP) carboxylase, has no oxygenase activity and has a higher affinity for CO<sub>2</sub> than Rubisco. In C<sub>4</sub> plants the mesophyll cells near the stomata at the surface of the leaf contain PEP carboxylase, but no rubisco. In C<sub>4</sub> plants, rubisco is found in the bundle sheath cells in the interior of the leaf and so the oxaloacetate, which is concentrated in the mesophyll cells of the leaf, is converted into aspartic acid or malic acid and is then transported to the bundle sheath cells, where CO<sub>2</sub> is released and re-fixed by the C<sub>3</sub> pathway. The extra steps involved in the C<sub>4</sub> pathway require more energy (adenosine triphosphate, ATP) and as C<sub>3</sub> plants do not require this extra energy, are more efficient than C<sub>4</sub> plants when they are grown under optimal environmental conditions. However, when grown under high temperatures, low nitrogen levels and/or low CO<sub>2</sub> concentrations, or especially when water is limited, C<sub>4</sub> plants are able to fix CO<sub>2</sub> more efficiently than C<sub>3</sub> plants (Wang et al., 2013). Some important C<sub>3</sub> crop plants include, maize (*Zea mays*), sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum officinarum*).

CAM photosynthesis is a mechanism whereby plants take up and store CO<sub>2</sub> at night and then use the stored CO<sub>2</sub> for photosynthetic carbon fixation during the day, when light is available. As few agricultural crops use CAM photosynthesis, pineapple is an exception, they are not considered important for food security under conditions of climate change.

For C3 crop plants grown under present day conditions, ambient CO<sub>2</sub> levels are sub-optimal and rubisco is not CO<sub>2</sub>-saturated because CO<sub>2</sub> levels are not high enough. Hence the competing oxygenation reaction is not completely inhibited and so photorespiration occurs, which leads to the loss of some organic carbon and energy. However, in the future increased atmospheric CO<sub>2</sub> concentrations could directly affect photosynthesis in C3 plants via a process known as the CO<sub>2</sub>-fertilization effect (CFE) [Wang et al. \(2013\)](#). In C3 crops, increased atmospheric CO<sub>2</sub> concentrations should lead to enhanced carboxylation and reduced oxygenation, so photorespiratory losses would be less and carbon uptake would be enhanced. Therefore C3 crop plants should show an increase in net primary productivity (NPP) and hence increased yields. In addition, if the efficiency of carbon fixation is increased, a plant's carbon demands could be met earlier and so plants growing under increased atmospheric CO<sub>2</sub> concentrations may reduce their stomatal apertures reducing stomatal conductance. This would result in lower water losses due to transpiration and improved water-use efficiency (WUE). Hence soil moisture could be retained and likelihood of crop plants being placed under water stress would be reduced.

In contrast to C3 plants, in C4 plants the C4 mechanism already results in 3 to 6 times higher than atmospheric CO<sub>2</sub> concentrations in the bundle sheath cells where rubisco is localized, which is sufficient to saturate rubisco and largely inhibit photorespiration. Hence, in theory, there would be no CFE for C4 crop plants. However, in C4 plants a reduction in stomatal conductance caused by the increase in atmospheric CO<sub>2</sub> concentrations may still occur, which would increase WUE and potentially this could increase crop yields ([Long et al., 2006](#)).

However, the potential benefit of CFE brought about by CO<sub>2</sub> enrichment is a very controversial topic. While recent studies ([Wang et al., 2013](#); [Degener, 2015](#)) have suggested that CFE will increase under drier and warmer climatic conditions, other studies on C3 plants have suggested that any benefits of future CFE could be reduced or lost completely due to the indirect effects of more extreme weather conditions. For example, [Parry et al. \(2004\)](#) predicted that any CFE yield gains could be cancelled out due to adverse climate-driven yield decreases. In a more recent study using data from temperate grassland plants grown for 16 years under "free-air CO<sub>2</sub> enrichment" conditions, [Obermeier et al. \(2016\)](#) showed that CFE was reduced or lost when conditions varied from "local average environmental conditions", e.g. were wetter, drier and/or hotter. As in the future the demand for fresh water supplies may limit the amount of water available for crop production and the prediction that the frequency and severity of extreme weather conditions is likely to increase under future climate scenarios ([IPCC, 2013](#)), the ability of CFE to improve crop productivity should be viewed with some caution.

### Adaptation of Plants to Increased Temperatures

While most crop plants can survive over a reasonably wide temperature range the optimal day and night temperatures for plant growth and maximal productivity vary greatly between crop species ([Poincelot, 1980](#)). Most cultivars of our main crop plants are adapted to temperate regions and have been bred to yield well when supplied with optimum levels of water and nutrients. Therefore, they do not perform well under the more stressful conditions associated with higher temperatures. The yields obtained from crop plants are heavily dependent on photosynthesis and the chloroplast thylakoid membrane, electron transport systems, light harvesting complexes and the activation status of rubisco are all especially sensitive to heat stress ([Sharkey, 2005](#)). With respect to photosynthetic and respiratory rates, optimum temperature ranges for photosynthesis for C3 crop plants are lower than those of C4 crop plants ([Sharkey, 2005](#)). So C4 photosynthesis would be beneficial for C4 crop plants cultivated under predicted future climates. However, in general the respiratory response of crop plants to temperature is logarithmic, so the potential does exist for a large decrease in net carbon fixation with increasing temperatures, as respiration rates could increase ([Sharkey, 2005](#)).

Data gathered over the past thirty years have already provided strong evidence that significant reductions in the yields of many important crops has occurred due to global warming and it has been predicted that further increases in global temperatures and more frequent heat-waves are likely to result in even greater yield losses in the future ([Long and Ort, 2010](#)). In a recent predictive study [Zhao et al. \(2017\)](#) used data from the published literature and a robust multi-method analysis, comprising four independent methods, to predict the vulnerability of four major crops, wheat, rice, maize, and soybean, to climate change. Globally these four crops provide more than two-thirds of the caloric intake in the human diet. The study predicted that without CFE and the development of cultivars adapted to effectively cope with increasing temperatures, for each 1 °C increase in the mean global temperature the global yields of wheat, rice, maize, and soybean would, on average, reduce by 6%, 3.2%, 7.4% and 3.1% respectively. Importantly they did also point out that their results were "highly heterogeneous across crops and geographical areas, with some positive impact estimates" ([Zhao et al., 2017](#)).

So how will increased temperatures influence plants and what general mechanisms do plants have to cope with temperature increases? From a physiological perspective, an increase in mean temperature could increase the rate of key biochemical processes in plants, but in all likelihood this effect would be reduced by increased transpirational cooling and acclimation responses. Leaving aside the increased likelihood of water deficit, the main danger for crop plants would be a greater frequency of hot days and/or higher temperature extremes. This could result in extended periods of heat stress and result in the induction of typical heat-shock responses. With respect to adaption to temperature extremes, plants possess a wide variety of mechanisms that enable them to grow at elevated temperatures. At the molecular level, plants possess a class of proteins called Heat-shock proteins (HSPs). These proteins are termed "molecular chaperones" as they are known to play an important role in preventing protein

misfolding and in stabilizing proteins under high-temperature induced stress (Jacob et al., 2017). In plants under heat-stress, protein misfolding is common and the genes encoding many HSPs are relatively rapidly upregulated at high temperatures. In addition to their roles under high temperature stress, HSPs/chaperones have been found to play multiple roles in protecting plants from other forms of stress including cold, drought, osmotic, salt, high-light, ultraviolet radiation, pathogen infection and oxidative stress (Swindell et al., 2007). For an in-depth review of plant HSPs readers are referred to Jacob et al. (2017).

In addition to high temperatures causing general stress responses in crop plants, another important factor that should also be considered is the acceleration of a crops development/life-cycle. For example, wheat is a temperate C3 grass species that produces its highest grain yields at temperatures below 26 °C. It is also very sensitive to heat stress and the impact on yields can be severe. Increasing temperatures have already been reported in most of the main wheat producing regions of the world (Asseng et al., 2011). Increasing mean temperatures have been shown to reduce grain yields, even for well-watered wheat crops, with plants most vulnerable to high-temperature induced yield losses at flowering and grain filling (Asseng et al., 2011). For wheat, high temperatures are known to accelerate plant development and hence reduce the growing season. This can limit the plants ability to produce and accumulate the carbohydrates needed for grain set and filling. In addition, heat stress just before flowering can result in floret sterility, which reduces the number of grains produced per plant and hence potential yields. How serious could higher global temperatures be for food production? In a review of the impact of temperature variability on wheat yields, Asseng et al. (2011) stated "With average temperatures and the frequency of heat events projected to increase world-wide with global warming, yield reductions due to higher temperatures during the important grain-filling stage alone could substantially undermine future global food security".

On a positive note, an increase in mean temperature and a reduction in the frequency of cold weather events, would reduce the need for chilling and freezing responses in crop plants, and could increase crop plant growth rates and extend the growing seasons in some parts of the world. However, for this to be of value a global re-evaluation of where we grow our staple food plants is required.

### Adaptation of Plants to Reduced Water Availability

Reduced soil water availability resulting in "drought stress" is becoming an increasingly common problem, due to a combination of climate change and depletion of ground water reserves, often due to excessive agricultural use. A lack of available water is, and is likely to remain so in the future, the main environmental factor that limits crop productivity and yields (Bray, 1997; Dai, 2012).

Depending on the duration and severity of the drought, crop plants can adapt to reduced water availability at the molecular, biochemical, physiological, and morphological levels. However, our main crop plants are not desiccation tolerant and so cannot survive once soil water levels drop below a critical threshold. In addition, adapting to lower soil water availability is costly to plants and so even drought tolerant plants will show reduced yields under drought conditions compared to yields when grown under conditions of optimum soil moisture. The timing of the drought event with respect to the developmental stage of a plant can also be critical, as plants are often more sensitive to drought stress when the drought event occurs during the plants reproductive phase, i.e. prior to or at flowering or seed production (Farooq et al., 2009).

With respect to adaptation to drought, plants have evolved a range of morphological, physiological, and biochemical mechanisms, which are often used together, to cope with reduced soil water levels. However, these mechanisms can result in lower leaf area indexes, lower vegetative biomass and hence reduced yields, compared to plants grown under conditions of optimal soil moisture. While a detailed coverage of the mechanisms associated with adaptation to drought is beyond the scope of this commentary (for brief reviews of how plants cope with drought please see Farooq et al., 2009; Osakabe et al., 2014) the key mechanisms include; smaller leaves, and slower growth rates (that result in a smaller plants), reduced stomatal densities and stomatal closure, osmotic adjustment and the production of compatible solutes, increased scavenging of reactive oxygen species (ROS), and the synthesis of proteins (e.g. dehydrins) that help plants reduce cellular damage due to water loss. The induction of these processes requires that plants are able to sense water loss and then via signaling pathways, which involve crosstalk between the pathways involved in plant responses to other stressors, regulate the expression of drought-responsive genes. Some of the signaling mechanisms that function in plants in response to water deficit include; Absciscic acid (ABA), strigolactones, ROS, sugar mediated pathways, and others. The development of cultivars, by manipulating the above mechanisms in our main crop species, that are both high-yielding under optimal conditions and are able to maintain good yields under drought stress conditions is essential for future food security.

### Can We Produce Enough Food to Feed a Growing Global Population in the Face of Climate Change?

At no time in history have plant scientists and plant breeders had available to them the range of tools with the potential to be applied to crop plant improvement that we have today. Using both conventional breeding tools and molecular marker-assisted breeding (MAB) the natural sources of genetic variation in many important crop plants and their wild relatives can still provide plant breeders with opportunities to identify novel alleles that can be used to produce abiotic stress tolerant cultivars of our main crops (Tester and Langridge, 2010; Huang and Han, 2014; Nunes-Nesi et al., 2016). While in the past this process would have required that numerous breeding lines be grown under a diverse range of environmental conditions, advances in rapid, low-cost sequencing and genotyping technologies and advances in DNA marker assays mean that cultivar development has the



potential to be more rapid than at any time in the past. In addition, although the contribution to agriculture to date has been very limited, the development of genetic engineering tools and the increasing use of gene editing have the potential to produce cultivars with tolerance to the environmental stressors associated with climate change (Barrangou and Doudna, 2016; Schiml and Puchta, 2016; Paul et al., 2017). High-throughput automated phenotyping technologies also have great potential to assist the development of cultivars more tolerant of these stressors (Cobb et al., 2013; Fiorani and Schurr, 2013).

In addition to breeding stress tolerant crop plants, other approaches should also be considering to improve future food security. For example, Davis et al. (2017) compared current and optimized crop distributions, for 14 major food crops, in terms of production and water use. They found that the current distribution of crops around the world was not optimal and identified alternative global crop distributions that they estimated could feed an additional 825 million people and reduce rainwater and irrigation water use by 14% and 12%, respectively.

However, food is not all about calories and Muller et al. (2014) expressed concern that current research on the potential impacts of climate change on food security, largely focuses on yields and calories, and not on nutritional quality. They state that, "Atmospheric CO<sub>2</sub> fertilization may go some way to compensating the negative impact of climatic changes on crop yields, but it comes at the expense of a deterioration of the current nutritional value of food". This is a very important and often overlooked point, as nutritional deficiencies are already a global problem. Future breeding efforts to produce crop plants that are more tolerant to climate change should pay careful attention not only to food quantity, but also to food (nutritional) quality.

## Conclusions

Above, each of the three main climatic factors that could limit crop plant productive have been briefly discussed individually and all have been shown to potentially influence either positively or negatively crop yields. However, as can be seen from the above sections, under natural conditions, these three climatic factors almost always occur together and interact with each other. As future crop growing conditions will likely expose plants to multiple climate change related factors simultaneously, with a sum negative influence on global agriculture, a critical area of future research is how can crop plants be adapted to produce high yields under multiple stressor scenarios? This is a very important, but under researched area. It is also very important to note that many factors other than climate change can influence food security e.g. customer demand, agricultural practices and especially local and global political shifts. However, what is clear is that an understanding of how climate change will influence our stable food plants is critical if plant breeders, farmers, and policymakers, are to work together to ensure future global food security.

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# Advancements in the Understanding of Pectin Methyltransferase Enzymes and Their Inhibitors for Use in Food Science Applications

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## Glossary

**Enzymatic processivity** ability of enzymes to catalyze several reaction cycles before dissociating from a substrate.

**Glycosidic bond** covalent bond linking two monosaccharides.

**$\beta$ -Helix fold** protein fold characterized by the arrangement of multiple  $\beta$ -strands in a helical configuration.

**Carbohydrate-binding enzymes** class of enzymes that specifically bind carbohydrates, mostly polysaccharides. This ability is ascribable to a particular part of the fold called carbohydrate-binding module and often characterized by a binding groove able to allocate a polysaccharide linear chain.

**De-methyl esterification** chemical reaction having as a substrate a methyl ester and leading to the removal of the methyl group and the creation of a carboxylic acid and methanol.

**Phytopathogen** a pathogenic organism attacking plant hosts.

**Hamiltonian** a function describing the total energy of a system.

**Monte Carlo simulations** simulation technique based on a class of algorithms that obtains numerical solutions through a repeated random sampling of specific observables.

**Multiple sequence alignments** alignment of more than two protein, DNA or RNA sequences.

**Co-evolutionary analysis** method to assess the degree of inter-dependence between molecules by mainly looking at their sequence and scoring the observed recurrence of particular residues along the aligned sequences.

## Introduction

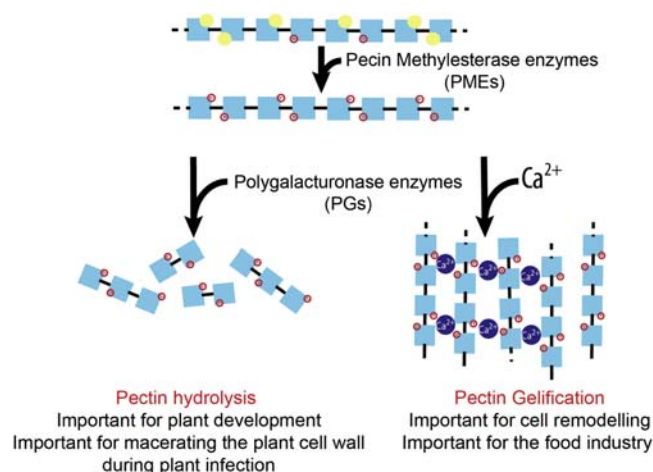
### Pectin Methyltransferase Enzymes, Their Role in Plant Physiopathology and Their Applications in Food Production

Pectin is the most abundant component of the plant cell wall and its modifications are a crucial factor in many life stages of plants. For this reason, the plant cell wall can be considered as a dynamic organelle rather than a simple barrier between the interior of cells and the outside world. From a structural perspective, pectin is a heteropolysaccharide composed by linear chains of homogalacturonan (HG) stretches constituted of  $\alpha$ -(1,4)-linked D-galacturonic acid monomers, branched by xylogalacturan or apiogalacturonan polysaccharides of various length. Besides HG, rhamnogalacturonan (RG) I or II are also present in variable quantities and are composed of a polymer of galacturonic acid-rhamnose disaccharides, which can be branched by neutral sugars such as D-galactose, L-arabinose and D-xylose (Darley, Forrester & McQueen-Mason, 2001; Fasoli et al., 2016; Gorshkova et al., 2013; Mohnen, 2008; Pauly and Keegstra, 2016; Ridley, O'Neill & Mohnen, 2001; Willats, McCartney, Mackie & Knox, 2001; Wilson et al., 2000; Zabackis et al., 1995; Zabolina, 2016).

The metabolism of pectin is extremely important as it regulates a variety of processes linked to both plant development and plant defense and is therefore tightly controlled via a cascade of enzymes (Cosgrove, 1993, 2005; Fry, 1995). Modifications occurring on the HG chains are particularly important as they regulate the assembly and disassembly of pectin and, in this way, the remodeling of the plant cell wall in physiological contexts and during plant infection, in which pathogens aim to create a breach into the cell wall by disrupting HG networks via pectin hydrolysis (Kubicek et al., 2014). Hydrolysis of pectin, however, can only occur after a step-wise modification of the monomeric units composing HG.  $\alpha$ -(1,4)-Linked D-galacturonic acid can exist as a methyl esterified monomer which can then be de-methyl esterified by pectin methyl transferase (PMEs) enzymes. The removal of methyl groups by PMEs transforms neutral HG chains into highly negatively charged polymers, which have different physico-chemical properties with respect to highly methylated pectin and serve different physiological purposes in the plant cell wall (Cosgrove, 2005). Pectin hydrolysis is initiated with the de-methyl esterification of HG chains, as pectinolytic enzymes called polygalacturonases (PGs) can only efficiently recognize de-methylated substrates (Arancibia and Motsenbocker, 2006; Babu and Bayer, 2014; Chen and Mort, 1996; Daas et al., 2001) (Fig. 1).

The activity of PMEs is of such critical importance to the physiopathology of plants that it is tightly controlled in the cell wall and inhibited in a timely manner by the expression of proteinaceous inhibitors called pectin methyl transferase inhibitors (PMEIs) (Giovane et al., 2004; Juge, 2006; S  n  chal et al., 2015). To give an idea of how important the activity of PMEs and PMEIs in plants is, it could be enough to mention that in *Arabidopsis thaliana* 66 different PME-PMEI pairs have been identified, with specificities and expression patterns still mostly unknown (Louv  t et al., 2006).

Importantly, PMEs are of extreme interest for the food industry in various manufacturing processes: for the production of pectin with desired characteristics, the fabrication of low sugar jams and jellies, the clarification of juices or the enhancement of fruit and vegetable firmness (Kohli et al., 2015; Kumar, 2016; Sharma et al., 2017).



**Figure 1** Schematic representation of pectin chemical modifications and breakdown. The modification of pectin is initiated by Pectin methyl transferase enzymes (PMEs) that remove methyl groups (yellow circles) from homogalacturonan chains: the linear polysaccharide of pectin. The exposed negative charges can be recognized by polygalacturonase enzymes (PGs) along the enzymatic cascade. PGs hydrolyze the glycosidic bond between sugar monomers and disassembly pectin. In the presence of divalent (mostly calcium) ions, HG chains modified by PMEs, can assemble by forming gels. Hydrolysis or gelification of pectin is crucially important for the development and, in some cases, infection of plants and is extremely useful to the food industry for several industrial processes, from juice clarification to the enhancement of fruit and vegetable firmness (see text for details).

Pectin is majorly responsible for the cloudiness of juices, which impacts both viscosity and sensory properties of a product. A strategy used to eliminate pectin during the treatment of juice is to favor its precipitation through the addition of PMEs.  $\text{CaCl}_2$ , which forms complexes with the negatively charged pectin (modified by PMEs), is subsequently added to the juice. Ultimately, upon binding  $\text{Ca}^{2+}$ , the pectin precipitates facilitating its removal.

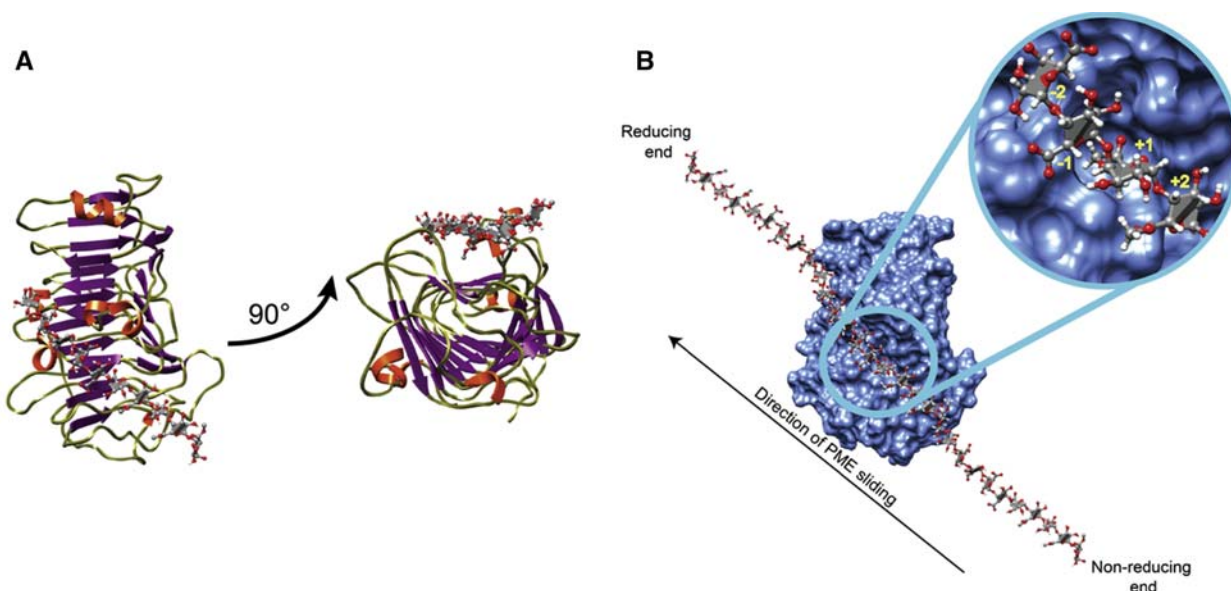
Alternatively, the combination of PMEs and PGs can also improve juice clarification through the hydrolysis of pectin polymers, thus creating shorter polymeric stretches. On the contrary, in order to preserve the firmness of the product by mimicking the polysaccharide networks that maintain the integrity of the plant cell walls, a similar treatment for fruit and vegetables is carried out. During post-harvest treatments of fruit and vegetables, which may include freezing, dehydrating or pasteurizing, the disruption of the cell wall leads to loss of cellular components and fluids and to the deterioration of product firmness. The use of PMEs and  $\text{CaCl}_2$  as previously described for the process of juice clarification is advantageous to avoid loss in firmness. Upon the removal of methyl groups from HG chains,  $\text{Ca}^{2+}$  ions bridge the negatively charged carboxylate groups of de-methylated HG chains favoring the formation of polymeric networks that, in this case, have the effect to preserve the firmness of the processed fruit or vegetable (Fig. 1). Isolated pectin, on the other hand, can be used as a thickening or gelling agent according to the degree of negative charge that it carries (Moreira et al., 2014; Urias-Orona et al., 2010).

Overall, the numerous uses of PMEs in the food industry make these enzymes particularly significant beyond their physiological role. However, a fine control of industrial processes that can be regulated by the use of PMEs can only occur once the biological principles regulating the activity of these enzymes and their inhibitors have been elucidated. Moreover, the rational design of new PMEs needs to be devoted to either enhance or inhibit their activity as a function of pressure or temperature, which may wildly oscillate during product manufacturing. Here I will illustrate the recent advancements made in our understanding of PMEs and will describe how the acquisition of knowledge about these enzymes can lead to the creation of engineered PMEs with desired functional and regulatory properties.

### The Structural and Dynamical Properties of PMEs Reveal Useful Strategies to Enhance Enzymatic Efficiency

The structure of all PME isoforms, so far characterized by means of X-ray crystallography or molecular modelling (Boraston and Abbott, 2012; Camardella et al., 2000; Ciardiello et al., 2008; D'Avino et al., 2003; Jimenez-Lopez et al., 2012; Jolie et al., 2010; Kent et al., 2016; Markovic et al., 2002; Teller et al., 2014), has revealed a triple  $\beta$ -helix as their characteristic three-dimensional fold. Triple  $\beta$ -helices are not rare in carbohydrate-binding enzymes as they allow the formation of a groove able to allocate linear polysaccharides. The binding groove can be then subdivided into a series of subsites, each one able to accommodate the single monomeric units composing the polysaccharide (Fig. 2).

Although the triple  $\beta$ -helical fold is common to all the PME structures known and to numerous other pectinases (Ciccarelli et al., 2002), a quick surf among the published structures reveals profound differences. A paradigmatic example of this is the structure of pmeA expressed in the bacterium *Erwinia chrysanthemum* in which the binding groove is flanked by two long flaps (Jenkins et al., 2001). The exact role of these flaps is still unknown but a plausible explanation for their presence is the bacterial need to avoid the inhibition of this enzyme by plant PMEIs, which act by sterically hindering the binding groove.



**Figure 2** Fold and substrate binding of Pectin Methyltransferase enzymes. (A) Structure of a pectin methyltransferase binding a HG decamer. The  $\beta$ -helix is composed, at each pitch, by the turn of three  $\beta$ -strands (purple) and is alternated with unstructured segments (loops).  $\alpha$ -helical segments are shown in orange, whereas loops are shown in ochre. The HG decamer binding the enzyme is shown using a “balls and sticks” representation and colored by atom type (carbon grey, oxygen red and hydrogen white). (B) Surface representation of a PME enzyme bound to a HG polymer. The arrow shows the directionality of processive PMEs, which slide along the substrate from the non-reducing to the reducing end of the polymer. The binding groove of PMEs is subdivided in smaller subunits called subsites and labelled as -2, -1, +1 and +2 (yellow labels in the inset). The catalytic triad responsible for the enzymatic modification of the substrate is located in the +1 subsite.

Overall, at least three main features of PMEs’ activity are interesting for the food industry. These are enzymatic efficiency, specificity and stability. I will therefore focus on these three features explaining the basis of PME action on pectin substrates and how the acquired knowledge can already be useful to further study and rationally modify these enzymes to meet the needs of industry.

Recently, combined experimental and computational efforts have revealed the basis of PMEs and PMEIs activity (Hocq et al., 2017; Kent et al., 2016; Mercadante et al., 2014; Mercadante et al., 2013; Senechal et al., 2017).

From a biophysical perspective, PMEs are extremely interesting machines. Many proteins, especially those involved in the duplication or translation of genetic material, bind linear polymers (for example, DNA, RNA or polysaccharides) and move along them to perform their action, effectively acting as molecular motors (Maga et al., 2000; Morales and Kool, 1999; Tsurimoto and Stillman, 1991). In order to do this, they mostly require an energetic co-factor, which commonly consists of adenosine triphosphate (ATP): they are ATP-dependent. The energetic co-factor acts as an adjuvant to perform useful work as these molecules need to bias thermal fluctuations that in the nanoscale are not negligible. To explain this concept I will refer to the so called Brownian ratchet, which is a thought experiment first proposed by Marian Smoluchowsky in 1912 (Smoluchowsky, 1912).

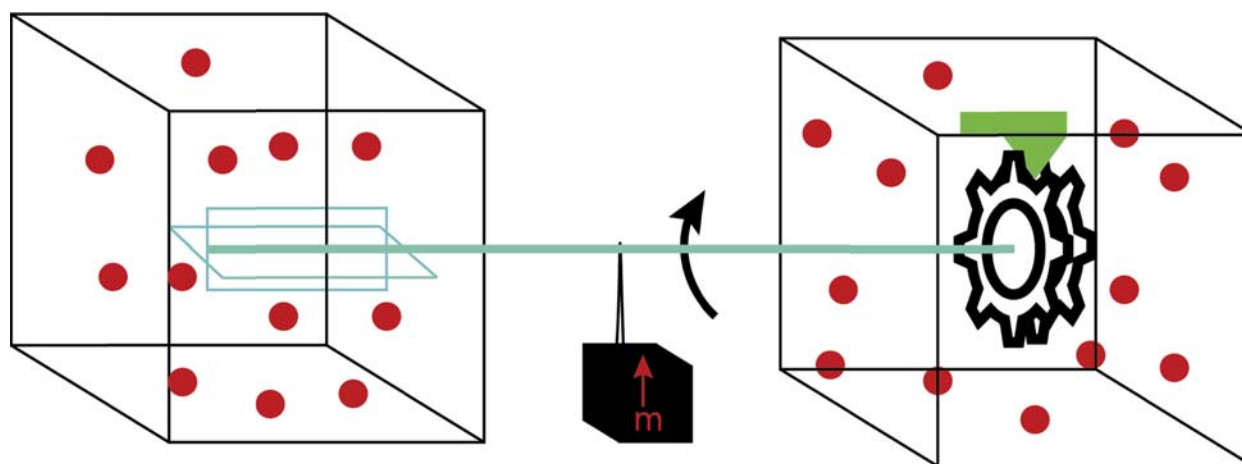
A Brownian ratchet is an imaginary device consisting of two chambers that contain gas particles heated at temperatures  $T_1$  and  $T_2$ , with  $T_1 = T_2$  as the boxes are in thermal equilibrium. In one of the chambers there is a paddle, small enough to be hit by the gas atoms and rotate. The paddle is then connected, through an axle, to a second chamber in which a dented wheel, blocked by a pawl, would rotate in a single direction as a consequence of the rotating paddle hit by the gas particles in the first chamber and the pawl impeding backwards movements in the second chamber (Fig. 3).

At first appearance, since the pawl would block the wheel from rotating back and forth, such a device would generate useful work at thermal equilibrium, thus clearly violating the second principle of thermodynamics. Years later Richard Feynman demonstrated that this is not possible, as the thermal fluctuations affecting the gas molecules would also similarly affect the pawl, which, subject to thermal motions of the same magnitude due to the thermal equilibrium, would fail to block the wheel from rotating backwards (Feynman, 1963). As a result, rather than continuously rotating in a single direction, the wheel would rotate back and forth.

*But what does a Brownian ratchet have to do with enzymes sliding on linear substrates?*

Since enzymes work in the nano-scale, their sliding along mono-dimensional chains is similarly affected by non-negligible thermal fluctuations that would therefore randomly produce sliding in both directions. Nevertheless, protein function can only be successfully performed when sliding is unidirectional. Therefore, the energy provided by the hydrolysis of ATP into ADP is commonly used to bias thermal fluctuations so that the sliding along a substrate occurs in only one direction and the molecule doesn’t slide backwards.

The evidence that PMEs can move along linear HG chains catalyzing several de-methyl esterification events before dissociating from the polymeric substrate is given by the observation that some PMEs produce blocks of de-methyl esterified substrates (Cameron et al., 2008; Grasdalen et al., 1996; Guillotin et al., 2007; Willats, 2001) (Fig. 2b). The ability to catalyze several reactions on a linear chain before dissociating from it, is called *processivity* (Berg et al., 2002).



**Figure 3** Schematic illustration of a Brownian ratchet. A Brownian ratchet is composed of two chambers in which gas particles (red circles) diffuse randomly according to Brownian motions. The two chambers are at thermal equilibrium (temperature in the two chambers is identical). By hitting the paddle in the first chamber, gas particles allow the rotation of the wheel in the second chamber. The pawl (green) insures that the wheel moves in a single direction as it blocks backwards movements. Ultimately, this generates useful work that in the example is represented by lifting a weight of a defined mass ( $m$ ). This thought experiment was formulated by Marian Smoluchowsky in 1912 to prove that work can be done in conditions of equilibrium and has been proven wrong by Richard Feynman, who noticed that the pawl in the second chamber will fail in impeding backward movements of the wheel as it will be affected by the same thermal fluctuations of the gas particles in chamber one, ultimately making impossible the achievement of fruitful work at equilibrium.

Processivity of PME is of great relevance to the food industry as blocky or randomly esterified pectins have different physico-chemical properties and can therefore be used for different purposes. In order to control the degree of methyl esterification in pectin, it is thus crucial to understand the basis of the processivity of PMEs. Even of more interest, PMEs do not require energetic co-factors to perform processive catalysis on HG chains, which brings the question of how these enzymes could perform useful work without an external energy source.

An interesting perspective on processivity, analyzed from a purely structural standpoint, has been provided by Breyer and Matthews, who analyzed the structure of numerous enzymes including those of many carbohydrate-binding proteins and featuring either open or closed substrate binding grooves (Breyer and Matthews, 2001). They concluded that structural asymmetry is key to achieve processivity, as it insures topological restraints along the binding interface and these restraints act like the pawl described in the Brownian ratchet formulated by Smoluchowsky (Smoluchowsky, 1912). For proteins featuring an open binding groove rather than a closed toroidal structure, an explanation for processivity is less straightforward and must occur through the binding of substrate moieties with different affinities along the groove: hence generating binding asymmetry (Breyer and Matthews, 2001). Structural or binding asymmetries can therefore be related to the thermal dis-equilibrium that a Brownian ratchet needs in order to work.

The triple  $\beta$ -helix fold of PMEs allows for the formation of an open groove in which HG substrates are allocated. Recent studies characterizing the binding of *E. chrysanthemum* PME to a variety of differently methylated HG oligomers have revealed binding preferences for either methylated or negatively charged sugar units in different subsites of the binding groove (Fries et al., 2007).

Within the binding groove, the subsites can be identified and labelled starting from the position of the catalytic site, which is conventionally placed in subsite +1. Consequently, the subsites flanking the catalytic triad towards the reducing end of the polysaccharide are numbered using the minus sign ( $-1$ ,  $-2$ ,  $-3$ , etc.) while those towards the non-reducing end of the polysaccharide are numbered using the plus sign ( $+2$ ,  $+3$ , etc.) (Fig. 2b). An analysis of the oligosaccharide conformations bound to the enzyme revealed that subsequent monomeric units along the groove point the carboxymethyl ester groups in opposite directions, suggesting that a simple sliding of the enzyme would not be sufficient to assure multiple catalytic events along the chain (Mercadante et al., 2013). Indeed, upon the de-methyl esterification of the first residue in subsite +1, the monomeric unit in subsite +2 would not face the catalytic triad, making catalysis impossible. Therefore, either the rotation along the glycosidic bond of adjacent monomeric HG units or the rotation of the enzyme around the bound chain needs to occur.

Although a structural analysis of PMEs can promote speculation regarding the mechanism adopted by these molecules to achieve processivity, only the analysis of their dynamics could shed direct light on it. Molecular dynamics (MD) simulations of *E. chrysanthemum* PME bound to differently methylated substrates have shown that the catalysis of a D-methyl galacturonate in the active site releases the potential energy necessary to achieve the rotation of the substrate in order to promote a second catalytic event. The de-methyl esterification reveals a negatively charged moiety, which is docked into a negatively charged pocket containing the catalytic triad. Upon catalysis, the repulsion between the two similarly charged moieties (the active site and the freshly generated carboxylate group) increases the potential energy of the system generating a flipping of the monosaccharides along the glycosidic bond linking them (Mercadante et al., 2013). As a consequence, the residue pointing to the methyl ester opposite to the catalytic triad in subsite +2 now points towards it (Video 1). Thus, the sliding of the substrate along the group would position the second methyl ester



in the right position and orientation for catalysis (Video 2). *What then drives the sliding?* The analysis of the electrostatic potential along the binding groove, observed in MD simulations once de-methyl esterification of the monomer docked in the +1 subsite has occurred, suggested that charges along the binding groove are asymmetrically distributed and that such asymmetry drives the sliding of the substrate. A positive patch in the -1 subsite “drags” the freshly de-methylated monomer docked in subsite +1 along the binding groove, ultimately promoting slithering (Mercadante et al., 2014). The processive activity of the enzyme is therefore promoted by an asymmetric chemical potential along the binding groove that allows first rotation and then sliding of the HG chain, finally achieving useful work meant as the unidirectional motion of the enzyme and the subsequent generation of blocky de-methyl esterified pectin. Using this knowledge, the rational engineering of PME by mutating critical residues favoring or disfavoring the above-described mechanism will be crucial to tune processivity and meet the needs of the food industry. Further research comparing the activity of PMEs that show different degrees of processivity will be invaluable.

### **The Molecular Basis of PME Specificity and the Possibility to Design PME Small-Molecule Inhibitors Starting From the Acquired Knowledge Regarding Enzymatic Processivity**

The discovered mechanism of processivity has laid the foundation to understand the specificity of processive PMEs for different substrates. Highly methylated substrates are not good substrates for processive PMEs, because, as evidenced by both structural and dynamical studies, the basis for processivity lies in the ability to bind partially de-methyl esterified substrates. This assures a differential affinity of binding for monomeric units in different subsites of the groove (Breyer and Matthews, 2001; Fries et al., 2007; Mercadante et al., 2014; Mercadante et al., 2013) and this asymmetry is crucial for enzymatic processivity. The mechanism describing processivity therefore explains why processive PMEs may act slower when incubated with highly methylated pectin while becoming progressively more efficient once the de-methyl esterification of the HG chains starts occurring. The mechanism by which a polymeric substrate bound by PMEs slides along the groove is of great importance for the design of small-molecule PME inhibitors, especially considering that the food industry has searched for these to finely control the activity of PMEs. Additionally, an efficient inhibition of PMEs by small-molecule compounds will be useful to investigate the role of specifically expressed PMEs in plants. Interestingly, the search for small-molecule PME inhibitors has focused towards green tea catechins, which have shown the ability to inhibit both isolated PMEs and PME extracts (Lewis et al., 2008), with these investigations having an empirical rather than a rational basis. Nevertheless, the knowledge about the mechanism adopted by the enzyme to achieve processivity, can be used to design substrate analogs that reduce the asymmetry required for the processive action of the enzyme and, in doing so, modulate catalytic efficiency. The design of efficient inhibitors can also be directed towards compounds that are hindered in their ability to rotate around the glycosidic bond, possibly modifying the linkage between D-galacturonate monomers. Overall, although more research is significantly needed, the studies that have elucidated the structural and dynamical basis of a few pectin methyl transferase enzymes from plants, bacteria and fungi, have paved the way for the design of new strategies to be used for the modulation of PME activity.

### **Enhancing the Thermal Stability of PMEs and Their Proteinaceous Inhibitors, for Their Use in the Food Industry**

In plants, the activity of PMEs is tightly regulated through the expression of PMEIs because of their importance in modifying the plant cell wall and consequently affect cellular development. A consequence of this is their redundancy within the proteome of plants. The exact role of all the expressed PME-PMEI pairs is unknown, however it has been described that the activity of different PME-PMEI pairs is dependent from micro-environmental conditions such as pH or ionic strength. The ability of PME-PMEI pairs to be sensitive to the micro-environment is extremely appealing to the food industry as the differential activity of PMEs through the use of their natural proteinaceous inhibitors can be explored according to the conditions in which a single industrial procedure is carried out.

The combined efforts of experiments and molecular simulations have recently explained the structural basis of this micro-environmentally controlled activity. The pH-dependent interaction of several PME-PMEI pairs has been assessed by means of MD simulations to pinpoint the structural and dynamical features of complex stability at different pH values. Simulations revealed that the sensitivity to changes of pH is mediated by the activity of a few protonatable residues at the binding interface (Hocq et al., 2017). The mutation of these residues decreased the stability of a PMEI from *A. thaliana* at pH5 (PMEI7). Mutated forms of the inhibitor were indeed unable to efficiently inhibit their target PME (PME3), transforming a pH-dependent PMEI into a pH-independent protein.

Besides the pH-controlled activity of PMEIs, one of the main interests of the food industry is inhibiting PMEs in industrial processes that undergo high temperatures and at which PMEIs are usually inactive. It is well known that few PMEs in crude fruit extracts are thermo-resistant and that the possibility to have thermally stable inhibitory counterparts (PMEIs) is a crucial advantage. Therefore, strategies for the design of thermally stable proteins can be particularly useful. In addition to the enormous, possibly unsurmountable difficulties in designing protein sequences *de novo*, the biggest limitation for the use of structure-based algorithms for the creation of more stable PMEIs is the lack of structural information. To date, only a few PME/PMEI structures have been resolved and the knowledge about their relative thermal stabilities is limited. On the other hand, the sequence space of these variants, due to the numerous molecular biology and proteomics studies on plant proteomes, has expanded the amount of sequences of expressed PMEs and PMEIs. It is therefore logical to start exploring the possibility to design more thermally stable PMEIs by looking into the sequence space and their co-evolution across many proteins of the same class (Tian and Best, 2017).



Recently, it has been suggested that the design of more stable proteins can be achieved by combining sequence information via multiple sequence alignments (MSA) to design co-evolutionary fit sequences. This approach has proven successful for some folds, such as those found in the GA, GB and SH3 domains (Tian et al., 2018).

Briefly, the design of a co-evolutionary fit sequence is based on extracting information regarding the preferential positions of each residue and its relative position with respect to every other residue, considering a multiple sequence alignment calculated from as many possible relevant sequences. This information is used to estimate the likelihood of a designed sequence using a likelihood estimator. The sequence design proceeds via the shuffling of residues through a Monte Carlo (MC) simulation, where the substitution of each residue is tested using the Metropolis criterion and guides the creation of an artificial sequence, to which an evolutionary Hamiltonian energy is assigned. The Hamiltonian energy has shown to negatively correlate with the designed proteins' ability to fold and with its thermal stability. Overall, the method has proven useful to design new, more stable proteins, and more importantly proteins still retaining their function (Tian et al., 2018). The stability of the newly designed proteins can then be tested using MD simulations run at different temperatures. Since short MD runs performed at high temperatures can be enough to assess stability and finally select a few candidates for experimental validation, a large number of designed sequences can be tested and this increases the probability to find designs with enhanced thermal stability.

Therefore, the application of this method can be extremely useful for the rational control of industrial processes mediated by pectin methyl esterase and other pectinases.

## Conclusions

Pectin methyl esterase enzymes are of enormous interest to both plant biology and food science for their role in the physiopathology of plants and their uses in various industrial applications. Thus, the control of their activity as a function of the micro-environment is particularly important. Recent advancements in the understanding of these enzymes, their mechanism of action and their inhibition by proteinaceous binding partners can fuel innovative research to enhance industrial applications of these enzymes and understand basic processes interesting to food science. Nevertheless, considering that the most recent advancements in understanding these molecular entities have come from single-molecules experimental and computational studies, a multidisciplinary approach that combines more traditional food science branches of research (analytical, textural or sensorial) with new biophysical strategies is needed and will prove beneficial for the improvement of tunability in industrial processes and the expansion of knowledge about food, with positive effects on the global economy.

## Supplementary data

Supplementary data related to this article can be found online at <https://doi.org/10.1016/B978-0-12-814026-0.21724-1>.

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# Addressing Global Protein Demand Through Diversification and Innovation: An Introduction to Plant-Based and Clean Meat

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## Glossary

**Cellular agriculture** a biotechnological process by which agricultural products (including meat, gelatin, leather, egg white, and others) are produced through techniques of cell culture instead of raising and slaughtering livestock

**Clean meat** genuine meat that is grown through culturing animal cells (cellular agriculture) rather than in a living animal

**Essential amino acid** an amino acid that is required for human growth and survival, but must be consumed as part of the diet because it cannot be synthesized by the body

**Extrusion** a physical process by which food ingredients are forced through an opening in a plate or die to shape the plant-based meat end product

**Green Revolution** the period of tremendous advancement in agricultural production realized between the 1930s and 1960s due to developments in agricultural science including fertilizers, pesticides, and crop engineering and selection

**Microcarriers** small spheres or beads used in bioreactor-based cell culture to support the growth of cells that require a surface on which to adhere

**Nucleotide** the base unit of DNA and RNA that is composed of a nitrogenous base, a five-carbon sugar, and one or more phosphate groups

**Plant-based meat** structured protein-based products created from non-animal ingredients designed to directly replace animal meat, including poultry and seafood, that are marketed as meat substitutes; also called meat analogues or meat alternatives

**Protein-energy malnutrition (PEM)** a type of malnutrition resulting from insufficient dietary consumption of protein and/or energy that predominantly affects children, due to their lower protein intake, and individuals in low-income countries who lack sufficient resources to consume a complete diet

**Recommended dietary allowance (RDA)** the average daily dietary intake level of a nutrient considered sufficient by the Food and Nutrition Board of the US Institute of Medicine to meet the requirements of 97.5% of healthy individuals

**Shear cell technology** a type of manufacturing method used to create structured plant-based meat that relies on two concentric cylinders moving tangentially to create laminar flow

**Tolerable upper intake level (UL)** the maximum daily intake for a nutrient that is unlikely to cause adverse health effects in most healthy people

## Dietary Protein

Despite Earth's tremendous biodiversity, the molecular composition of life is relatively simple. Only a handful of chemical elements make up the vast majority of all living matter: carbon, nitrogen, oxygen, and hydrogen are the most biologically abundant elements. These elements combine in unique and varied ways to form the nucleotides, amino acids, carbohydrates, and lipids that comprise cellular structure and perform all functions central to the maintenance and propagation of life (Voet et al., 1999).

Humans require a steady input of the above four elements — along with others such as calcium, phosphorus, and potassium, and trace elements such as iron, copper, and zinc — in order to survive and grow. We acquire these essential nutrients from our food, where they exist in more complex macromolecular structures such as proteins, carbohydrates, and fats which must then be processed by our bodies into smaller bioactive components. Proteins, which are the most abundant component of cells other than water, are our body's primary nitrogen source. Unlike carbohydrates and fats, ingested proteins are not stored by the human body but are immediately broken down into amino acids that can be incorporated into newly synthesized proteins, used for the production of other cellular components, or lost through excretion, oxidation, or conversion to metabolic waste products. Approximately 43% of the protein in the human body is present in skeletal muscle, and about half of the body's protein content is comprised of only four proteins: myosin, actin, collagen, and hemoglobin (Otten et al., 2006).

Protein is clearly essential, but how much do humans require on a daily basis? The answer varies based on an individual's metabolic demand and efficiency of utilization of dietary protein. Physiological characteristics that influence these properties include age, lifestyle factors such as physical activity level, special conditions such as pregnancy and lactation, and certain diseases and metabolic conditions that may impact the body's ability to properly metabolize specific amino acids (World Health Organization, 2007). In the US, the Institute of Medicine's Food and Nutrition Board, which is part of the National Academies of Sciences, Engineering, and Medicine, serves as the authoritative body for establishing dietary intake recommendations that guide health professionals in assessing both individuals' and populations' nutrient needs. The Acceptable Macronutrient Distribution Range (AMDR) for protein is

5%–20% of total calories for children 1–3 years of age, 10%–30% of total calories for children 4–18 years of age, and 10%–35% of total calories for adults older than 18 years of age (Otten et al., 2006). The recommended dietary allowance (RDA) for both adult males and females is 0.8 g of protein per kilogram of body weight per day (Otten et al., 2006). This translates to an RDA of 56 g of protein per day for a 70 kg (154 lb) adult male and an RDA of 46 g of protein per day for a 57 kg (126 lb) adult female. The dietary reference intake recommendations for total protein do not include a tolerable upper intake level (UL) because insufficient evidence exists to determine whether excess total protein (amino acid) intake poses a risk of adverse effects for most healthy people (Otten et al., 2006; World Health Organization, 2007).

Data from the National Health and Nutrition Examination Survey (NHANES), a survey conducted by the Centers for Disease Control and Prevention (CDC) among a nationally representative sample of about 5000 persons each year, demonstrate that dietary protein consumption among US adults (15.9% of calories from protein for males and 15.5% for females) is well within the stated AMDR (Wright and Wang, 2010). However, these daily average protein consumption levels translate to about 99 g for males and 68 g for females, suggesting a high likelihood of higher-than-recommended levels of protein intake. On a global level, data from the UN Food and Agriculture Organization suggest that over the past several decades, the world has experienced a steady increase in protein supply and intake among both developed and developing countries.

So why does this matter? If protein is an essential macronutrient and there is no UL, shouldn't this increase in protein consumption be viewed as a beneficial trend? Are there implications to rising global protein demand that extend beyond human health impacts? From a global perspective, are there any negative consequences stemming from our hunger for protein? Certainly in developing countries that disproportionately experience the burden of deaths attributable to protein-energy malnutrition (PEM) (Grover and Ee, 2009), one would expect that increased protein consumption is desirable. But is it leading to better health outcomes? We explore these questions next.

### Not All Proteins Are Created Equally

Protein quantity is not the only consideration when analyzing dietary protein intake. Protein quality must also be taken into account. The primary intrinsic determinant of protein quality is amino acid composition. Humans cannot synthesize all of the amino acids our cells use to synthesize proteins and thus we must obtain the non-synthesizable (essential) amino acids from dietary sources. Additional determinants of protein quality include factors within foods that impact digestibility and bioavailability of the protein's amino acids (Wu, 2013).

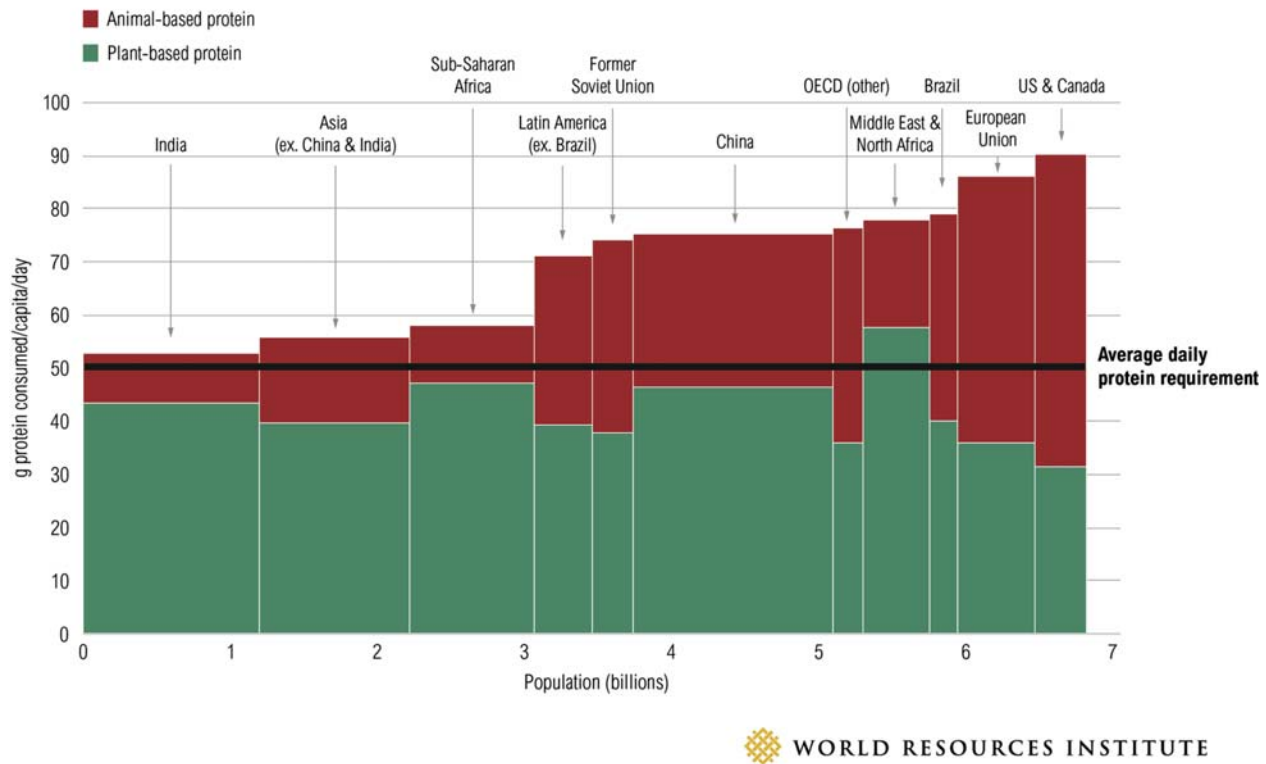
Protein quality varies by source and according to subsequent processing and cooking methods. While certain foods may contain low amounts of one or more of the essential amino acids — for example, many cereal grains naturally contain lower amounts of lysine, methionine, histidine, and tryptophan (Koehler and Wieser, 2013) — eating a variety of foods ensures that adequate amounts of each amino acid are consumed. One reason why PEM is still a major concern for developing countries despite the increase in overall protein consumption is because there is a lack of diversity in the diet. There is a common belief that diets reliant on proteins from plant sources are inferior for human health compared to diets rich in animal-based protein sources. However, scientific evidence supports the nutritional adequacy of well-balanced plant-based diets (Sanders, 1999), and a growing body of work points to the health benefits of reducing consumption of animal-based foods in favor of plant-based foods (Richter et al., 2015; Song et al., 2016; Kahleova et al., 2018).

Fig. 1 depicts daily per capita protein consumption across the world's regions dichotomized by whether the protein comes from plant sources or animal sources. On average, people worldwide are consuming more protein than physiologically needed. Not surprisingly, developed countries usually over-consume to a much larger extent than developing countries. Globally almost 60% of protein intake comes from plant sources. What is particularly striking, however, is the percentage of daily protein intake that comes from animal sources versus plant sources when comparing developed regions to developing regions. While India and Sub-Saharan Africa rely on plant-based protein for over 80% of their daily intake, the US and Canada consume approximately two-thirds of their daily protein intake from animal sources, which is the equivalent of almost 60 g of animal protein per capita per day on average.

As developing regions gain wealth, they experience a nutrition transition defined by a shift to a more Westernized diet reliant on animal products and higher in saturated fat, sugar, and refined foods and lower in fiber (Popkin et al., 2012). Thus, demand for animal-based protein is likely to increase substantially as developing countries continue to move through these demographic transitions and their associated epidemiology.

This is concerning for multiple reasons. From a public health perspective, though humans have consumed meat for millennia, overconsumption of animal products is linked to obesity, cardiovascular disease, Alzheimer's disease, and several other chronic conditions currently plaguing developed countries (Grant, 2014; Levine et al., 2014; Wolk, 2017). From an environmental viewpoint, the production of animal protein through industrialized animal agriculture is an inefficient, resource-intensive process. Growing evidence suggests that our current methods of animal protein production are not sustainable, especially as we experience population growth and increasing demand for protein, particularly animal protein (Walker et al., 2005; Gerber et al., 2013; Harwatt et al., 2017). The ultimate question we must answer, then, is how can we feed the world's growing population with safe, nutritious, and accessible protein-rich foods produced through systems that do not negatively impact global health, the climate, and scarce natural resources?

## People Are Eating More Protein than They Need—Especially in Wealthy Regions



**Figure 1** Per capita daily protein consumption by world region and protein source (plant-based vs. animal-based). Figure created by and reprinted with permission from the World Resources Institute.

### Food for Thought: Protein Product Innovation

In the study, “Shifting Diets for a Sustainable Food Future,” the World Resources Institute estimates we will need 70% more food to meet global demand in 2050, compared to 2006. It is unlikely that improvements in agricultural productivity alone will be able to close this gap, given yields would have to increase 33% faster than they did during the Green Revolution. The authors suggest that closing this 70% food gap will require both productivity increases and dietary shifts away from consumption of animal-based proteins, due to the fact that the production of animal-based foods involves substantially more resources and generates more environmental stress than the production of plant-based foods (Ranganathan et al., 2016).

Nevertheless, decades of work by scientists, public health authorities, environmentalists, and others to persuade people to consume more plants and less meat have not put a dent in meat consumption. Despite rising awareness of the global impacts of our dietary choices, consumers continue to base their purchasing decisions primarily on price, taste, and convenience (Glanz et al., 1998). Quite simply, reducing meat consumption is intractable for most people due to a lack of appetizing and affordable products that could serve as alternatives to conventional meat products. The challenge, then, is to innovate and bring to market diverse protein alternatives that are as delicious, price-competitive, and convenient as the animal-based food products currently on the market. Doing so would make the healthy and sustainable choice the default choice.

The remainder of this chapter focuses on two types of products that exhibit tremendous potential for becoming the protein foundation of a sustainable global food system. We refer to these products as plant-based meat and clean meat. After defining these terms, we discuss the current challenges and research gaps that must be addressed in order to bring appetizing, affordable, and accessible plant-based and clean meat products to market.

### What Is Plant-Based Meat?

Plant-based meats are structured plant-derived products designed to directly replace animal meat — including poultry and seafood — and that are marketed as meat substitutes. These meat substitutes can also be called meat analogues or meat alternatives. The name plant-based meat is sometimes used to refer to fungus-derived products, though this is not the most accurate terminology given that fungi are not technically plants. Products such as seitan – a mixture of water and the wheat protein gluten that when



cooked has a meat-like look and texture – are traditional foods in some cultures and are also included as plant-based meat because they are a central component of many meat analogues and are marketed as such. Similarly, veggie burgers — even those that do not simulate meat (e.g., black bean burgers) — are included in this definition because they serve as a direct replacement for a beef, chicken, or fish patty.

While plant protein isolates, concentrates, and textured vegetable protein (TVP) are typically used to make plant-based meat, such ingredients should not be confused with plant-based meat itself, which is the final consumer product designed to replace meat directly. TVP is extruded (or otherwise textured by kneading or mixing) protein in the form of small chunks or shreds and is not typically seasoned or prepared for direct consumption. Although protein is the primary ingredient consideration during the creation of plant-based meat, the final products are typically a mix of multiple types of plant-based ingredients, including other nutrients such as fats, carbohydrates, and fiber. Converting these mixtures of plant protein ingredients into plant-based meat usually requires processing in an extruder or forming machine, with additional formulation and flavoring to create an appetizing plant-based meat product. Optimal proteins for plant-based meat applications are those that can be structured such that the final product recaptures the texture and mouthfeel of meat. These attributes can be quantitatively described using food science functional metrics like gelling and crosslinking capacity for structuring and water-holding and fat-holding capacity for juiciness.

To effectively compete with animal-based meat products, the plant-based meat industry must expand and innovate to develop a variety of replacements that are as appetizing and affordable as conventionally produced animal meat. Fortunately, opportunities for innovation in the plant-based meat industry are even vaster than for the conventional meat industry. Relatively few species comprise the vast majority of all animals that are bred, raised, and killed for meat. Each species produces many different types of meat, some of which is sold relatively unprocessed while a significant amount of meat is ground, minced, or processed in other ways. But ultimately, the conventional meat industry is inherently constrained by limitations in the diversity, anatomy, and physiology of the animals it uses, whereas plant-based meat companies can innovate beyond mere replication of the limited types of animal meat currently manufactured. In [Fig. 2](#), we visualize categories of meat replacements that represent areas for plant-based meat product innovation that align with this premise.

## What Is Clean Meat?

Unlike plant-based meat, clean meat is genuine animal meat that can replicate the exact sensory and nutritional profile of conventionally produced meat. Clean meat is comprised of the same cells — and even the same myosin and actin proteins responsible for muscle cell contractility — that comprise the overall three-dimensional structure of muscle tissue in living animals, but clean meat is produced without the need to raise and slaughter whole animals. The process is inherently much more efficient than conventional production methods because resources and inputs are not being expended to grow parts of the animal that are not consumed. Furthermore, clean meat eliminates the need for massive amounts of crops to be grown and fed to livestock, only a small fraction of which are converted to meat as the vast majority are expended through the animal's metabolic processes.

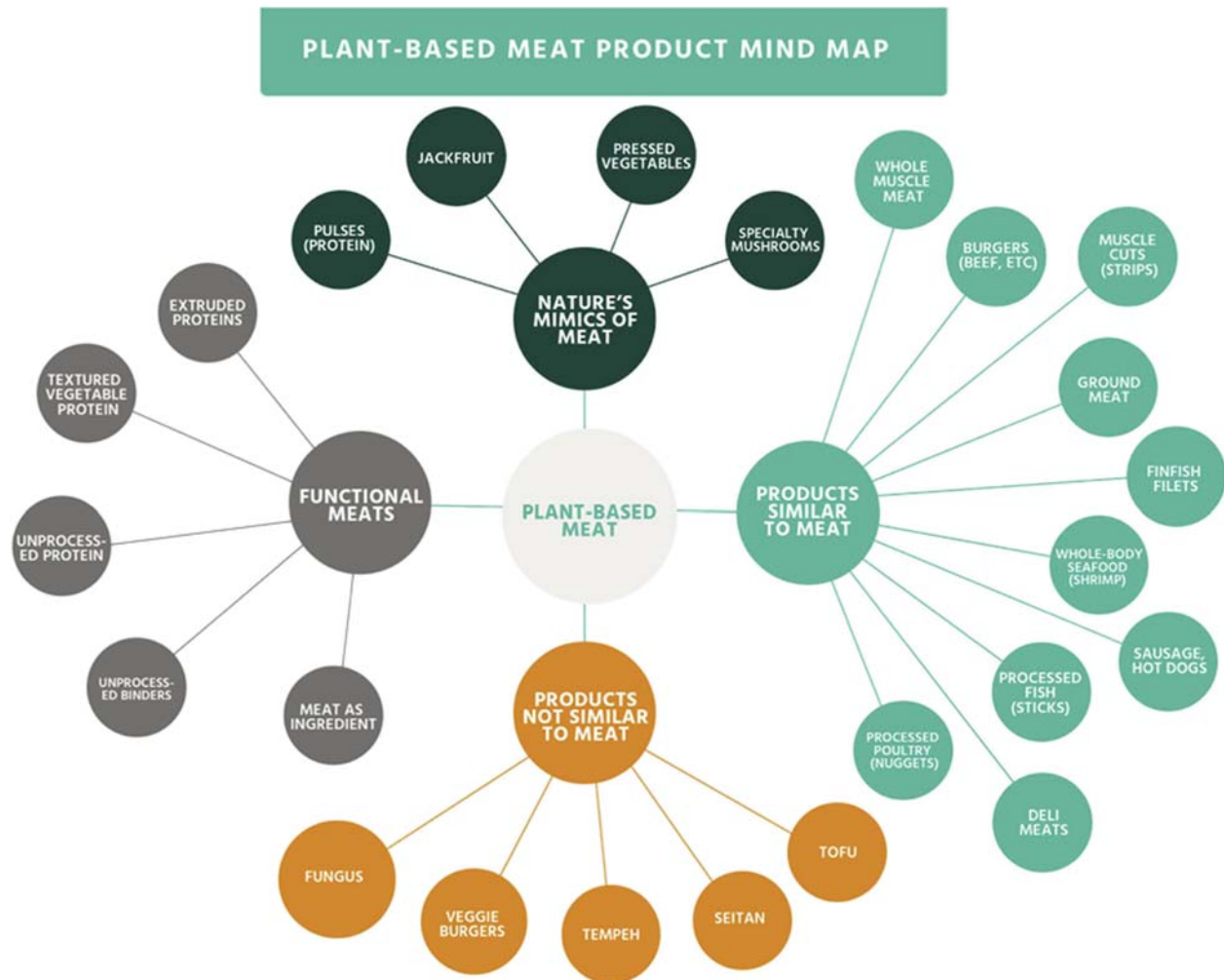
So how is clean meat actually produced? Clean meat relies on advances in cell culture and tissue engineering — many of which were developed for biomedical applications like cell therapy and organ implants — to directly grow the types of cells that comprise meat. Meat is essentially just a collection of muscle cells, fat cells, and connective tissue cells, all of which can be grown outside the body of an animal in controlled, enclosed environments. In general the process involves collecting a small number of animal cells from an agriculturally relevant species (cow, pig, chicken, turkey, fish, etc.) and proliferating them in a bioreactor where they are fed a nutrient broth media to supply them with the ingredients they need to survive. The cells then undergo a differentiation process during which they mature into the muscle cells, fat cells, and connective tissue cells that comprise meat. These two stages (proliferation and differentiation) may or may not be spatially separated, but they will almost certainly be temporally distinct as the signals and environments that encourage cellular proliferation differ from those that induce differentiation ([Specht, 2018](#)). [Fig. 3](#) provides a graphical representation of this process.

At first glance, plant-based and clean meat may seem like two distinct solutions to sustainable protein production. However, they are better represented as two ends of a continuous spectrum ([Fig. 4](#)) exhibiting overlap among the raw materials and ingredients used, and products are developed that incorporate both plant-based and clean meat ingredients. Clean meat and other cellular agriculture products will likely find an early market entry point as a high-value ingredient in foods that are predominantly plant-based. Because the taste profile of meat is highly complex and the texture and mouthfeel are specific to each type and cut of meat, incorporating clean meat into plant-based products may make it easier to create products that meet the sensory expectations of consumers without sacrificing health and environmental impact profiles.

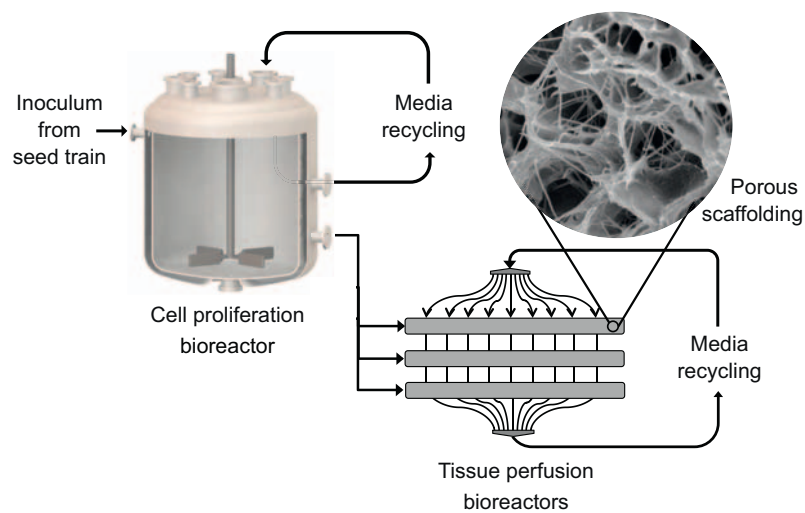
## Current Research and Development Needs for Plant-Based Meat

The concept of plant-based meat is not a modern development. Various forms of plant-based meat have been made for centuries, with early forms predating modern food processing techniques like extrusion by nearly a millennium. The prevalence and variety of plant-based meat has steadily increased over time, culminating in an explosion of innovation throughout the sector during the last decade. Despite this long history and exciting present development, the future success of plant-based meat depends heavily on

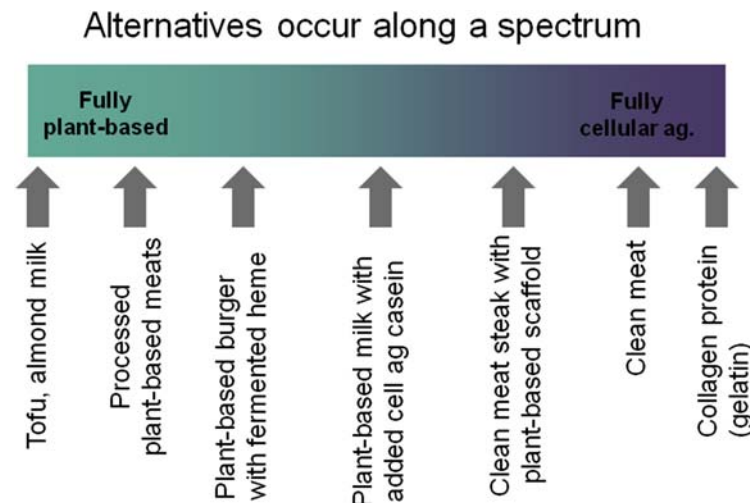




**Figure 2** Plant-based meat product mind map.



**Figure 3** Schematic of clean meat production.



**Figure 4** Spectrum showing progression of foods from fully plant-based to fully cellular agriculture.

continued research and technological development in ingredient sourcing, raw material isolation and functionalization, product formulation, and manufacturing technologies.

### Ingredient Sourcing

The vast majority of commercially available plant-based protein ingredients come from only 2% of the 150 plant species on which today's global food supply depends (Vernooij, 2003). A significant pool of potential plant protein sources is thus available for exploration, and this does not even take into account the nearly 250,000 additional plant species not currently used in large-scale agriculture. Innovation opportunities in this area include expanding and diversifying our use of plant protein sources, determining which sources are best suited for particular plant-based meat products, and ensuring that the proteins from these novel sources are optimized specifically for plant-based meat rather than for other plant-based foods that do not require proteins with the specific structural properties needed for meat analogues. While protein is often the primary ingredient consideration for meat analogues, it is also important to consider plant-based sources of fats and other ingredients that contribute to the sensory and nutritional profiles of plant-based meat products. Finally, all decisions related to plant sources for plant-based meat applications must be made within the context of agronomy so that the desirability of the plant's end-use characteristics is balanced with its suitability for production in particular geographic regions and climates.

### Raw Material Isolation and Functionalization

Some meat analogues, such as those made from jackfruit or certain types of mushroom, require minimal post-harvest processing of the source plant or fungus. However, many other plant-based meat products currently on the market rely upon plant protein concentrates or isolates as raw materials. To obtain refined ingredients such as proteins, fats, and starches from plants, the source plant is typically milled and processed to acquire more purified and concentrated ingredient fractions. The inherent characteristics of the proteins, such as molecular weight and amino acid composition, determine how well each protein concentrate or isolate will perform in the final product. The quality and performance of plant-based meat would be greatly advanced by implementing environmentally friendly methods for protein conditioning, which increase the desirable functional traits of a protein, such as gelation capacity, solubility, and fat adsorption. Moreover, the idea of processing plants into functional fractions instead of highly purified fractionates of single types of macromolecules may be beneficial not only in making the production of plant-based meat products more efficient, economical, and sustainable, but also for the sensory profiles of the products themselves (van der Goot et al., 2016).

### Product Formulation

Product formulation is a complex process that includes the culinary art of flavor selection, consideration of nutrition and ingredient interactions, and testing for desired texture and sensory attributes. Plant-based proteins—especially hydrolyzed proteins, which are more soluble and thus exhibit greater functionality—are often prone to bitter or “beany” off-flavors. This could be addressed by creating new bitter-blocking agents as part of a larger opportunity for developing new flavoring components, particularly those derived from natural sources and specifically designed to address the challenges of working with plant-based proteins. The selection

and incorporation of flavoring in the production process is vital for creating plant-based meat that replicates the sensory palette of animal meat.

Formulation must also consider the desired nutritional qualities of the plant-based meat product. In some cases, the goal is to replicate as precisely as possible the nutritional composition of animal-based meat. In other cases, product developers may intentionally alter the nutritional profile of the plant-based meat product in beneficial ways. For example, while animal-based meat contains no fiber, enhancing natural plant fiber incorporation into plant-based meat products without sacrificing taste and texture would provide a nutritive advantage to these products compared to animal-based meat. Given the complexity of product formulation, this process should include both culinary experts and food scientists.

### Manufacturing Technologies

After successful formulation, the mixture of ingredients that will become the final plant-based meat product must be structured and shaped into an appropriate form. Methods for transforming plant-based ingredient mixtures into meat analogues include a variety of manufacturing processes, such as stretching, kneading, shear-cell processing, press forming, folding, layering, and extrusion. Each of these processes can produce unique forms and textures of plant-based meat and affect the nutritional quality of the final product. Furthermore, different plant proteins perform differently in each of these manufacturing processes due to their unique compositions and functional properties. Therefore, it is essential to understand how particular plant proteins and combinations of plant proteins from various sources react to specific production techniques. Additionally, another manufacturing (and formulation) challenge involves identifying the optimal methods and timelines for combining proteins with other ingredients to ensure that the process is scalable and does not compromise the nutritional quality or sensory aspects of the end product. For example, how and when should fats, vitamins, and carbohydrates be mixed with the protein(s) to ensure that the end product cooks properly, possesses the desired nutritional profile, taste, and texture, and does not fall apart while eating? Critical needs exist for optimizing current technologies and developing novel manufacturing techniques for making plant-based meat, with particular emphasis on technologies that require minimal resource use, produce minimal waste, and could be easily adapted for use in rural areas and developing countries.

### Current Research and Development Needs for Clean Meat

Although plant-based meat analogues have been produced, sold, and consumed for millennia, clean meat has yet to be commercialized. In fact, the first clean meat prototypes have been produced only in the past five years. Despite the recent emergence of this industry, it is advancing rapidly due in large part to its ability to adapt underlying technologies from the fields of tissue engineering, stem cell biology, and cell-based therapeutics (Specht et al., 2018). Leveraging decades of research and billions of dollars of public investment into these relevant fields effectively provides the clean meat industry with an accelerated path to commercialization. The primary technological challenges currently facing the clean meat industry include further development and refinement of agriculturally relevant cell lines, cell culture media, scaffolding, and bioreactors. We briefly discuss these below. Because no clean meat products yet exist on the market, regulatory approval and consumer acceptance also need to be addressed as the technology develops (Specht, 2018).

#### Cell Lines

Clean meat production begins with obtaining primary cells and, in some cases, developing cell lines for the desired agriculturally relevant animal species. Generating a cell line means isolating a population of cells that is stable and capable of long-term proliferation in culture. In other words, the cell line must behave in a consistent and predictable way through many generations, while ideally maintaining an unlimited capacity to divide and efficiently differentiate. Creating cell lines for agriculturally relevant species that can be banked and then shared with researchers and companies around the world will limit duplication of effort from each individual researcher creating his or her own cell lines, and it will also enable more informative comparisons across different labs and food companies as there will be consistency in the starting cell line material.

#### Cell Culture Media

Just like cells inside an organism, cells in culture require nutrients to grow. Cell culture media is a nutrient solution containing salts, pH buffers, and the building blocks of cellular structures like proteins and fats. Animal cell culture media typically contains molecules called growth factors, which are signalling molecules that direct the cells to behave in certain ways. The cell culture media is likely to be a driving factor of clean meat cost at scale; as such, innovation in media ingredients and media recycling processes is a crucial need for commercialization of clean meat products that are cost-competitive with meat products currently on the market.

## Scaffolding

Scaffolds provide a support structure for cellular adherence. In the simplest case, microcarriers within a stirred bioreactor may act as scaffolds during cell proliferation. For more complex, structured products, the scaffold requirements are much more demanding and must be integrated within the final product. The material must be edible and/or biodegradable, inexpensive, derived from abundant sources, and amenable to large-scale fabrication. While the first clean meat products that come to market may not require significant scaffolding if they are primarily ground meat products or simply designed to be used as ingredients in other products, scaffolding supporting sophisticated, highly structured products incorporating multiple cell types — such as chicken breasts, pork chops, and steaks — will ultimately be required by the industry.

## Bioreactors

Bioreactors are the machines in which every other element in clean meat production is contained: where cells proliferate, and where the maturation from cells to meat occurs. As with media, clean meat bioreactors will have unique design requirements depending on the stage of production. Proliferation bioreactors are already widely used in industries ranging from pharmaceuticals to food ingredients so it is likely that more of the innovation in clean meat bioreactor technology will be needed for the maturation or differentiation step of the clean meat production process as opposed to the proliferation stage. Creating bioreactors or alternative technologies that enable easy harvest of the final meat product and require minimal inputs to function will help advance the clean meat industry. Simultaneous and coordinated advancement of all four of the above primary technology areas for clean meat (cell lines, cell culture media, scaffolding, and bioreactors) will be the most efficient way to move this industry forward, as these components all interact in complex ways. For example, cell culture media will need to be optimized for different cell lines; bioreactors must produce fluid dynamics that are tolerable by the cells; and parameters of the media like pH and density may affect the stability of the scaffold.

## Conclusion

Meat has long been synonymous with protein in Western culture and animal protein provides the vast majority of dietary protein intake in the US as the world population expands and developing countries gain greater wealth, the global demand for animal protein continues to increase. To meet this growing demand for animal protein, our food system has shifted to large-scale, industrialized methods of raising farm animals for food. The small-scale, regenerative farming of our grandparents' generation now exists for only a tiny fraction of animal protein production and typically commands premium prices, rendering it inaccessible to a large fraction of the population.

Yet there is growing recognition that our current methods of industrialized animal agriculture are not sustainable. Emerging innovation in two specific areas — plant-based meat and clean meat — show promise for revolutionizing our food system. These areas represent both tremendously profitable market opportunities and substantial promise for addressing the urgent challenges facing our global food supply. For this to happen expeditiously, there is an urgent need for additional technical talent and expertise from around the world to enter these industries; the next generation of scientists and entrepreneurs will play a critical role in advancing the quality, cost, and availability of plant-based and clean meat products, positioning them as economically viable and sustainable sources of protein. If successfully implemented on a global scale, a food system with a much greater reliance on plant-based and clean meat has the potential to sustainably feed 9.7 billion people by 2050, mitigate climate change and other pressing environmental problems, reduce animal suffering, and decrease global public health issues such as antibiotic resistance, zoonotic threats, and diet-related chronic disease.

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 The Good Food Institute <http://www.gfi.org/>.  
 World Resources Institute <http://www.wri.org/>.

# Anthocyanidins and Anthocyanins

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## Overview

Anthocyanidins (or glycosidic forms, anthocyanins) are members of the flavonoid class of secondary metabolites and highly important water-soluble pigments in plants. They are polyhydroxy or polymethoxy derivatives of the flavylium cation (2-phenylbenzopyrylium ion), containing a C-15 backbone structure arranged in two C-6 benzyl rings (A and B) and a heterocyclic C-3 ring (C) (Zaffino et al., 2015). More than 700 compounds have been described in the literature (Andersen and Jordheim, 2013) and mainly derive from six anthocyanidins: cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin (Kahkonen and Heinonen, 2003). Their structure is shown in Fig. 1.

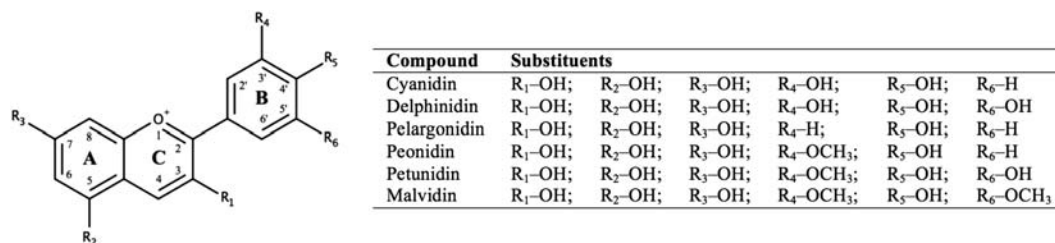
In plants, anthocyanins accumulate in vacuoles of certain cells in vegetative and reproductive organs (Mushtaq et al., 2016) and are responsible for functions involved in the preservation and protection against biotic and abiotic stress. For instance, they act as sunblock that protects the plant against photo-oxidation (Feild et al., 2001) and exhibit antioxidant activity by scavenging reactive oxygen species generated during normal plant metabolism (Nakabayashi et al., 2014). Human consumption of anthocyanins has been associated with reduced risk of degenerative diseases (Cassidy et al., 2013), although a considerable gap still exists between *in vitro* study results and those confirmed *in vivo* (Celli et al., 2016).

## Chemistry of Anthocyanins

Owing to their physicochemical properties (e.g., poor stability and low solubility), anthocyanidins (aglycone) are most often found in nature as salts of their glycosidic forms, known as anthocyanins (ACNs). Glycosylation results in the formation of an intramolecular H-bonding network that helps stabilize the ACN molecule, in addition to improve its water solubility (Borkowski et al., 2005). These ACNs can differ in relation to the type, number, and position of the sugar moieties attached to the main backbone. Glucose, galactose, rhamnose, arabinose, xylose, and rutinose are the sugar groups frequently attached to anthocyanidins by substitutions of the hydroxyl groups in positions 3 (i.e., 3-glycosides) and 5 (i.e., 3,5-diglycosides). Other structures identified include 3-diglycosides, 3-diglycoside-5-monoglycosides, and 7-glycosides. In certain cases, these sugar groups can be esterified (acylated) with organic aliphatic or aromatic acids (e.g., caffeic, ferulic, cinnamic, malonic, acetic, and malic acids). Acylation with organic acids can also improve ACN stability; however, unlike glycosylation, acylation reduces their water solubility (He and Giusti, 2010).

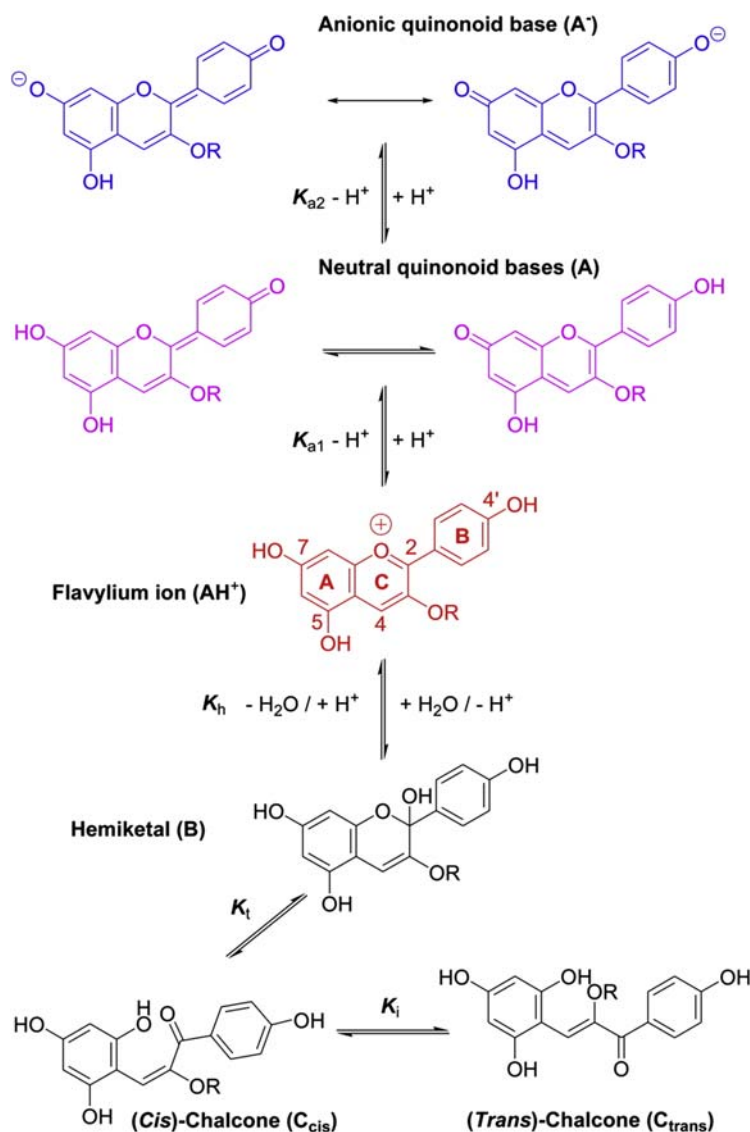
In addition to their chemical structure, the stability of ACNs is also affected by variations in pH, temperature (Sui et al., 2014), oxygen (Weber et al., 2017), light (Mahdavi et al., 2016), water activity (Tonon et al., 2010), and the presence of other compounds (e.g., ascorbic acid) (Guldiken et al., 2017). Overall, ACNs are unique flavonoids as they can rearrange and form resonant structures with changes in the pH, which affects their color and stability. The different forms observed for ACNs are the flavylium cation ( $AH^+$ , orange/red), quinoidal base (A, blue/violet), carbinol pseudobase (B, colorless), and chalcone (C, yellow), shown in Fig. 2.  $AH^+$  predominates at very acidic conditions (generally below pH 2) and it is the most stable form. At mild acidic conditions, this cation is converted into bases by deprotonation of one of the three most acidic phenolic OH groups (in C7, C4', or C5, in this order). When the pH is close to neutrality, A can lose another proton and form its ionized form,  $A^-$ . It is also possible that, above pH 2,  $AH^+$  becomes hydrated at C2 forming B that rapidly equilibrates with  $C_{cis}$ , which is itself in a slow equilibrium with  $C_{trans}$  (Trouillas et al., 2016). In general, the ACN stability is significantly compromised at pH values close to neutrality. It is also interesting to note that these forms can coexist in equilibrium, as shown in Fig. 3.

Modifications of the main ACN structure can also modify the color exhibited. For instance, an increase on the number of hydroxyl and methoxyl substitutions has been associated with blue and red hues, respectively (Delgado-Vargas et al., 2000), whereas purplish shades were observed with substitution on position 5 or acylated with cinnamic acids (Stintzing et al., 2002). However, variations in pH and structural transformations alone are not sufficient to explain the diversity of ACN-based hues found



**Figure 1** Basic structure of the anthocyanidins (left) and substituents of the six major types of molecules found in nature (right). The numbering systems presented is the most commonly accepted for these compounds.



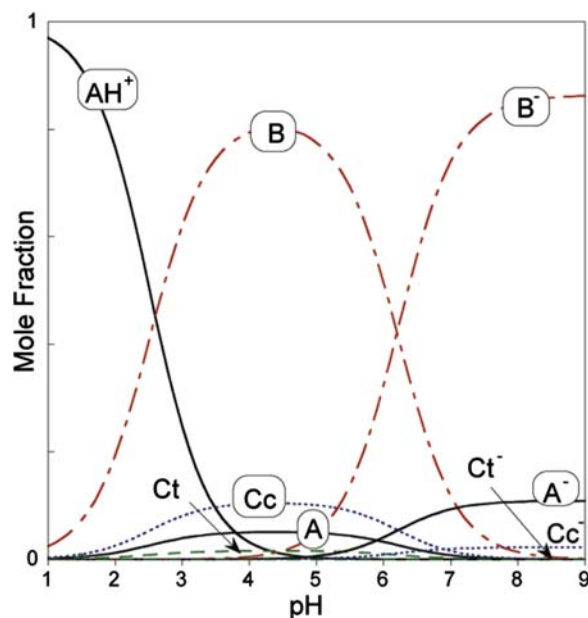


**Figure 2** Resonant structures of ACNs as a function of the pH.  $K_a$ ,  $K_h$ ,  $K_t$ , and  $K_i$  refer to the acidity, hydration, hemiketal-chalcone tautomerization, and *cis-trans* chalcone isomerization thermodynamic constants. Reproduced with permission from Trouillas et al. (2016), © 2016, American Chemical Society.

in nature. In fact, these compounds can be associated with other polyphenols (Wigand et al., 1992) and/or metals (Buchweitz et al., 2013) to produce a variety of hues and improve their stability *in vivo*. A unique feature of ACNs is the copigmentation phenomenon that serves to protect the flavylium cation from nucleophilic attack and, consequently, stabilize color by weak association with polyphenols (Teixeira et al., 2013), carbohydrates (Fernandes et al., 2016), among others. Additionally, ACNs can donate protons to free radicals, thus preventing their propagation, or chelate metal ions that can lead to the formation of these radicals, which are the underlying mechanisms of their antioxidant activity.

## Extractions From Food Sources

ACN-rich extracts can be prepared from fresh, frozen, or dried plant materials. Examples of plant materials rich in ACNs include blueberry (Skrede et al., 2000), elderberry (Veberic et al., 2009), purple corn (Pedreschi and Cisneros-Zevallos, 2007), among others. The particle size of source materials is an important factor during extraction; they are often milled or ground to increase available surface area and, consequently, the amount of compound extracted. Liquid nitrogen can be used during the grinding step to reduce ACN degradation. Since these compounds are susceptible to degradation by various factors, considerable care should be taken when designing and carrying out the extraction procedure.



**Figure 3** Graphic representation of the mole fractions of catechin-(4,8)-malvidin-3-*O*-glucoside structures (from red wine), with variations of pH. Abbreviations: flavylium cation,  $AH^+$ ; quinoidal base and its ionized form, A and  $A^-$ , respectively; hemiketal or carbinol pseudobase and its ionized form, B and  $B^-$ , respectively; *cis*-chalcone,  $C_{cis}$ ; *trans*-chalcone,  $C_{trans}$ . Reproduced with permission from Nave et al. (2010), © 2010, American Chemical Society.

Extraction procedures are commonly classified as solid or liquid extraction. Solid extraction is applied to liquid matrices, although typically only during purification rather than extraction due to saturation of the absorbents. Liquid extraction yields good recovery of ACNs and is the most-used technique to extract from fruit samples. In general, ACNs are commonly extracted with acidified water and polar organic solvents (methanol, ethanol, and acetonitrile) due to their hydrophilic nature. More recently, the use of deep eutectic solvents (e.g., lactic acid-glucose and choline chloride-malic acid mixtures) has been proposed as a green alternative to extract ACNs in favor over conventional (and often toxic) organic solvents (Dai et al., 2016). The extraction system can also interfere with subsequent analytical procedures, which is an important consideration when designing a study. For example, Myjavcova et al. (2010) noticed that less polar solvents (ethanol and acetone) used for the extraction of ACNs from haskap berries compressed the Sephadex LH-20 gel used for extract purification. This resulted in longer retention times when the fractions were analyzed by high-performance liquid chromatography and, additionally, the co-extraction of impurities. This study also noted that acetone had a low extraction efficiency and formed ACN-derived complexes (5-methyl-pyranoanthocyanin) which were not found in fresh fruits (Myjavcova et al., 2010).

In relation to the techniques, various methods have been developed to increase the efficiency of liquid extractions, decrease the processing time, and minimize the use and exposure to organic solvents. Examples are supercritical fluid extraction (Maran et al., 2014), pressurized liquid extraction (Feuereisen et al., 2017), microwave-assisted extraction (Pap et al., 2012), and ultrasound-assisted extraction (Celli et al., 2015).

### Proposed Mechanisms of Action

Several studies have found a positive link between ACNs and health-promoting properties, mostly in *in vitro* and animal studies. A potential cause for the gap that exists between the results obtained *in vitro* and the clinical practice would be the short residence time of the parent ACNs in their absorption window and in sites in the body where their stability is favored (Celli et al., 2016). Although the stomach is not often considered an absorption site of exogenous molecules, there is a considerable amount of evidence supporting the role of this organ in ACN absorption (Talavera et al., 2003) via bilitranslocase carrier (Passamonti et al., 2003), in addition to the small intestine (Mueller et al., 2017). The compounds that are not absorbed are extensively degraded by changes in pH and metabolized by host enzymes or intestinal microbiota, and eliminated mainly through feces and urine.

As with other flavonoids, ACNs are most known for their antioxidant activity, by scavenging free radicals or metals that could generate such radicals. Structure-antioxidant activity relationships have been strongly demonstrated by earlier researchers (Azuma et al., 2008; Rice-Evans et al., 1996). More specifically, the antioxidant activity of anthocyanidins greatly depends on the functional groups present on the B-ring and as well as different glycosylation patterns. Generally, the antioxidant activity increases with an increasing number of hydroxyl substituents present on the B-ring. Wang et al. (1997) reported that glycosylation of cyanidin to cyanidin 3-glucoside improved the antioxidant activity, whereas glycosylation of malvidin and pelargonidin to the corresponding

3-glucosides decreased the activity. On the other hand, antioxidant action resulting from differences in glycosylation can be closely related to the assay used. [Kahkonen and Heinonen \(2003\)](#) studied six common anthocyanidins and their glycosidic forms in three lipid-containing models and found that most ACNs and their aglycones exerted strong antioxidant activity in emulsions and human low-density lipoprotein (LDL), but weak activity or even pro-oxidation in bulk oil.

ACNs have additionally been associated with antidiabetic by activation of AMP-activated protein kinase (AMPK) ([Takikawa et al., 2010](#)), anti-inflammatory by downregulation of cyclooxygenase (COX)-2 ([Pereira et al., 2017](#)), and neuroprotective properties by improving the glutamatergic neurotransmission ([Shah et al., 2015](#)), as well as the prevention and improvement of cardiovascular diseases by increasing the superoxide dismutase antioxidant activity ([Wang et al., 2017](#)). A summary of health beneficial properties and some of the proposed mechanisms of action based on *in vitro* and *in vivo* studies is provided in [Table 1](#). In one example, ACN-rich extracts from maqui berry were shown to ameliorate diabetic conditions *in vitro* by reducing glucose production and increasing its uptake ([Rojo et al., 2012](#)). Compared to insulin, ACNs were shown to have a mild effect on the downregulation of the gene for glucose-6-phosphatase (G6Pase), an enzyme that catalyzes glucose-6-phosphate hydrolysis to liberate free glucose into circulation. However, significant increases in glucose uptake were observed in cells treated with ACNs alone or in combination with insulin and it has been suggested that ACNs may sensitize the cells to insulin ([Martineau et al., 2006](#); [Rojo et al., 2012](#)).

On the prevention and amelioration of cardiovascular disease, it has been shown that ACNs can inhibit 15-lipoxygenase and xanthine oxidase that produce reactive oxygen species in vascular cells ([Bräunlich et al., 2013](#)). Furthermore, one *in vitro* study ([Edirisinghe et al., 2011](#)) demonstrated that ACNs from black currant enhance the activation of endothelial nitric oxide synthase (eNOS), which also has positive effects on the cardiovascular system through increased nitric oxide production. In a study using rats, [Liu et al. \(2015\)](#) showed that animals treated with blueberry ACNs presented an attenuation of mean arterial blood pressure (by approximately 15 mmHg) and heart rate (around 100 bpm) after cyclophosphamide-induced cardiac injury. The beneficial effect of ACNs on blood pressure was observed in a cross-sectional study with 1898 women, where high intake of these compounds was associated with a significantly lower blood pressure ([Jennings et al., 2012](#)).

**Table 1** Health-related properties of anthocyanins and proposed mechanisms of actions

Type of study	Property	Mechanism of action	Reference
<i>In vitro</i>	Antidiabetic	Increase of glucose uptake by improving insulin sensitivity. Downregulation of glucose-6-phosphatase (G6Pase)	<a href="#">Rojo et al. (2012)</a>
	Anti-inflammatory	Increase of glucagon-like peptide (GLP)-1 secretion Downregulation of inflammatory signaling pathway Inhibition of nuclear translocation of pro-inflammatory molecules in macrophages	<a href="#">Kato et al. (2015)</a> <a href="#">Sogo et al. (2015)</a> <a href="#">Lee et al. (2014)</a>
	Prevention and amelioration of cardiovascular diseases	Inhibition of 15-lipoxygenase and xanthine oxidase Increase of protein kinase B (Akt) and endothelial nitric oxide synthase (eNOS) phosphorylation	<a href="#">Bräunlich et al. (2013)</a> <a href="#">Edirisinghe et al. (2011)</a>
	Neuroprotection	Suppression of dopaminergic cell death and release of nitrite from microglial cells Improved cell viability, normalized the mitochondrial membrane potential and calcium levels, and reduced neuronal cell death	<a href="#">Strathearn et al. (2014)</a> <a href="#">Badshah et al. (2015)</a>
<i>In vivo</i>	Antidiabetic	Activation of AMPK and expression of glucose membrane transporters. Downregulation of G6Pase Activation of insulin receptor phosphorylation, increasing glucose uptake, and prevented apoptosis of pancreatic cells	<a href="#">Takikawa et al. (2010)</a> <a href="#">Nizamutdinova et al. (2009)</a>
	Anti-inflammatory	Reduction of pro-inflammatory molecules (interleukin-6, monocyte chemoattractant protein-1, and tumor necrosis factor- $\alpha$ ) Downregulation of inducible nitric oxide synthase (iNOS) and COX-2	<a href="#">Sogo et al. (2015)</a> <a href="#">Pereira et al. (2017)</a>
	Prevention and amelioration of cardiovascular diseases	Attenuation of mean arterial blood pressure (by ~5–15 mmHg) and heart rate (by ~100 bpm) after cyclophosphamide-induced cardiac injury. Improvement of cardiac dysfunction and left ventricular hypertrophy Reduction of cytokine levels (by almost 100 pg/mL) and increase of superoxide dismutase antioxidant activity (by ~3 U/mL) after exposure to fine particulate matter	<a href="#">Liu et al. (2015)</a> <a href="#">Wang et al. (2017)</a>
	Neuroprotection	Reduction of glutamate-induced neurotoxicity and increased the content of glutathione and other endogenous antioxidants Improved glutamatergic neurotransmission, synaptic dysfunction, and neural apoptosis by stimulating specific pathways in the hippocampus	<a href="#">Shah et al. (2016)</a> <a href="#">Shah et al. (2015)</a>

Additionally, ACNs have been demonstrated to have neuroprotective effects. In one pig-based study (Kalt et al., 2008), blueberry ACNs (1.3–8.5 to 6.6–42.3  $\mu\text{mol/kg}$  of body weight) were shown to be distributed to various organs and tissues following prolonged consumption, including the eyes (average 1.58 pmol/g of tissue, in a fresh weight basis), liver (1.30 pmol/g), brain cortex (0.878 pmol/g), and cerebellum (0.664 pmol/g); even when consumed for just a short period of time (four weeks). In the brain, ACNs can suppress the dopaminergic cell death via modulation of mitochondrial dysfunction and the release of pro-inflammatory nitrite from microglial cells, both of which are important factors in the development and progression of Parkinson's disease (Strathearn et al., 2014). In this and other health beneficial contexts, it should be noted that most of the benefits identified in *in vitro* or animal studies have not been confirmed in clinical trials.

## Conclusion

Continuing public concern over the healthfulness and nutritional quality of foods and other products intended for human consumption will inevitably continue to drive growing demand for the utilization and study of anthocyanins. The structural diversity of these flavonoids already makes them suitable in a range of applications. Still, this diversity leaves significant room for research and product-specific developments aimed at improving the stability and functionality of the many specific variations available. Furthermore, increasing reports highlighting new health benefits anthocyanins have to offer consumers will certainly lead to growth in the importance in a range of industries.

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## Anti-cancer Foods: Flavonoids

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### Diet and Diseases

The increasing embrace of preventive, dietary and alternative approaches to cancer therapy stem from the failure of conventional methods to stem the tide of the disease or provide reliable and effective treatment outcomes. Worldwide, cancer remains a leading cause of mortality with developing countries where the disease was hitherto not common now witnessing very high incidence and prevalence. One of the reasons adduced for this trend is the increasing shift from traditional to western diets. This partly makes cancer a diet-related disease, and the focus on anticancer functional foods and nutraceuticals is, therefore, plausible.

The notion that foods may possess the ability to prevent disease and or be used as treatment of ailments dates back a couple millennia. Hippocrates proclaimed some 2500 years ago, "Let food be thy medicine and medicine be thy food" (Wildman, 2000). Recent experimental, epidemiological and clinical evidences have established negative correlations between the quality of diet and incidence, mortality and morbidity from chronic and non-infectious diseases (Bontempo et al., 2015; Reedy et al., 2014; Thorburn et al., 2015; Siri-Tarino and Krauss, 2016; Lange et al., 2017; Yeh et al., 2016; Taylor et al., 2017; Mahady and George, 2016; Imperatore et al., n.d.). Moreover, the choice of diet after cancer diagnosis may improve survival (Playdon et al., 2017).

### Dietary Phytochemicals and Cancer

Dietary phytochemicals have been shown to be the major components in diets conferring these observed health benefits and their chemopreventive, carcinopreventive and anti-cancer properties have been demonstrated in several studies. A large number of dietary phytochemicals have been demonstrated to have anti-proliferative, anti-metastatic, antioxidant, reactive oxygen species (ROS)-inducing, anti-angiogenic and pro-apoptotic effects including efficacy in targeting cellular molecules and pathways implicated in malignancy. Combinational therapy of phytochemicals with standard therapeutic drugs has also been proposed (Banudevi et al., 2015; González-Vallinas et al., 2013; Ma and Adjei, 2009). In addition, observational studies have revealed an inverse relationship between consumption of polyphenol-rich diets and the incidence of cancer (Rosato et al., 2016; Filomeno et al., 2015).

### Flavonoids

#### Biological Activities of Flavonoids

Polyphenolic compounds, or polyphenols, are polyhydroxylated phytochemicals, of which the two main classes comprise flavonoids and phenolic acids (Lotito and Frei, 2006). Flavonoids are a large family of polyphenolic compounds that have a common chemical structure. With their broad spectrum of pharmacological activities and ubiquity in the plant kingdom, they are arguably the class of phytochemicals eliciting the most interest as well as the most promising in cancer chemoprevention through dietary and herbal interventions.

Flavonoids are a broad and biologically active class of polyphenolic compounds. They are one of the largest and most widespread groups of plant secondary metabolites, with marked antioxidant properties (Corradini et al., 2011). The number of individual molecules in this class of phytochemicals has been estimated to be over 10,000 (Amawi et al., 2017; George et al., 2017). Other pharmacological effects ascribed to flavonoids include antimicrobial (Thongnest et al., 2013; Bahrin et al., 2014; Iranshahi et al., 2015), hepatoprotective (Farombi et al., 2009; Olaleye et al., 2014a; Vázquez-Flores et al., 2017), renoprotective (Cai et al., 2017; Athira et al., 2016), antidiabetic (Keshari et al., 2016; Unnikrishnan et al., 2014; Wang et al., 2017), protection against reproductive dysfunctions, cardioprotective (Olaleye et al., 2014b; Testai, 2015; Lv et al., 2017), anti-arthritis (Somasundaram et al., 2013; Li et al., 2015; Liu et al., 2017), neuroprotective and anti-neurodegenerative (Choudhary et al., 2011; Nabavi et al., 2015; Ayuso et al., 2017; Falode et al., n.d.; Preethi Pallavi et al., 2018), gastroprotective (Mota et al., 2009; Antonisamy et al., 2016; Hari Babu et al., 2010; Olaleye and Akinmoladun, 2013), antigenotoxic and antimutagenic (Nwankwo et al., 2000; Snijman et al., 2007; Zarev et al., 2017; Serpeloni et al., 2015; de Carvalho-Silva et al., 2014); and antiproliferative, antimetastatic and carcinoprotective (George et al., 2017; Yang et al., 2008; Rengarajan and Yaacob, 2016; Klimaszewska-Wiśniewska et al., 2017) properties. Epidemiological studies have established inverse relations between flavonoid intake and the incidence of cancer (Zamora-Ros et al., 2014; Petrick et al., 2015).



## Flavonoid-Rich Foods

Flavonoids are ubiquitously present in fruits and vegetables and are the most abundant species of phytochemicals in human diets with their mean daily intake in the diet estimated to be up to approximately 500 mg (Havsteen, 2002; Burkard et al., 2017; Dai and Mumper, 2010). Being phytochemicals, they cannot be synthesized by humans or animals and can only come from plant sources (Kumar and Pandey, 2013). Flavonoids occur either as the aglycones or glycosides in nature. Generally, the aglycones show better activity as glycosylation renders the flavonoid more polar and less reactive (Corradini et al., 2011). Table 1 shows some exceptionally rich dietary sources of flavonoids.

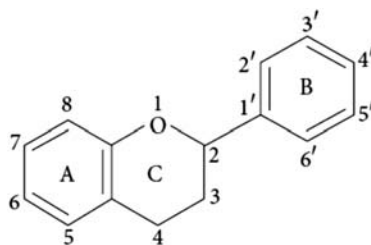
## Chemistry

Flavonoids are synthesized via the phenylpropanoid pathway and are localized inside vacuoles in virtually every part of a plant (Catarino et al., 2016). As depicted in Fig. 1, the basic structure is a 15-carbon benzopyranone or benzopyran in which the three-carbon bridge between the phenyl groups is commonly cyclised with oxygen forming a C<sub>6</sub>–C<sub>3</sub>–C<sub>6</sub> flavan nucleus. Simply put, the flavonoid backbone consists of two benzene rings (A and B) linked via a heterocyclic pyrene ring (C) (Corradini et al., 2011; Kumar and Pandey, 2013; Beltz et al., 2006).

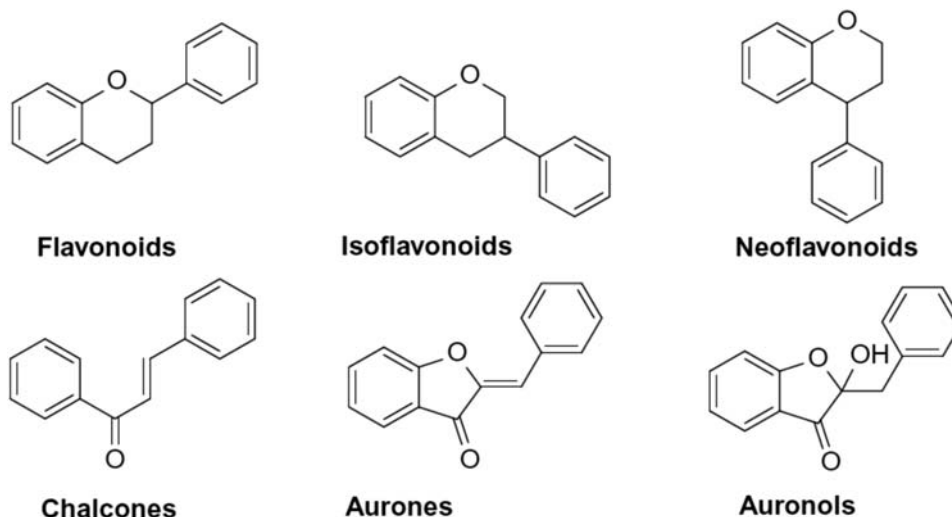
The main subclasses of flavonoids are flavones, isoflavones, flavanones, flavonols, flavanols (also called catechins), and anthocyanidins (Fig. 2). As shown in Fig. 3, the various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings (Kumar and Pandey, 2013). Although most flavonoids have their Ring B attached to the C<sub>2</sub> position of Ring C, some flavonoids such as isoflavones and neoflavonoids, whose Ring B is connected at the C<sub>3</sub> and C<sub>4</sub> position of Ring C, respectively, also occur

**Table 1** Selected flavonoid-rich foods

<i>Food</i>	<i>Main flavonoids</i>	<i>Flavonoid subclass</i>	<i>References</i>
<b>Fruits</b>			
Apple	Quercetin, Myricetin, Kaempferol, Epicatechin, Catechin, Epigallocatechin gallate	Flavanols	Lotito and Frei, 2006; Hooper et al., 2008; Macready et al., 2014; Bertoia et al., 2016
Pears	Quercetin, Myricetin, Kaempferol, Epicatechin, Catechin, Epigallocatechin gallate	Flavanols	Lotito and Frei, 2006; Bertoia et al., 2016
Grapes	Quercetin, Myricetin, Kaempferol, Epicatechin, Catechin, Epigallocatechin gallate	Flavanols	Lotito and Frei, 2006
	Cyanidin, Malvidin, Delphinidin, Pelargonidin	Anthocyanidins	Hooper et al., 2008; Macready et al., 2014; Bertoia et al., 2016
Berries	Quercetin, Myricetin, Kaempferol, Epicatechin, Catechin, Epigallocatechin gallate	Flavanols	Lotito and Frei, 2006
	Cyanidin, Malvidin, Delphinidin, Pelargonidin	Anthocyanidins	Hooper et al., 2008; Macready et al., 2014; Bertoia et al., 2016; Kozłowska and Szostak-Wegierek, 2014
Citrus fruits	Naringenin, Hesperetin, Eriodictyol	Flavanones	Lotito and Frei, 2006; Hooper et al., 2008; Macready et al., 2014; Kozłowska and Szostak-Wegierek, 2014
<b>Vegetables</b>			
Spinach	Quercetin, Myricetin, Kaempferol	Flavanols	Lotito and Frei, 2006
Broccoli	Quercetin, Myricetin, Kaempferol	Flavanols	Lotito and Frei, 2006; Macready et al., 2014
Onions	Quercetin, Myricetin, Kaempferol	Flavanols	Lotito and Frei, 2006; Hooper et al., 2008; Macready et al., 2014; Kozłowska and Szostak-Wegierek, 2014
Hot peppers	Quercetin, Myricetin, Kaempferol	Flavanols	Lotito and Frei, 2006
	Apigenin, Luteolin	Flavones	Macready et al., 2014; Bertoia et al., 2016; Kozłowska and Szostak-Wegierek, 2014
Cherry tomatoes	Quercetin, Myricetin, Kaempferol	Flavanols	Lotito and Frei, 2006
Sweet potato	Quercetin, Myricetin, Kaempferol	Flavanols	Lotito and Frei, 2006
<b>Others</b>			
Black tea	Quercetin, Myricetin, Kaempferol, Epicatechin, Catechin, Epigallocatechin gallate	Flavanols	Lotito and Frei, 2006; Hooper et al., 2008; Kozłowska and Szostak-Wegierek, 2014
Coffee	Quercetin, Myricetin, Kaempferol	Flavanols	Lotito and Frei, 2006
Red wine	Quercetin, Myricetin, Kaempferol, Epicatechin, Catechin, Epigallocatechin gallate	Flavanols	Lotito and Frei, 2006
	Cyanidin, Malvidin, Delphinidin, Pelargonidin	Anthocyanidins	Hooper et al., 2008
Dark chocolate	Quercetin, Myricetin, Kaempferol, Epicatechin, Catechin, Epigallocatechin gallate	Flavanols	Lotito and Frei, 2006; Hooper et al., 2008
Cocoa powder	Quercetin, Myricetin, Kaempferol	Flavanols	Lotito and Frei, 2006; Hooper et al., 2008



**Figure 1** Core structure of flavonoids – the flavan nucleus. Source: S. Kumar, A.K. Pandey, Chemistry and biological activities of flavonoids: an overview, Scientific World Journal. 2013 (2013) 162750. <https://doi.org/10.1155/2013/162750>.



**Figure 2** Major categories of flavonoids. Source: Catarino, M.D., Talhi, O., Rabahi, A., Silva, A.M.S., Cardoso, S.M., 2016. The antiinflammatory potential of flavonoids. Stud. Nat. Prod. Chem. 48, 65–99.

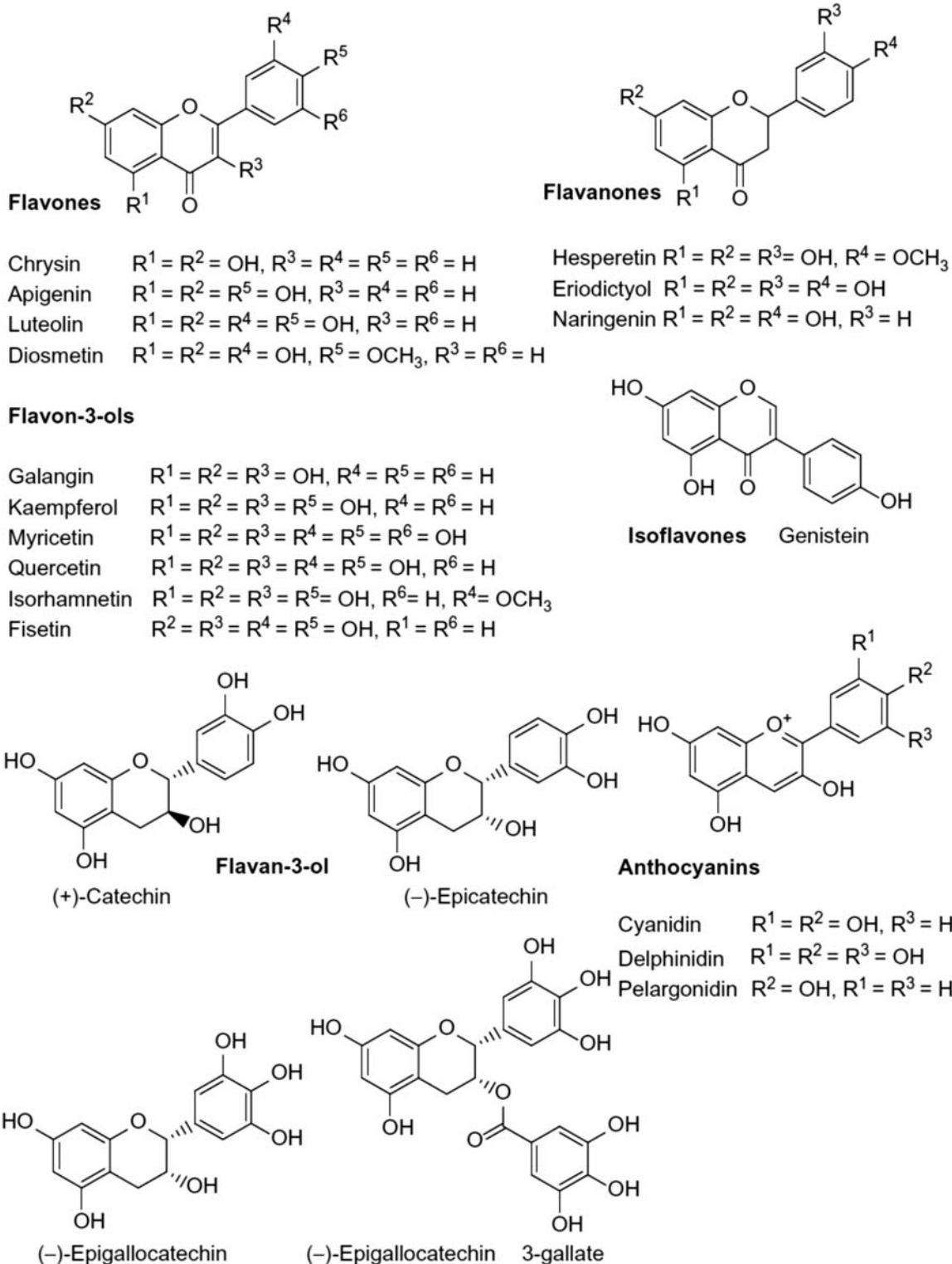
plants. Chalcones (flavan-opened chain compounds) though lacking the heterocyclic Ring C, or 5-membered C-ring derivatives aurones and auronols are still categorized as members of the flavonoid family (Beltz et al., 2006; Cheng et al., 2016). These basic structures of flavonoids are aglycones; however, in plants, most of these compounds exist as glycosides. Biological activities of these compounds, including antioxidant activity, depend on both the structural difference and the glycosylation patterns.

Flavones, flavonols, flavanones and flavanols flavonoid subgroups are the most common, and almost ubiquitous, throughout the plant kingdom. Flavones and their 3-hydroxy derivatives flavonols, including their glycosides, methoxides and other acylated products on all three rings, make this the largest subgroup among all polyphenols. The most common flavonol aglycones, quercetin and kaempferol, alone have at least 279 and 347 different glycosidic combinations, respectively. Isoflavones are mostly found in the leguminous family of plants and have great impact on human health since beans are a major part of the diet in many cultures. Neoflavonoids are not often found in food plants while the open-ring chalcones are found in fruits such as apples (Cheng et al., 2016).

### Anticancer Property of Flavonoids

Flavonoids can exhibit anticarcinogenic effects due to their antioxidant property, ability to regulate signaling pathways involved in carcinogenesis, interaction with proteins that control cell cycle progression and effective modulation of the wingless-related integration site (Wnt) signaling pathways in which most conventional therapeutics are ineffective (Ramos, 2008). Flavonoids can interfere with all three stages of cancer: initiation, development and progression by modulating cellular proliferation, differentiation, apoptosis, angiogenesis as well as metastasis (Kris-Etherton et al., 2002). Moreover, the chemopreventive effect of dietary flavonoids is quite specific as cancerous cells have been shown to be more sensitive to polyphenol actions than normal cells (Brglez Mojzer et al., 2016).

Polyphenols, including flavonoids have some distinct properties that contribute to their efficacy as anticancer agents. They are reported to differentially show cytoprotective effects toward normal cells and cytotoxic effects toward cancerous cells and to regulate



**Figure 3** Main classes of flavonoids and examples. Source: Catarino, M.D., Talhi, O., Rabahi, A., Silva, A.M.S., Cardoso, S.M., 2016. The anti-inflammatory potential of flavonoids. *Stud. Nat. Prod. Chem.* 48, 65–99.

growth factor-receptor interactions and cell signaling cascades that can induce cell cycle arrest and have impact on cell survival and apoptosis of cancerous cells (Ramos, 2008; Tabrez et al., 2013). Depending on their concentration, target molecules, cell type and other conditions, they can induce apoptosis through pro-oxidative action that is exerted instead of their anti-oxidative action (Brglez Mojzer et al., 2016; Beltz et al., 2006). In addition, polyphenols help to establish the body's immune system by inhibiting

angiogenesis necessary for tumor growth and act also as anti-inflammatory agents (Tabrez et al., 2013). In the final stages of cancer, polyphenols attenuate the adhesiveness and invasiveness of cells therefore reducing their metastatic potential (Brglez Mojzer et al., 2016).

### Anticancer Mechanisms of Flavonoids

A large amount of data exists on the biochemical and molecular mechanisms of the carcinoprotective property of flavonoids. Some general properties of flavonoids which underlie their biological activities are also applicable to their anticancer effect but other unique mechanistic aspects are also involved. The tumour microenvironment is dynamic and complex and phytochemicals, including flavonoids have been widely documented to directly or indirectly target multiple signaling pathways and networks in cancer cells (Cheng et al., 2016).

### Modulation of Redox Balance

Flavonoids are potent antioxidants *in vitro*, mainly due to their low redox potential and ability to donate electrons or hydrogen atoms. For example, catechins have a redox potential of +0.53–0.57 V, which from a thermodynamic standpoint enables them to protect urate (+0.59 V), but not ascorbate (+0.28 V), from oxidation by peroxy radicals (+1.06 V). The redox potentials of quercetin and (–)-epigallocatechin gallate (EGCG) are even lower (+0.33 and 0.43 V, respectively), which places them close to ascorbate in the biological antioxidant network (Lotito and Frei, 2006; Bors et al., 2001; Jovanovic and Simic, 2000).

Cancer is characterized by increased aerobic glycolysis and oxidative stress. The link between ROS and cellular transformation was first identified in 1981, when it was found that insulin elevated intracellular  $H_2O_2$  levels and increased tumour cell proliferation suggesting the potential efficacy of antioxidant therapy. In addition, commonly used radio- and chemotherapeutic drugs can influence tumor outcome through ROS modulation. However, it is now apparent that the link between oxidative stress and cancer is far from simplistic (Roleira et al., 2015). ROS could promote or act as barrier to tumorigenesis depending on their levels. The redox status of cancer cells usually differs from that of normal cells with cancer cells exhibiting elevated ROS levels because of metabolic and signalling aberrations. This is said to influence development of powerful antioxidant mechanisms and alternative pathways by which pre-neoplastic cells evade the deleterious consequences of large amounts of ROS accumulation without compromising the energy demand required by cancer cells. At low to moderate levels, ROS may contribute to tumour formation either by acting as signalling molecules or by promoting the mutation of genomic DNA. For instance, ROS can stimulate the phosphorylation of mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK), cyclin D1 expression and JUN N-terminal kinase (JNK) activation (Gorrini et al., 2013; Sosa et al., 2013). Based on the foregoing, it has been opined that targeting enhanced antioxidant defence mechanisms may represent a strategy that can specifically kill cancer cells, including tumour initiating cells TICs, while sparing normal cells (Gorrini et al., 2013). Supplemental antioxidants have become an important strategy in ROS-scavenging, and therefore cancer chemoprevention. Some antioxidants can also act as potent pro-oxidants, sensitizing cancer cells to chemotherapy (El Bairi et al., 2017). Flavonoids also act by inhibiting prooxidant enzymes. Downregulation of superoxide-producing NADPH oxidase 1 (NOX 1) reversed tumor growth while its overexpression is associated with tumor progression (Fukuyama et al., 2005; Arnold et al., 2007). In addition, lipoxygenases (LOX), cyclooxygenases (COXs), and xanthine oxidase (XO) are metalloenzymes whose catalytic cycle involves ROS such as lipid peroxy radicals, superoxide, and hydrogen peroxide. Flavonoids may exert part of their anti-oxidant and anti-inflammatory activities via direct inhibition of these prooxidant enzymes (LOXs, COXs, and XO), which are also important inflammatory cascades (Batra and Sharma, 2013). ROS function as secondary messengers in several pathways that contribute to increase in cell proliferation, resistance to apoptosis, in addition to activation of proto-oncogenes such as cFOS, cJUN and cMyc. In human hepatoma cells, ROS modulated the expression of cFOS and cJUN through PKB pathway (Liu et al., 2002). The inhibitory property of flavonoids against prooxidant enzymes can, therefore be intertwined with other distinct inhibition mechanisms such as the anti-inflammatory mechanism.

### Induction/Inhibition of Drug Metabolizing Enzymes

Flavonoids are good substrates and inducers of phase II enzymes. The capacity of flavonoids to induce detoxifying enzymes is regarded as a major mechanism by which flavonoids protect against mutagens and carcinogens. Flavonoids can interact with phase I xenobiotic metabolizing enzymes (such as various isoforms of the heme-containing cytochrome P450 (CYP) monooxygenases), which has been associated with the generation of oxidative stress in the liver and involved in the activation of procarcinogens (Lotito and Frei, 2006; Martín and Ramos, 2016; Hodek et al., 2009). Flavonoids also modulate the detoxification of carcinogens through induction of phase II enzymes such as UDP-glucuronyl transferase, quinone reductase and glutathione S-transferase (Cederbaum and Muriel, 2017). Flavonoids can affect CYP activity in several ways including regulation of gene expression and direct binding to the enzyme. For example, quercetin binds to aryl hydrocarbon receptor (AhR) as an agonist and stimulates gene expression for CYP 1A1 with a parallel increase in CYP 1A1-mediated O-deethylation of 7-ethoxyresorufin in human breast cancer cells. Flavones and flavonols, also directly bind to several CYP isoforms (1A1, 1A2, 1B1, 3A4) involved in xenobiotics metabolism and inhibit enzyme activity. Flavonoids can positively influence cellular level of UDPglucuronosyltransferases (UGT), sulfotransferases (SULT), glutathione transferases (GST), N-acetyltransferases, which are all phase II enzymes. For example, increased mRNA levels, protein

expression and enzyme activity of UGT isoform 1A1 by chrysin, apigenin, baicalein, diosmetin, quercetin and kaempferol has been reported in human Hep G2 cells and human colon carcinoma cells (Batra and Sharma, 2013; Sugatani et al., 2004).

### Genome Stabilization

Carcinogenesis involves genetic and epigenetic changes that lead to the initiation, promotion, and progression of cancer. DNA damage, which could be caused by exposure to ultraviolet radiation (UVR), ionizing radiations (IR), mutagenic chemicals, environmental agents, therapeutic agents or diagnostic imaging instruments can lead to carcinogenesis. Maintaining genomic integrity is, therefore highly important for the organism (George et al., 2017; Weitzman et al., 2010). Several studies have shown that dietary flavonoids prevent DNA damage thereby contributing to genomic stability and cancer prevention. For instance, quercetin, quercitrin, isoquercitrin and rutin isolated from the leaves of *Scutia buxifolia* L. were found to protect against chromosome damage in cultured human lymphocytes by retaining mitotic index (at a concentration of 50 µg/mL) and genomic stability (at the concentration range of 1–100 µg/mL) against H<sub>2</sub>O<sub>2</sub>-induced toxicity (Boligon et al., 2012).

### Inhibition of Multidrug Resistance

Typically, in cancer cells, there is overexpression of P-glycoprotein (Pgp) or multidrug resistance associated protein (MRP) which is a group of ATP-dependent transmembrane transporters capable of expelling a wide variety of chemically unrelated drugs used in cancer therapy at the expense of ATP hydrolysis. This phenomenon is known as multidrug resistance (MDR) and its inhibition to prevent drug efflux has potential clinical applications during cancer therapy. Quercetin (10, 30 or 100 µM) and Kaempferol (10 or 30 µM) in different *in vitro* experimental set-ups, were shown to inhibit P-Glycoprotein function and expression (Batra and Sharma, 2013; Chen et al., 2010; Kioka et al., 1992; Limtrakul et al., 2005).

### Anti-angiogenesis

Flavonoids inhibit angiogenesis and this activity can cause non-diffusion of nutrients and oxygen to rapidly growing cancerous cells due to anti-angiogenic properties and hence lead to cell death. Inhibition of angiogenesis could be through the inhibition of PKs. Luteolin was found to inhibit VEGF-induced angiogenesis and endothelial cell survival and proliferation by targeting phosphatidylinositol-3-kinase action (Tosetti et al., 2002; Bagli et al., 2004). He et al. (2011) indicated that hispidulin targets the VEGF receptor 2-mediated PI3K/Akt/mTOR signaling pathway in endothelial cells, leading to the suppression of pancreatic tumor growth and angiogenesis.

### Induction Apoptosis and Cell Cycle Arrest

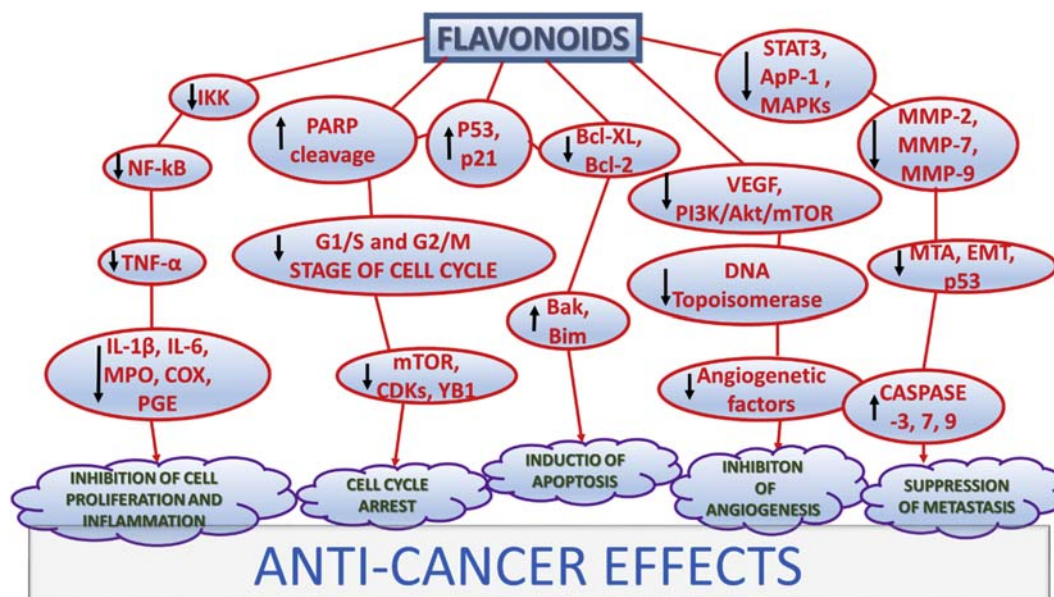
Poly (adenosine diphosphate-ribose) polymerase (PARP) is involved in DNA repair, genomic stability and apoptosis. Flavonoids induce the cleavage of PARP which contributes to cell cycle arrest. Also, the tumour suppressor gene, p53 plays a pivotal role in controlling the cell cycle, apoptosis, genomic integrity, and DNA repair (Caltagirone et al., 2000) by acting as transactivator or as transrepressor (Ho et al., 2005). After activation, p53 can bind to regulatory DNA sequences and activate the expression of genes involved in cell cycle inhibition (p21, reprimin, cyclin G1, GADD45), apoptosis (PERP, NOXA, PUMA, p53AIP1, ASPP1/2, Fas, BAX, PIDD) and genetic stability (p21, DDB2, MSH2, XPC). Epigallocatechin 3-gallate (EGCG) activated p53 and BAX in breast carcinoma cells. Using MDA-MB-468 human breast cancer cell line as an *in vitro* model of ER-negative breast cancers, it was found that treatment with EGCG resulted in dose-dependent (5–80 µg/mL) and time-dependent (24–72 h) inhibition of cellular proliferation and cell viability in MDA-MB-468 cells with decrease in cell viability associated with the induction of apoptosis (Roy et al., 2005). Genistein (100 µM) induced G2/M arrest and apoptosis in human malignant glioma cell lines by activating p53 and p21 (Schmidt et al., 2008). Flavonoids may block one or more steps in the NF-κB signaling pathway. The NF-κB target genes influenced by the flavonoids include inhibition of Bcl-2 and Bclx(L), cyclin D1, matrix metalloproteinases (MMP), and VEGF. Flavonoids have been found to suppress activator protein-1 (AP-1) activation and modulate AP-1 target genes (Batra and Sharma, 2013; Schmidt et al., 2008; Li et al., 2006).

Summarily, flavonoids act on specific tumor and stromal targets which could be grouped as membrane-bound receptor kinases (HGF/c-Met, human epidermal growth factor receptor and insulin growth factor receptor pathways), intracellular signalling kinases (Src, PI3k/Akt/mTOR, and mitogen-activated protein kinase pathways), epigenetic abnormalities (DNA methyltransferase and histone deacetylase), protein dynamics (heat shock protein 90, ubiquitin-proteasome system), and tumor vasculature and micro-environment (angiogenesis, HIF, endothelium, integrins) (Ma and Adjei, 2009) (Fig. 4).

### An Overview of the Interaction of Some Dietary Flavonoids With Enzymatic and Molecular Targets

Many studies have reported the anticancer property of flavonoids and flavonoid containing food and the list continues to grow almost on a daily basis. An exhaustive or detailed treatment is not possible within the scope of this article.





**Figure 4** Scheme showing regulatory processes, proteins, factors and signalling pathways inhibited (↓) or activated (↑) by flavonoids leading to anti-cancer effects. Flavonoids interact with multiple cellular and molecular targets thereby regulating a wide variety of cell function. Key: **IKK**, I kappa B kinase; **NF-κB**, nuclear factor kappa-light-chain-enhancer of activated B cells; **TNF-α**, tumor necrotic factor alpha; **IL-1,6**, interleukin 1,6; **MPO**, myeloperoxidase; **COX**, cyclooxygenase; **PGE**, postaglandin E; **PARP**, poly (ADP ribose) polymerase; **G1/2 and G2/M**-gap 1-synthesis stage and gap 2-mitosis stage of cell cycle; **mTOR**, mechanistic target of rapamycin; **CDK**, cyclin-dependent kinase; **CDKI**, CDK inhibitor; **YB1**- Y box binding protein 1; **Bcl-2**, B-cell lymphoma-2; **Bcl-xL**, B-cell lymphoma-extra-large; **Bak**, Bcl-2 homologous antagonist/killer; **BIM**, Bcl-2-like protein 11; **MMP 2,7 and 9**, Matrix metalloproteinases 2,7 and 9; **MTA**, metastasis-associated protein; **EMT**, epithelial-to-mesenchymal transition; **p53**, protein 53 gene.

### Cocoa Flavonols

A lot of studies have reported the anticancer property of flavonoids and flavonoid containing food. [Martín and Ramos \(2016\)](#) reported that cocoa and its flavanols modulate the first steps of carcinogenesis by protecting against cell damage via direct free radical scavenging and metal-chelation, alteration of pro-carcinogenic metabolism by inhibiting phase-I drug-metabolising enzymes (cytochrome P450, CYP) and/or activating phase II conjugating-enzymes, and induction of pro-oxidation.

### Chrysin

Cotreatment with 20 and 40 mg/kg (p.o.) of the flavonoid, chrysin for 16 weeks suppressed N-nitrosodiethylamine-induced and ferric nitrilotriacetate-promoted renal carcinogenesis in male Wistar rats. Chrysin treatment resulted in marked decline in renal hyperplasia, renal ornithine decarboxylase activity and protein expression of proliferating cell nuclear antigen, iNOS, COX-2 and secretion of proinflammatory cytokines, all of which are traditional markers of inflammation and tumor promotion. Chrysin treatment maintained antioxidant armory and suppressed activation of redox active transcription factor NF-κB ([Srivastava et al., 2016](#)). Chrysin was also found to induce aryl hydrocarbon receptor-dependent apoptotic cell death in human colorectal cancer cells involving up-regulation and activation of the tumor necrosis factor-mediated transcriptional pathway ([Ronnekleiv-Kelly et al., 2016](#)).

### Curcumin

[Srinivasan \(2017\)](#) elaborated on the antimutagenic and carcinoprotective properties of culinary spices like turmeric, garlic, ginger, and black cumin. These spices showed chemopreventive effects against cancers of the skin, forestomach, pancreas, liver, colon, and oral cancer in experimental models by impeding cell division and promoting apoptosis in cancerous cells as well as regulating inflammation and immunocompetence. Curcumin of turmeric, in particular, was noted to impede carcinogenesis at all three stages and mediate its anticarcinogenic effect partly through its inhibition of the transcription factor NFκB and inhibition of pro-inflammatory pathways as well as by inducing apoptosis and suppressing proliferation and angiogenesis. Anti-metastatic action of curcumin includes inhibition of transcription factors and their signalling pathways (e.g., NFκB, ApP-1 and STAT3), inflammatory cytokines (CXCL1, CXCL2, IL-6, IL- 8), proteases (uPA, MMPs), protein kinases (MAPKs, FAK), regulation of miRNAs (miR21, miR181b) and heat shock proteins (H1J1). The author suggested that use of spices as food adjuncts could, therefore be a promising approach to reduce the risk of cancer and these spices deserve to be considered as nutraceuticals for deriving anticancer influences. In a rat bladder carcinogenesis model, curcumin (0.2 mL, 240 μM) administered intravesically every other day for a total of seven doses, inhibited N-methyl-N-nitrosamine-induced urothelial tumor development via suppression of IGF2 and IGF2-mediated PI3K/AKT/mTOR signalling pathway ([Tian et al., 2017](#)).



### Fenugreek

Similarly, both *in vitro* and *in vivo* studies have alluded to the anticancer property of Fenugreek [*Trigonella foenum-graecum* Linn (Fabaceae)], a seed spice which is also employed for medicinal purposes in many traditional systems. Fenugreek flavonoids such as quercetin, luteolin, vitexin cinnamate, vicenin and isovitexin are believed to support the anticancer activity of the herb. Fenugreek alcohol seed extract (100 and 200 mg/kg) administered for five days inhibited experimental Ehrlich ascites carcinoma in mice. In another experiment, 400 mg/kg methanol extract of Fenugreek ameliorated skin papillomas in Swiss albino mice (El Bairi et al., 2017). Naringenin was reported as one of two main constituents in Fenugreek seed extract with anticancer activity through p53, Bax, and PCNA upregulation in a caspase-3 dependent manner (Khalil et al., 2015).

### Hesperidin

Siddiqi et al. (2015) provided evidence suggesting that hesperidin was a potent chemopreventive agent against renal carcinogenesis possibly by virtue of its antioxidant properties and by modulation of multiple molecular pathways. They found that hesperidin (100 and 200 mg/kg, administered by oral gavage to male Wistar rats for 16 weeks protected against DEN-initiated and Fe-NTA-promoted renal carcinogenesis in a dose dependent manner. Hesperidin was found to induce caspase-3, caspase-9, and bax expression and downregulate bcl-2, NFκB, iNOS, TNF-α, and PCNA expression. Histopathological findings further revealed hesperidin's chemopreventive efficacy shown by the complete restoration of renal morphology at the dose of 200 mg/kg.

### Kaempferol

Kaempferol, initially discovered in *Camellia sinensis* (tea tree) and abundant in different genera of plants such as *Capparis spinosa* (Capers) and *Crocus sativus* (Saffron) has been described as a valuable functional food ingredient with a broad range of therapeutic applications (Kashyap et al., 2017). Anticancer mechanisms of kaempferol have been demonstrated to include cell cycle arrest and apoptosis, anti-angiogenesis effect through alteration of the VEGF expression and possible down-regulation of ERK-NFκB-cMyc-p21-VEGF signalling pathway, antimetastasis effect through mechanisms including suppression of STAT3 and downregulation of MMP-2 & 9, ERK ½ and p38. In an *in vivo* study, 28 days post-treatment with kaempferol (100 mg/kg p.o.) showed ameliorative effects in AFB<sub>1</sub>-induced experimental hepatocarcinogenesis in male Wistar rats (Kulanthaivel et al., 2012).

### Apigenin

Many fruits and vegetables such as parsley, celeriac, celery and chamomile tea are rich in apigenin. Apigenin demonstrated ability to halt cancer growth in a wide range of cancers including liver, pancreatic, colorectal, blood, prostate, breast, lung, thyroid, skin, neck and head, and bone cancers by a variety of mechanisms. Examples include reduction of Snai1 and NF-κB expression, leading to reversal of increased epithelial-mesenchymal transition (EMT) marker levels, improved cellular adhesion, controlled actin polymerization and cell migration, and inhibited invasion and migration in experimental liver cancer. Others include inhibition of STAT3 signalling evident from up-regulated levels of cleaved caspase-8 and caspase-3, and provoked cleavage of PARP. Another mechanism involved induction of extrinsic apoptosis, blockage of the phosphorylation of JAK2 and STAT3 in addition to inhibition of CoCl<sub>2</sub>-induced VEGF secretion and decreased nuclear staining of STAT3 in breast cancer cells. Apigenin also acted as an anti-cancer agent through binding with IKKα, attenuation of IKKα kinase activity and suppression of NF-κB/p65 activation leading to inhibition of cell proliferation, invasiveness and decrease in tumor growth in experimental prostate cancer. Lastly, apigenin stimulated the mitochondrial pathway of apoptosis, modified expression of apoptotic proteins, as well as significantly up-regulated the expression of cytokine genes IL17F, LTA, IL17C, IL17A, and IFNβ1 in experimental pancreatic cancer (Salmani et al., 2017). Apigenin and quercetin (25 and 50 mg/kg) were equipotent in dose-dependent inhibition of melanoma growth following their intraperitoneal administration at the time of i.m. injection of B16-BL6 cells into female C57BL/6N syngeneic mice. EGCG at 50 mg/kg was found to be more effective than apigenin and quercetin (Caltagirone et al., 2000). Also, 50 mg/kg apigenin administered i.p. to female Sprague Dawley rats for 21 days prevented the development of medroxyprogesterone acetate accelerated 7,12-dimethylbenz(a)anthracene-induced mammary tumors in Sprague Dawley rats (Mafuvadze et al., 2011) while 20 mg/kg apigenin administered i.p. to C57BL/6 mice showed anti-tumoral effect in malignant mesothelioma induced by transplanting mice with MM# 40a cells forming ascites (Masuelli et al., 2017). In another study, inclusion of 0.1% apigenin and 0.02% naringenin in diet of male Sprague-Dawley rats for 10 weeks suppressed colon carcinogenesis in azoxymethane-treated rats (Leonardi et al., 2010).

### Quercetin

Quercetin is a prominent member of the flavonoid family and a medically important dietary antioxidant. Quercetin is found in various medicinal plants and commonly consumed vegetable and fruits such as onions, apples and berries. The anticancer property of quercetin is due to binding to cellular receptors and proteins, which modulates various cell signalling mechanisms. It also involves inhibition of enzymes responsible for the activation of carcinogens. Treatment with 25 μM Quercetin inhibited the phosphatidylinositol 3-kinase (PI3K)-Akt/PKB (protein kinase B) pathway in cancer cells which suggests that this compound may have therapeutic benefit against tumorigenesis and cancer progression. In an *in vivo* experiment, Swiss albino mice with Ehrlich Ascites Carcinoma (EAC) cells-induced tumor showed a remarkable reduction in tumor volume when orally administered with 6 doses of quercetin (1 mg/kg) on every third day, after the 10th day of treatment. Xenograft NOD/SCID female mice post-treated with quercetin (30 mg/kg p.o.) for two weeks following implantation of human gastric carcinoma SNU791 cells for 2 weeks as well, showed inhibition of the human gastric carcinoma as from day 5 of treatment with the flavonoid. In a model of pancreatic cancer, quercetin (1%) administered orally in the diet, inhibited orthotopic tumor growth and induced tumor cell apoptosis in mice. Quercetin

extensively downregulated the potential oncogenic MAPK signalling *in vivo*. Both Raf and MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK) act as direct targets for quercetin, leading to the potential to decrease MEK1 activity. Quercetin binds with SAPK/ERK kinase 1 (SEK1), c-Jun N-terminal kinase 1/2 (JNK1/2), MEK1 and ERK1/2 thereby modulating reprogramming events related to gene expression, potentiation of cellular growth, and antiapoptotic properties. Other mechanisms adduced for the anticancer property of quercetin include induction of senescence, inhibition of telomerase, induction of apoptosis even in cells which are resistant to TNF-related apoptosis-inducing ligand (TRAIL), and inhibition of the aryl hydrocarbon receptor (AhR), which is a ligand-gated transcription factor that is activated by the interaction with synthetic and natural chemicals (Culati et al., 2006; Srivastava et al., 2016; Lee et al., 2016; Angst et al., 2013; Kumamoto et al., 2009; Ashida et al., 2000; Khan et al., 2016; Murray et al., 2014).

#### **Garcinia kola Biflavonoid Complex (Kolaviron)**

Kolaviron is a biflavonoid complex, extracted from *Garcinia kola* seeds, containing GB1, GB2 and kolaflavanone. The anticancer potential of kolaviron, especially its protective potential in hepatic cancer has been highlighted in several studies. Kolaviron showed chemopreventive effect on aflatoxin B1 (AFB1) - induced toxicity. AFB1 is a potent hepatocarcinogen in experimental animals and is also a human carcinogen (Eaton and Gallagher, 1994; International Agency for Research on Cancer, 1993). Studies of the chemopreventive effects of kolaviron on AFB1-induced genotoxicity in rats showed that co-treatment of rats with 500 mg/kg kolaviron 30 min before and then after AFB1 administration inhibited the induction of micronucleated polychromatic erythrocytes. In addition, kolaviron inhibited AFB1 induction of markers of hepatic oxidative damage (Farombi et al., 2005). Rats administered kolaviron alone showed significant elevation in the activities of phase 2 enzymes—glutathione S-transferase, uridyl glucuronosyl transferase and NADH:quinone oxidoreductase. Also, *Garcinia* biflavonoid inhibited aflatoxin B1-induced genotoxicity in HepG2 cell. Kolaviron specifically induced CYP 3A4 activation. In addition, GST isozymes  $\alpha$ -1 and  $\alpha$ -2.2 were also induced, which signified molecular mechanisms of action of antigenotoxic properties of kolaviron via induction of phase 2 detoxifying enzymes. Studies have shown that kolaviron suppressed the expression of COX-2 and iNOS and the DNA binding activity of NF- $\kappa$ B and AP-1 induced by dimethyl nitrosamine (DMN) (Gonzales et al., 2016). Kolaviron demonstrated antiproliferative effect by suppressing the growth and survival of both colon adenoma (LT97) and carcinoma cells (HT29) (Farombi and Owoeye, 2011).

#### **Myricetin**

Myricetin is a flavonol with powerful antioxidant activity. It has been shown to have a therapeutic effect in different cancers both *in vitro* and *in vivo*. It exerts apoptotic effects in combination with TRAIL or by other mitochondrial-dependent pathways, as well as inducing G2/M cell cycle arrest (Siegelin et al., 2009; Zhang et al., 2008). Several myricetin-derived, synthetic flavonoids with improved antioxidant properties, specific mitochondrial targeting and optimised physicochemical properties and drug-like attributes have been developed. They showed more potency and enhanced potential as antitumour agents than myricetin. Among them, Oncamex was the most potent compound. Oncamex was capable of specific mitochondrial delivery and redox modulation. It specifically targeted the mitochondrial compartment, with rapid delivery and stable accumulation. *In vivo* study in mice implanted with the MDA-MB-231 breast cancer xenograft showed that treatment with Oncamex (25 mg/kg/day, i.p.) over 14 days inhibited tumour growth, reducing tissue viability and Ki-67 proliferation, with no signs of untoward effects on the animals. Oncamex exerted its antitumour effect through induction of cytotoxicity and apoptosis, induced gene expression changes related to cell cycle and apoptosis regulation and inhibited tumour proliferation and viability (Martínez-Pérez et al., 2016).

#### **Tea Catechins**

Tea, made from the dried leaves of the plant *C. sinensis*, is the most popular beverage worldwide after water. The major catechins in green tea are (–)-epigallocatechin-3-gallate (EGCG) (–)-epigallocatechin (EGC) (–)-epicatechin-3-gallate (ECG) and (–)-epicatechin. Among tea catechins, EGCG is the most effective in reacting with the majority of ROS. EGCG has been shown to bind directly to several receptors and signalling molecules, and to inhibit the functions of key receptors, kinases, proteinases and other enzymes thereby affording protection against cancer. Inclusion of <0.5% tea polyphenols or EGCG in drinking water of experimental animals for multiple weeks have been reported to inhibit various types of experimental carcinogenesis (Yang et al., 2009). Cancer treatment using a combination of EGCG and anticancer drugs have been reported to produce remarkable results (Suganuma et al., 2011).

#### **Fisetin**

Fisetin (10–80  $\mu$ M) was reported to bind to tubulin and stabilize microtubules with binding characteristics far superior than paclitaxel. It enhanced tubulin polymerization and increased  $\alpha$ -tubulin acetylation and MAP-2 and MAP-4 expression. Fisetin arrested cells in the G2/M phase of cell cycle, inhibited cell proliferation, invasion and migration. In addition, this polyphenol was found to inhibit the viability, colony formation and decrease the expression of P-gp in multidrug-resistant cancer cell line NCI/ADR-RES (Mukhtar et al., 2015). Results of experiments by Meng et al. (2016) provided insight into antitumor, anti-proliferative and the induction of apoptosis efficacy of fisetin against ovarian cancer *in vitro* and *in vivo*. Data obtained suggested a safe promising therapeutic potential of fisetin in ovarian cancer treatment. In their athymic nude rat model, under the influence of fisetin, tumor volume and tumor mass were significantly decreased. Treatment with fisetin resulted in a significant decrease of Bcl-2 and a significant increase of Bax.

### Multiple Dietary Flavonoids

Kuo et al. (2016) proposed dietary flavonoid supplements to be the key to improving the efficacy of chemotherapy and reducing cancer recurrence rate as well as side effects from chemotherapy. They reported that dietary flavonols (kaempferol, fisetin, quercetin), anthocyanidin (cyanidin), flavanol ((-)-catechin, EGCG), isoflavone (genistein), and flavanone (naringenin) which are common flavonoids in the diet could inhibit Chk1 phosphorylation and decrease clonogenic cell growth once breast cancer cells receive ultraviolet irradiation, cisplatin, or etoposide treatment. They also proposed that since the ATR-Chk1 pathway mainly involves response to DNA replication stress, flavonoid derivatives can reduce the side effect of chemotherapy by improving the sensitivity of cycling cells.

### Concluding Remarks and Outlook

Overwhelming evidences from epidemiological, *in vitro* and *in vivo* studies reveal the anticancer potential of dietary flavonoids and the mechanistic routes of their chemopreventive property. Therefore, consumption of flavonoid-rich foods is recommended as it will go a long way in reducing the scourge of the various cancers plaguing the world today. It is apparent that some mechanistic aspects of the action of flavonoids still need to be clearly delineated and properly elucidated so that health benefits inherent in this unique class of bioactives can be fully unlocked. However, the enormous volume of research works that are on-going and the impressive outputs and important discoveries being made as a result of these investigations present a positive outlook in the fight against the disease. There should be focus on discovery/formulation of carcinoprotective flavonoid-based nutraceuticals and functional foods. Efficient and effective synergy of flavonoid-based interventions with orthodox cancer therapeutics for optimum results in the battle against the disease should be further explored.

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## Antihypertensive Foods: Protein Hydrolysates and Peptides

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### Glossary

**Antihypertensive** The ability of an agent to reduce high blood pressure below the pathological level

**Bioactive** Compounds that induce physiological changes when present within the body

**Diastolic blood pressure** The pressure exerted on vascular walls when the heart muscles relax in-between beats

**Membrane ultrafiltration** The use of membranes with specific molecular weight cut-offs to separate protein hydrolysates into peptide fractions with defined sizes

**Oral gavage** A method that involves inserting a hollowed plastic or metal tube in the throat of a rat (or mouse) for direct deposition of a liquid sample into the oesophagus

**Peptides** Protein fragments released during proteolysis

**Protein hydrolysates** A mixture of peptides obtained after protein hydrolysis; the peptides differ according to chain length, amino acid sequence, amino acid composition and bioactive properties

**Proteolysis** Breakdown of food proteins into smaller fragments and free amino acids using enzymes, acids or bases

**Renin-angiotensin-aldosterone system** A cascade of enzyme-catalyzed reactions that regulates mammalian blood pressure through vascular contractions and control of blood sodium ion level

**Systolic blood pressure** The pressure exerted on vascular walls when the heart muscles contract to pump blood

### Nomenclature

**ACE** Angiotensin-I converting enzyme

**DBP** Diastolic blood pressure

**GIT** Gastrointestinal tract

**HPH** Hemp seed protein hydrolysate

**RAAS** Renin-angiotensin-aldosterone system

**RBPH** Rice bran protein hydrolysate

**RP-HPLC** Reverse-phase high-pressure liquid chromatography

**SBP** Systolic blood pressure

**SHR** Spontaneously hypertensive rats

**UF** Ultrafiltration

### Introduction

High blood pressure is a precursor of the pathological condition called hypertension, which can become fatal if left untreated or improperly managed with appropriate therapeutic interventions (WHO, 2013). Hypertension exists when the systolic blood pressure (SBP) exceeds 140 mmHg and the diastolic blood pressure (DBP) is  $\geq 90$  mmHg (Pickering et al., 2005). The mammalian blood pressure is controlled principally by the renin-angiotensin-aldosterone system (RAAS), which consists of a cascade of reactions that eventually lead to production of angiotensin II, the main vasopressor agent (Acharya et al., 2003). Activities of the RAAS begin with conversion of angiotensinogen (a 55 kDa protein secreted by the liver) into an inactive angiotensin I, which is the rate-determining reaction catalyzed by renin (EC 3.4.23.15). Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) then removes the C-terminal dipeptide (His-Leu) of angiotensin I to form angiotensin II, which binds to receptors on blood vessels to cause contractions that maintain blood flow and normal blood pressure (Acharya et al., 2003). However, under disease conditions or as a result of aging, genetic disposition, diet or environment, the rate of RAAS reactions become very high with excessive angiotensin II production (Allen et al., 2012; MacMachon et al., 1990; Pickering et al., 2005). Additionally, ACE also hydrolyzes and inactivates bradykinin (a protein that enhances blood vessel dilation), which leads to reduced vasodilation (Acharya et al., 2003). Therefore, excessive activities of renin and ACE lead to higher-than-normal levels of angiotensin II, which cause high rate of vasoconstriction but reduced vasodilation and consequently high blood pressure. Angiotensin II also enhances aldosterone activity to cause excessive reabsorption of sodium that leads to enhanced water retention, increased blood volume and ultimately contributes to the development of high blood pressure (Acharya et al., 2003). Hence, clinical blood pressure management involves the use of compounds that reduce activities of RAAS enzymes, mainly renin and ACE to produce and maintain homeostatic levels of angiotensin I,

angiotensin II or aldosterone. These blood pressure control agents are mainly pharmaceutical agents (drugs) that are based on synthetic compounds with inhibitory effects against renin and ACE activities (Acharya et al., 2003). However, the use of antihypertensive drugs is accompanied by negative side effects such as persistent dry cough (Yesil et al., 1994), angioedema (Gunkel et al., 1996; Vleeming et al., 1998), teratogenicity (Cooper et al., 2006; Pryde et al., 1993), and erectile dysfunction (Blumentals et al., 2003; Fogari and Zoppi, 2002). These undesirable effects are believed to be the main reasons for lack of compliance with physician-prescribed medication doses by patients, which then leads to increased intensity of hypertension and associated deteriorations in health quality (Bangalore et al., 2007; Flack et al., 1997). Therefore, recent trends have involved the search for and development of natural antihypertensive compounds that are less likely to cause severe negative side effects while maintaining therapeutic efficiency. One of the likely alternatives to drugs is food protein digests that contain short-chain peptides with affinity for renin and ACE. The short-chain peptides are preferred because of the increased potential for high rate of absorption from the gastrointestinal tract into blood circulation (Grimble et al., 1987; Roberts et al., 1999). Previous reports have also confirmed the absorption of food protein-derived di- and tripeptides from the gastrointestinal tract into human blood circulatory system following oral consumption (Foltz et al., 2007; Matsui et al., 2002). In addition, *in vivo* suppression of renin and ACE activities as well as angiotensin II concentration has also been demonstrated as a mechanistic explanation for observed blood pressure reducing effects (Brasil et al., 2018; Fernández-Musoles et al., 2013; Girgih et al., 2014a,b; Onuh et al., 2016). Therefore, peptides could function effectively as antihypertensive agents as a result of proven absorption properties. However, rather than depend on *in vivo* gastrointestinal enzyme digestion through feeding of whole proteins, these peptides are created mostly by custom *in vitro* digestion of food proteins (Chen et al., 2013, 2017; Guo et al., 2017; Garcia-Tejedor et al., 2015; Pattarayangsakul et al., 2017; Udenigwe et al., 2017). *In vitro* food protein digestion avails researchers the opportunity to produce specific range of peptides using a variety of highly efficient microbial enzymes that are not present within the gastrointestinal tract (GIT) such as alcalase (Chen et al., 2013), neutrase and flavourzyme (Uluko et al., 2013), or protamex, savinase and corolase (Garcia-Mora et al., 2015). This approach has enabled the creation of a wide variety of antihypertensive food protein hydrolysates.

### Production and Efficacy Evaluation of Antihypertensive Food Protein Hydrolysates

Within the primary structure of food proteins are encrypted specific amino acid sequences that have bioactive properties because of their ability to influence various physiological conditions. These sequences, which are called cryptides (encrypted peptides) or bioactive peptides will be inactive when presented as part of the protein primary structure but become active when released through enzyme or acid-based hydrolysis (Mora and Hayes, 2015; Udenigwe, 2013). Enzyme hydrolysis is usually preferred because of the high specificity and reproducibility of the reaction when compared to the non-specific nature of acid hydrolysis. However, a recent work has shown that acid hydrolysis of hemp seed proteins yielded specific bioactive peptides that can be produced with a high degree of reproducibility (Orio et al., 2017). The hemp seed protein (5 g) was mixed with 25 mL of 6 M HCl, stirred at 110 °C for 6 h, cooled, and followed by additions of 4 M NaOH and then 1 M Na<sub>2</sub>CO<sub>3</sub> under stirring to reach pH 5.8; the digested mixture was freeze-dried as the protein hydrolysate. *In vitro* evaluation showed that the acid-digested hemp seed protein hydrolysate (aHPH) inhibited ACE activity up to ~45% at 1.1 mg/mL. Further separation and purification of aHPH led to identification of the following four peptide sequences that also inhibited *in vitro* activity of ACE: GVLY, IEE, LGV, and RVR. At 1 mg/mL or less, GVLY, IEE, LGV, and RVR had ACE inhibitory values of 95, 21, 95, and 93%, respectively.

However, the most popular means of food protein hydrolysate production involves the use of proteases to reduce protein size and lead to a peptide-enriched product. Table 1 shows the antihypertensive activities of various food protein hydrolysates obtained from enzymatic hydrolysis of proteins. To measure antihypertensive effects, the protein hydrolysates are usually administered to spontaneously hypertensive rats (SHR) by oral gavage followed by blood pressure measurements using the tail-cuff or telemetry method. It is evident from the results that the effective dose differs, which is a reflection of the differences in the types and levels of bioactive peptides present within each hydrolysate. The differences in antihypertensive efficacy could also be due to the nature of starting materials, since primary structure will vary between proteins. But the low doses ( $\leq 1000$  mg/kg SHR body wt) indicate feasibility for commercial exploitation as alternatives to antihypertensive drugs. Based on the animal-to-human dose translation (Reagan-Shaw et al., 2007) these protein hydrolysate doses are equivalent to 1–10 g/day for a typical 70 kg human being, which can be readily achieved. In addition to the effect on SBP, food protein hydrolysates also can reduce DBP, mean arterial blood pressure and heart rate (Li et al., 2011; Lin et al., 2017; Neves et al., 2017; O’Keeffe et al., 2017).

The wide variation in blood pressure-reducing effects as evident in Table 1 also suggests that conditions during protein hydrolysis are very vital for producing strong antihypertensive protein hydrolysates. During *in vitro* enzymatic protein hydrolysis, the protease is added to a protein substrate that can range from seed flours (<30% protein content) to defatted oilseed meals (30%–50% protein) or concentrates (50%–90% protein) and isolates (>90% protein) as well as animal protein preparations (Chen et al., 2017; Eckert et al., 2018; Girgih et al., 2014a,b; Garcia-Mora et al., 2015; Guo et al., 2017; Udenigwe et al., 2017; Uluko et al., 2013). Protein content of the raw material is important to the quality of the protein hydrolysate because it determines the level of soluble non-peptide contaminants that can reduce efficacy as antihypertensive agents. Therefore, in general, higher purity protein materials (concentrates and isolates) are more frequently used than seed flours for protein hydrolysate production. There is now a wide choice of enzymes available for protein hydrolysis, ranging from microbial to gastrointestinal enzymes. The purity of the enzyme is not as important as that of the protein raw material because of the low enzyme to substrate ratios normally used (1:10 to 1:200). However, the protease preparation must be standardized for activity in order to ensure consistency in quality of

**Table 1** Blood pressure-reducing properties of typical food protein hydrolysates after oral administration to spontaneously hypertensive rats

Sample	Dose (mg/kg body wt)	Maximum SBP reduction (mmHg)	References
Hen protein hydrolysate (pepsin digested)	200	27	Udenigwe et al., 2017
Hen protein hydrolysate (pepsin + pancreatin digested)	200	36	Udenigwe et al., 2017
Bamboo shoot peptide preparation	20	12	Liu et al., 2013a,b
Bamboo shoot peptide preparation	50	20	Liu et al., 2013a,b
Bamboo shoot peptide preparation	100	25	Liu et al., 2013a,b
Porcine gelatin hydrolysate	50	29	O'Keeffe et al., 2017
Canola protein hydrolysate	200	34	Alashi et al., 2014
Rapeseed protein hydrolysate	100	24	He et al., 2013a,b
Casein protein hydrolysate	10	28	Yamada et al., 2013
Dairy whey protein hydrolysate	240	32	Wang et al., 2012
Shrimp protein hydrolysate	900	35	Zhang et al., 2009
Hemp seed protein hydrolysate	200	30	Girgih et al., 2011
Flaxseed protein hydrolysate	200	29	Nwachukwu et al., 2014
Seaweed protein hydrolysate	50	34	Fitzgerald et al., 2014
Boarfish protein hydrolysate	200	37	Hayes et al., 2016
Almond protein hydrolysate	800	21	Wang et al., 2011
Fermented milk	Ad libitum feeding	21	Sipola et al., 2002
Pistachio protein hydrolysate	1000	22	Li et al., 2014

the protein hydrolysates from batch to batch. Some of the mostly commonly-used proteases include alcalase, flavourzyme, neutrase, papain, pepsin, trypsin, chymotrypsin, thermolysin (thermoase), and pancreatin (Aluko, 2015). The enzyme choice is important because each has a unique proteolytic activity, which can determine the type and efficacy of peptides liberated from the protein substrate. Therefore, the degree of hydrolysis (DH) will vary according to enzyme type, hence the activity of resultant protein hydrolysates will differ. DH is determined as the ratio of number of peptide bonds broken by the enzyme (measured as quantity of free amino groups) to the total number of peptide bonds present in the original protein using the pH-Stat (Gong et al., 2017) or colorimetric (Adler-Nissen, 1979; Guan et al., 2018; Nielsen et al., 2001) methods. Thus, in most cases, ACE inhibition will increase as the DH becomes higher until a maximum inhibition level is reached (Hall et al., 2018), beyond which the inhibition could actually decrease (Garcia-Mora et al., 2014; Huang et al., 2014). The decrease in ACE inhibition after maximum inhibitory level has been reached could be due to excessive structural degradation of active peptides when protein hydrolysis (hence DH) proceeds beyond the optimum point. DH will also increase but eventually reach a plateau or even start to decrease with increases in hydrolysis time, substrate concentration and enzyme-to-substrate ratio (Huang et al., 2014; Rutella et al., 2016). However, the DH due to hydrolysis time had better correlation to ACE-inhibitory activity of the hydrolysate when compared to the relationships of ACE inhibition with DH calculated from variations in substrate concentration or enzyme-to-substrate ratio (Huang et al., 2014). Production of small size peptides also increases with length of digestion time (You et al., 2010), which enables formation of low molecular weight peptides that have bioactive properties and are suitable for absorption from the GIT.

One of the most used enzymes for protein hydrolysate production is alcalase because it is a non-specific protease that cleaves at random points with high degree of efficiency and produces smaller-sized peptides than most other proteases. Another commonly used enzyme is flavourzyme, which contains endo- and exoproteinases that are suitable for trimming of peptides to reduce peptide size but the product will have a higher content of free amino acids when compared to other protease preparations. Others include trypsin, which is highly specific for peptide bonds formed by arginine and lysine and will produce longer peptide sequences than alcalase or flavourzyme. In contrast, pepsin and chymotrypsin have high specificity for peptide bonds formed by aromatic amino acids or those with bulky side groups. Thermolysin is fairly unique because of the ability to release peptides that contain branched-chain amino acids. Irrespective of the enzyme choice, protein hydrolysis can be performed using one or more enzymes, depending on the type of target product. Each enzyme reaction must be performed at the optimum temperature and pH in order to realize the full potential of the proteolytic activity. The significance of enzyme choice was evident in the work of Huang et al. (2014), which compared the ACE-inhibitory activity of wheat germ protein hydrolysates produced from six different enzymes (trypsin, neutrase, chymotrypsin, pepsin, alcalase, and papain). The alcalase hydrolysate had the highest ACE inhibition followed by chymotrypsin, neutrase, papain, trypsin and pepsin in decreasing order.

### Single Enzyme Digestion

The simplest hydrolysis protocol involves addition of a single enzyme to the protein substrate, which is usually dispersed in water, heated to the desired temperature and then adjusted to the optimum pH for the enzyme reaction (Ruiz-Ruiz et al., 2013; Senaphan et al., 2018). Some investigators use protein substrates that have been dispersed in buffers instead of water (Aiello et al., 2017; del Castillo et al., 2007; Guan et al., 2018; Hall et al., 2018; Vasquez-Villanueva et al., 2018). The use of buffer has the advantage of resisting pH change during proteolysis and therefore, will require minimal pH adjustment. However, the buffer also introduces

a high level of salt in the final protein hydrolysate, which may not be desirable for taste and could introduce impurities that are originally present in the buffer salts. After the protein has been dispersed in water or buffer with adjusted temp and pH, the enzyme is added at a predetermined level or a range may also be used. Digestion is then allowed to proceed for a predetermined period that could range from 1–24 h. During digestion, peptide release is accompanied by  $H^+$  production, which causes pH to fluctuate from the original set value. In order to maintain the original set pH, dilute acid or alkali is usually added at intervals; this is more frequent during the first 30 min of digestion when proteolysis rate is very high and copious amounts of  $H^+$  are released within a short period of time. However, as digestion proceeds, less amounts of  $H^+$  are released and the pH fluctuation becomes less frequent, hence longer intervals will be required for pH adjustment. It is also important to use a thermostated hot plate to maintain the temperature throughout the digestion period. At the end of the enzyme hydrolysis period, the digest can be cooled to room temperature and freeze-dried as a protein hydrolysate. Using this method, a rice bran protein hydrolysate (RBPH) was produced and tested for cardiovascular benefits in Sprague Dawley rats that developed hypertension after feeding on a high-carbohydrate and high-fat diet. Daily oral administration of 500 mg RBPH/kg rat body weight led to significantly lower systolic and diastolic blood pressure values, which were better than the 250 mg dose (Senaphan et al., 2018). However, rats that consumed the 250 mg dose had significantly lower blood pressure values than those that were not given the RBPH treatment. Oral administration of the RBPH also led to reductions in pulse pressure and heart rate of the rats, which suggest ability of this product to ameliorate hypertension progression.

But post-digestion processing protocols might be used to enhance bioactive quality of protein hydrolysates. First, after cooling to room temperature, the digest is adjusted to a pH value that is close to the isoelectric point (pI) of the protein substrate. At the pI, the protein is mostly insoluble and this causes the undigested proteins to precipitate out of solution and leave behind in solution, the desired short-chain peptides. After adjusting to the pI, the digest is then centrifuged and the supernatant (contains the antihypertensive peptides) is freeze-dried for further testing while the precipitate (contains undigested proteins) is discarded. Second, the digest is heated to  $>90^\circ C$  before cooling to room temperature and freeze-dried or adjusted to the pI, centrifuged and supernatant freeze-dried. Advantage of post-digestion heating is the ability to inactivate the proteolytic enzymes and prevent further protein/peptide degradation that could inactivate already-formed antihypertensive peptides. Heating also enhances coagulation and insolubility of the undigested proteins, which increases efficiency of the subsequent centrifugation step to separate peptides from undigested proteins.

### Multiple Enzyme Digestion

Protein digestion can also be accomplished with the addition of more than one protease either simultaneously or consecutively (Aluko, 2018). For simultaneous hydrolysis, the enzymes must have similar optimum operating conditions, which ensures that each protease will work to the maximum potential as part of the enzyme cocktail. In contrast, if the enzymes have different optimum operating conditions such as pepsin (pH 2.0–3.0) and pancreatin (pH 7.0–8.0), protease addition is done one at a time in a pre-determined sequence. For example, pepsin will first be added and digestion conducted for a given period, then the mixture is adjusted to pH 7.0–8.0 and pancreatin added to continue the digestion. The consecutive use of pepsin and pancreatin (or trypsin) is sometimes used to simulate GIT digestion with or without addition of bile salts; pepsin addition simulates stomach digestion while pancreatin or trypsin is used to simulate intestinal digestion (Aiello et al., 2017; Girgih et al., 2011; Jakubczyk et al., 2017; Pattarayingsakul et al., 2017; You et al., 2010). Using this consecutive digestion method, antihypertensive protein hydrolysates were prepared from hemp seed proteins and shown to reduce SBP of SHR by up to 30 mmHg after 8 h of oral administration (Girgih et al., 2011). The hemp seed protein hydrolysate (HPH) had inhibitory activities against RAAS enzymes with  $IC_{50}$  values of 1.89 and 0.67 mg/mL against renin and ACE, respectively. Mechanism of *in vitro* inhibition was mixed-type, which indicates that the hemp seed peptides were bound to the active and non-active sites of ACE and renin enzyme molecules. During an 8 week feeding trial, the HPH prevented hypertension development in SHR (Girgih et al., 2014a). This long-term feeding trial confirmed that blood pressure reductions were due to reductions in the plasma levels of ACE and renin, which indicate that the HPH peptides were absorbed from the GIT. Canary seed protein was also subjected to pepsin + pancreatin consecutive hydrolysis to yield a hydrolysate with comparable ACE-inhibitory activity as the product made with single enzyme (trypsin) hydrolysis (Estrada-Salas et al., 2014). However, the advantage of pepsin + pancreatin digestion is that the peptides may not undergo further structural degradation within the GIT when ingested. Therefore, preservation of structural properties may enhance the antihypertensive properties of protein hydrolysates produced from pepsin + pancreatin digestion. In a variation of the simulated GIT digestion, pepsin hydrolysis was followed by trypsin hydrolysis to produce ACE-inhibitory peptides that reduced blood pressure of SHR (Guo et al., 2017). Simulated GIT digestion of foxtail millet also produced protein hydrolysates that reduced the systolic blood pressure of SHR (up to 29 mmHg) in addition to significant reductions in plasma ACE activity and angiotensin II level (Chen et al., 2017). The beneficial effect of double enzyme digestion has also been shown with spent hen meat where consecutive treatment with pepsin and pancreatin produced a protein hydrolysate with ACE-inhibitory  $IC_{50}$  value of 0.42 mg/mL when compared to the 0.65 mg/mL for the pepsin-only digest (Udenigwe et al., 2017). More importantly, the protein hydrolysate from pepsin + pancreatin hydrolysis reduced systolic blood pressure of SHR by  $\sim 36$  mmHg, which is significantly better than the  $\sim 27$  mmHg for the pepsin hydrolysate. Casein hydrolysis with thermolysin + alcalase, thermolysin + proteinase K, alcalase + proteinase K and papain + proteinase K all produced protein hydrolysates with stronger ACE-inhibitory activity than when individual enzymes were used (Lin et al., 2018). However, even though consecutive hydrolysis with two enzymes may produce higher DH (Jakubczyk et al., 2017) it does not always lead to the production of stronger ACE-inhibitory peptides as evident in the digestion of egg yolk proteins where an  $IC_{50}$  value of 1.1  $\mu g$  was obtained for single enzyme in comparison to

5.3 µg for 2 enzymes (Eckert et al., 2018). Similarly, casein hydrolysis with thermolysin + papain, alcalase + trypsin and papain + trypsin produced protein hydrolysates with similar or weaker ACE-inhibitory activity when compared to hydrolysates from individual enzymes (Lin et al., 2018). Therefore, the ACE-inhibitory beneficial effect of using 2 enzymes for protein hydrolysis is dependent on the type of enzyme combination.

If a third or more enzyme use is desirable for protein hydrolysis, appropriate pH and temperature adjustments can be made prior to further proteolysis. Irrespective of whether simultaneous or consecutive hydrolysis is conducted with multiple enzymes, the digest is processed using similar protocols as already described for the single enzyme digestion.

### Other Proteolysis Methods

The original discovery of food protein-derived ACE-inhibitory peptides was made during milk fermentation (Nakamura et al., 1995). When administered to SHR, the so called 'sour milk' was found to reduce blood pressure (Nakamura et al., 1995; Sipola et al., 2002). Investigation of the active components led to the discovery of two main peptides, IPP and VPP that were liberated from milk proteins by proteases produced by fermenting microorganisms. A recent work has also shown that milk fermentation with yeast (*Kluyveromyces marxianus*) can produce peptides with ACE-inhibitory activity (Li et al., 2015). VLSRYP and LRFF were the two main peptides identified from the yeast-fermented milk; both peptides inhibited ACE activity in a competitive manner. Camel milk fermentation with lactic acid bacteria led to increased ACE-inhibitory activity of the peptides, which was directly related to higher proteolytic activity as incubation period increased (Solanki et al., 2017). Kefir, another type of fermented milk has been shown to contain ACE-inhibitory peptides (Quiros et al., 2005). Oral administration of the soluble portion of kefir to SHR produced significant reductions in mean arterial pressure and heart rate in addition to a lower plasma ACE concentration (Brasil et al., 2018). Fermentation of pea seed proteins was performed with *Lactobacillus plantarum*, which led to production of ACE-inhibitory peptides (Jakubczyk et al., 2015). Fermentation for 7 days led to the production of pea peptides with an ACE-inhibitory IC<sub>50</sub> value of 0.19 mg/mL. Buckwheat sprout was also fermented with *L. plantarum* and after 2 weeks, the soluble product had antihypertensive effects in SHR at a very low dose of 0.010 mg/kg body weight (Nakamura et al., 2013). The fermented buckwheat sprout caused reductions in ACE activity within several SHR organs, including heart, kidneys, lungs, and the liver. The active compounds in the buckwheat sprout were identified as six main peptides: FQ, VAE, VVG, WIFR, DVWY, and FDART all of which reduced ACE levels in the SHR heart and liver (Koyama et al., 2014). Oral administration of fermented soybean-derived peptides to human subjects produced significant reductions (mean = 9.69 mmHg) when compared to 2.91 mmHg for the control group after 8 weeks (Kwak et al., 2013). However, even though the soybean peptides had *in vitro* ACE inhibition (Shin et al., 1995, 2001), there were no significant changes in the plasma level of this enzyme after the 8 weeks of human intervention trial. Therefore, the antihypertensive mechanism of action of the soybean peptides was attributed to reduction in oxidative stress as evident in the significant increase in superoxide dismutase and decrease in lipid peroxides as represented by malondialdehyde concentration.

Post-mortem muscle hydrolysis can be used to generate peptides with potential antihypertensive properties. For example, cow muscles (*longissimus thoracis* and *semitendinosus*) were aged for 1, 10 and 20 days followed by extraction of generated renin and ACE-inhibitory peptides (Fu et al., 2017). Extracts from raw and cooked muscles were also compared for enzyme inhibition capacity. The results showed significant increases in potency of the muscle-generated peptides against renin and ACE activities as aging progressed from 1 to 20 days. Moreover, cooking also enhanced potency of the peptides, which suggests higher extraction efficiency as a result of temperature treatment. Microbial-fermented sausages also contained peptides with capacity to inhibit ACE activity and could potentially be used as an antihypertensive food (Mejri et al., 2017). Generally, the ACE-inhibitory activity of aqueous extracts of the fermented sausages increased with length of ripening, which suggest time-dependent microbial breakdown of the meat proteins.

### Post-hydrolysis Processing

A crude protein hydrolysate produced from single or multiple enzyme digestion contains several protein fragments, which could be dominated by active or non-active peptides. Therefore, in order to enhance antihypertensive activity, the crude protein hydrolysate can be subjected to post-hydrolysis refining such as membrane ultrafiltration or column chromatography to produce fractions with superior properties. Post-hydrolysis processing is also required for peptide purification that enables isolation of homogenous preparations and identification of amino acid sequence. In all the separation protocols, activity of the fractions are then compared with that of the crude protein hydrolysate in order to determine fractionation efficiency.

### Membrane Ultrafiltration (UF) Processing

Protein hydrolysate separation using UF membranes has become an integral part of the production of antihypertensive peptides. This is because the process is size-based and enables separation of protein hydrolysates into fractions with distinct but broad range of peptide sizes, usually from <1 kDa up to 10 kDa. The process is based on evidence indicating small peptides are more rapidly absorbed than big peptides. Therefore, membrane UF offers the opportunity to collect high amounts of small size peptides and test the effect of peptide size on antihypertensive activity. For example, walnut protein hydrolysate was separated using a 3 kDa membrane into a permeate (<3 kDa), which led to a higher protein recovery (82%) when compared to 18% for the >3 kDa retentate (Liu et al., 2013a). A <3 kDa peptide fraction of pea protein hydrolysate had significant blood pressure-reducing effects during



short-term (24 h) and long-term (8 weeks) oral administration to rats (Li et al., 2011). The <3 kDa pea peptides had *in vitro* inhibitions of ACE and renin but only renin expression was ameliorated during the long-term *in vivo* rat study, which suggest that this product may target renin to lower blood pressure. The ACE-inhibitory activity of egg yolk peptides was improved after ultrafiltration where the >5 kDa fraction had a significantly lower IC<sub>50</sub> value of 0.84 µg in comparison to 1.1 µg for the protein hydrolysate (Eckert et al., 2018). Similarly, a <3 kDa rice peptide fraction had a significantly lower ACE-inhibitory IC<sub>50</sub> value of 0.28 mg/mL when compared to 0.84 mg/mL and 1.57 mg/mL for 3–10 and >10 kDa fractions, respectively (Chen et al., 2013). The <3 kDa peptide fraction of fermented sausage peptides also had stronger ACE inhibition than the >3 kDa peptides (Mejri et al., 2017). Using a papain-digested bovine blood proteins, UF produced a low molecular fraction (<1 kDa) that exhibited stronger ACE and renin inhibitions than high molecular weight (>1 kDa) peptides (Lafarga et al., 2015, 2016). In contrast, the ACE and renin-inhibitory activities of lima bean peptides was not dependent on small size as the >3 kDa fraction was a stronger inhibitor than the <3 kDa fractions (Ciau-Solis et al., 2018).

Since UF is compatible with large-scale production, the process can be used to obtain refined antihypertensive peptide fractions that can be readily commercialized. UF can be conducted using a single membrane, which produces a permeate (flow-through peptides) and a retentate (peptides bigger than the membrane pore size). The permeate and retentate are then tested for antihypertensive effects in comparison to the crude protein hydrolysate. However, narrower peptide size fractions can be obtained by the use of multiple membranes to collect only permeates or retentates (Aluko, 2018). For permeate collection, the process begins with the smallest membrane size, e.g. 1 kDa, which produces a permeate that consists essentially of <1 kDa peptides. The retentate is then passed through a membrane of bigger pore size, e.g. 3 kDa, which will enable collection of permeate peptides with 1–3 kDa sizes. The 3 kDa membrane retentate can then be passed through a 5 kDa to obtain a permeate containing 3–5 kDa peptides; the process can be continued using the 5 kDa retentate with a bigger pore size membrane whose retentate can be processed with an even bigger size membrane and so on until the maximum desirable size membrane has been used. To perform retentate only collection, the process is reversed such that the biggest size membrane is used first (Aluko, 2018). For example, the crude protein hydrolysate will be passed through a 10 kDa membrane and the retentate collected as >10 kDa peptides. The 10 kDa permeate is then passed through a 5 kDa membrane to obtain a retentate that contains 5–10 kDa peptides. The permeate from 5 kDa membrane can be passed through a 3 kDa membrane and the retentate collected as the 3–5 kDa peptides. Passing the 3 kDa membrane permeate through a 1 kDa membrane will then yield 1–3 kDa peptides in the retentate while the final permeate will contain <1 kDa peptides. Irrespective of the membrane process used, the permeates or retentates are analyzed for antihypertensive activities in comparison with the crude protein hydrolysate. Overall, the permeate collection route is more convenient and avoids contamination since the retentate is never collected but simply passed over another membrane. In contrast, if the retentate method is to be used, the ultrafiltration container needs to be washed and cleaned after each collection and prior to setting up the next membrane.

### Column Chromatography Processing

In general, column chromatography is used as a tool for peptide purification rather than to collect peptide fractions that represent the final antihypertensive product. The most commonly used chromatographic methods include reverse-phase HPLC (RP-HPLC), size-exclusion (SEC) and ion-exchange (IEC). In RP-HPLC, the peptides are separated according to net hydrophobicity whereby less hydrophobic peptides are eluted first while strongly hydrophobic peptides come out last. Therefore, the use of RP-HPLC produces peptide fractions with a wide variation in hydrophobicity, which offers the potential of different ability to inhibit ACE activity in comparison to the crude hydrolysate. This was demonstrated by the work of Eckert et al. (2018) who showed that RP-HPLC fractions of egg yolk peptides had significantly lower ACE-inhibitory IC<sub>50</sub> value (~0.024 µg) when compared to 1.1 µg and 0.84 µg for the crude hydrolysate and membrane fraction, respectively. A RP-HPLC fraction of rice peptides had an ACE-inhibitory IC<sub>50</sub> value of 0.02 mg/mL, which was significantly lower than the 0.28 and 0.46 mg/mL for the <3 kDa fraction and protein hydrolysate, respectively (Chen et al., 2013). Hydrophobicity was shown to contribute to renin-inhibitory property of seaweed peptides as the most hydrophobic fraction (last fraction to elute from the reverse-phase column) had the strongest activity (Fitzgerald et al., 2012). The ACE-inhibitory activity of RP-HPLC-fractionated olive seed peptides was also positively related with hydrophobicity because the IC<sub>50</sub> value decreased as retention time increased (Vasquez-Villanueva et al., 2018).

In contrast, SEC separates the peptides according to size with bigger peptides eluting first followed by smaller-size peptides. SEC separation of egg yolk protein hydrolysate produced 5 fractions with the smallest size peptides (fraction #5) having significantly higher ACE-inhibitory activity (Eckert et al., 2018). A similar result showing higher ACE inhibition by smaller-size peptides from SEC separation has been reported for walnut protein hydrolysate (Wang et al., 2014) and bamboo shoot peptides (Liu et al., 2013b). In contrast, the bigger-size peptides from an insect protein hydrolysate (Pattarayingsakul et al., 2017), rapeseed (He et al., 2013b) and fermented bean (Jakubczyk et al., 2017) exhibited higher ACE-inhibitory activity after SEC separation. The results suggest that peptide size alone does not dictate ACE inhibition but the influence of amino acid composition and sequence arrangement is also important. IEC can utilize a positively-charged column resin (anion exchanger) or a negatively-charged resin (cation exchanger). Negatively charged or positively-charged peptides will bind to the anion-exchange or cation-exchange column and be eluted according to their binding strength. Using either RP-HPLC, SEC or IEC methods, fractions are collected at pre-determined intervals based on elution volume or time. The fractions are usually concentrated in a vacuum rotary evaporator, then freeze-dried and assayed for antihypertensive activities using *in vitro* or *in vivo* methods. The most active fraction or



fractions will normally be subjected to further column separations until pure peptides are obtained, which can then be subjected to amino acid sequence analysis, usually involving mass spectrometry protocols.

## Structure and Function of Antihypertensive Peptides

Protein hydrolysates contain a complex mixture of peptides, which can be inactive or active as antihypertensive agents. Therefore, identification of active peptide sequences involves series of separation and isolation protocols that involve one or more of the post-hydrolysis protocols discussed in the preceding section. For example, in order to identify the active peptide sequences responsible for the antihypertensive activities of an alcalase rapeseed protein hydrolysate, samples were subjected to 3 rounds of consecutive RP-HPLC separation (He et al., 2013a). Three main ACE- and renin-inhibitory peptides (LY, TF and RALP) were identified from the rapeseed protein hydrolysate by tandem mass spectrometry. RALP ( $IC_{50}$ , 0.97 mM) was the most active against renin while LY ( $IC_{50}$ , 0.11 mM) had the strongest ACE inhibition. Oral administration of the peptides to SHR reduced SBP by 26, 16 and 12 mmHg for LY, RALP and TF, respectively. Therefore, the results show correlation of *in vitro* inhibition of the RAAS enzymes with actual *in vivo* effects since LY and RALP had the highest inhibitions and renin when compared to TF. In a separate approach, the pepsin + pancreatin hydrolysate of rapeseed protein was first separated by SEC followed by two rounds of RP-HPLC purification of the most active fraction (He et al., 2013b). GHS, a novel tripeptide was subsequently identified by tandem mass spectrometry with  $IC_{50}$  values of 0.52 and 0.32 mg/mL against ACE and renin activities, respectively. GHS also produced antihypertensive effects with a maximum SBP reduction of 17 mmHg reached 6 h after oral administration to SHR. Table 2 shows a list of food protein-derived peptides that have been reported to produce antihypertensive effects through oral consumption. It is evident from the data that there is a wide variation in the efficacy of peptides with those having same chain length differing in blood pressure-lowering effects. Therefore, the structural characteristics, especially arrangement of amino acids seem to be important determinants of ACE inhibition potency and antihypertensive efficacy.

Previous works have used statistical modeling tools to investigate the structural requirements of ACE-inhibitory peptides. Based on partial least square regression modeling, Wu et al. (2006a,b) determined the positional arrangements of peptides containing 2–10 amino acids with respect to potency as ACE inhibitors. Major findings suggest that dipeptides require amino acids with hydrophobic or bulky side groups while tripeptides require an aromatic amino acid in the C-terminal, a positively-charged residue in the middle and a hydrophobic residue in the N-terminal (Wu et al., 2006a). For longer peptides, the C-terminal tetrapeptide residue was determined to be the major determinant of ACE-inhibitory potency but type and arrangement of the four amino acids depend on peptide length (Wu et al., 2006b). This was supported by a subsequent report that found tetrapeptides contain enough structural features to bind to ACE and inhibit enzyme activity because presence of additional amino acid residues did not confer significant inhibition advantage (Zhou et al., 2013). A recent work suggested that hydrophobicity, steric and electronic properties of amino acids of dipeptides are important for ACE inhibition (Deng et al., 2017). Likewise, the C-terminal amino acids contribute more than the N-terminal residues to the inhibition of ACE activity by peptides. The work of Sun et al. (2017) also suggests that the C-terminal residues are important for ACE inhibition, especially when the peptides contain amino acids with hydrogen bond acceptor groups, which facilitates bond formation the enzyme. But as shown in Table 2, the peptide chain length may not be relevant to blood pressure-reducing efficacy. For example, increasing chain length was reported to produce better antihypertensive effects (Tavares et al., 2012). Girgih et al. (2014b) also showed that tetra and pentapeptides had stronger antihypertensive effects than a tripeptide. Meanwhile, DPYKLRP produced almost twice the SBP-reduction of RPYL when orally administered to SHR at the same dose (Garcia-Tejedor et al., 2015). It is possible that the long-chain peptides are broken down into smaller peptides within the GIT or blood such that the resultants fragments are also active, which potentiates antihypertensive activity through synergistic effects. This is illustrated by HLPLP, which was shown to be fragmented into HLPL, LPLP and HLP by rat plasma peptidases, all of which had SBP-lowering effect when orally administered to SHR (Sanchez-Rivera et al., 2016). In contrast, PLP and LPL produced SBP reductions that were similar to HLPLP (Sanchez-Rivera et al., 2016), which suggest that longer chains are not necessarily always more antihypertensive. Apart from peptide chain length, the type of amino acid may also be important for peptides of the same chain length as evident in the higher SBP-reducing ability of VWP when compared to VNP (Chen et al., 2013).

A major issue with bioactive peptides is whether absorption actually occurs as part of the antihypertensive mechanism (Matsui, 2018). However, several works have demonstrated the presence of peptides in the blood of animals (Matsui et al., 2004; Sontakke et al., 2016) and humans (Foltz et al., 2007; Iwai et al., 2005; Ohara et al., 2007; Matsui et al., 2002; Shigemura et al., 2011, 2018) after oral consumption. These findings are not surprising because the presence of peptide transporters within intestinal cells have been confirmed (Ding et al., 2015; Hanh et al., 2017; Meredith and Price, 2006; Xu et al., 2018). Moreover, peptides may also cross the intestinal barrier into blood by diffusion through the paracellular route. Therefore, the ability of an orally ingested peptide to cross into blood circulation is dependent mainly on resistance to gastrointestinal enzymes and not transportability (Matsui, 2018). While detection in the plasma has not been proven for most peptides, there is strong evidence of antihypertensive effects after oral administration to SHR (Table 2). Moreover, various works have also shown that food protein-derived peptides lower activity of RAAS enzyme in the blood. For example, oral administration of HPH to SHR led to >50% reduction in ACE and renin activities in the blood, which were positively correlated to blood pressure reductions (Girgih et al., 2014a). Similarly, the soluble portion of kefir (contains peptides) reduced the serum ACE activity of SHR down to the normal level present in non-hypertensive rats (Brasil et al., 2018). Meanwhile, blood ACE and angiotensin II levels were significantly reduced after oral ingestion of lactoferrin hydrolysate by SHR (Fernández-Musoles et al., 2013). Overall, the presence of peptides within the blood coupled with measured

**Table 2** Blood pressure-reducing properties of typical food protein-derived peptides after oral administration to spontaneously hypertensive rats

<i>Peptide</i>	<i>Dose (mg/kg body wt)</i>	<i>Maximum SBP reduction (mmHg)</i>	<i>References</i>
RYDF	5	10	Guo et al., 2017
YASGR	5	25	Guo et al., 2017
GNGSGYVSR	5	31	Guo et al., 2017
RPYL	10	8	Garcia-Tejedor et al., 2015
DPYKLRP	10	15	Garcia-Tejedor et al., 2015
VNP	5	25	Chen et al., 2013
VWP	5	35	Chen et al., 2013
DY	10	18	Liu et al., 2013a,b
YLVR	10	40	Liu et al., 2018
YLVR	20	34	Liu et al., 2018
YLVR	50	19	Liu et al., 2018
PLP	7	21	Sanchez-Rivera et al., 2016
LPL	7	22	Sanchez-Rivera et al., 2016
HLP	7	15	Sanchez-Rivera et al., 2016
LPLP	7	16	Sanchez-Rivera et al., 2016
HLPL	7	19	Sanchez-Rivera et al., 2016
HLPLP	7	21	Sanchez-Rivera et al., 2016
IRW	3	10	Majumder et al., 2013
IRW	15	22	Majumder et al., 2013
IRLIIVLMPILMA	50	33	Fitzgerald et al., 2014
WYT	30	13	Girgih et al., 2014a,b
WVYY	30	34	Girgih et al., 2014a,b
SVYT	30	24	Girgih et al., 2014a,b
PSLPA	30	40	Girgih et al., 2014a,b
IPAGV	30	36	Girgih et al., 2014a,b
IHRF	15	39	Kontani et al., 2014
DKVGINYW	5	15	Tavares et al., 2012
DAQSAPLRVY	5	10	Tavares et al., 2012
KGYGGVSLPEW	5	20	Tavares et al., 2012
MAW	10	13	Balti et al., 2012
AHSY	10	14	Balti et al., 2012
VYAP	10	22	Balti et al., 2012
VIIF	10	19	Balti et al., 2012
KRVIQY	10	23	Muguruma et al., 2009
VKAGF	10	17	Muguruma et al., 2009

decreases in ACE, renin and angiotensin II levels confirm that most orally-fed peptides are absorbed into the blood circulatory system from where they exert antihypertensive effects.

## Conclusions

Food proteins remain a veritable source of bioactive peptides that could make significant contributions to human health care management and delivery. This is especially the case in developing countries where access to life-saving antihypertensive drugs are limited. Since protein hydrolysis is a fairly straightforward and well-established procedure, bioactive peptides can be readily produced and marketed as antihypertensive peptides. Moreover, the wide choice of protein materials coupled with availability of cost-effective microbial proteases provide the necessary impetus for continued investigation and development of potent antihypertensive peptides. While potency level remains below that of antihypertensive drugs, bioactive peptides can be used at high doses to prevent or suppress hypertension. In this regard, peptides that modulate activities of the RAAS have been proven to be potentially powerful antihypertensive tools. Protein hydrolysates remain the cheapest products that meet commercialization needs but purified peptides offer the potential to develop strong antihypertensive peptidomimetics for the nutraceutical industry. However, there are still several regulatory hurdles that limit widespread marketing and utilization of antihypertensive peptides. To overcome these hurdles, more extensive human intervention trials are required that will confirm utility of bioactive peptides as efficient antihypertensive agents. Moreover, further knowledge of the structural requirements that potentiate peptide-dependent inhibition of RAAS enzyme activities (especially renin and ACE) are required to develop the next generation of antihypertensive food protein hydrolysates and peptides.

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## Anti-Obesity and Anti-Diabetes Foods: High Fibre Diets

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### Glossary

**Acidosis** A condition when the body fluids or tissues become highly acidic

**Dietary fibers** The indigestible part of plants which is fermented by gut bacteria

**Functional food** Food with health-promoting or disease preventing properties

**Inflammation** A condition when parts of the body become red, swollen, painful, and hot, resulting from injury, infection, or allergen contact

**Metabolic syndrome** A group of health conditions: high blood pressure, high blood sugar, excess body fat around the waist, and abnormal cholesterol or triglyceride levels

**Microbiota** Microorganisms inhabiting a particular niche

**Paleo diet** The diet that is composed of fruits, vegetables, nuts, roots, and meat

### Introduction

Metabolic syndrome (MetS), which has surged in the past decades, encompasses a large repertoire of disorders, including obesity and diabetes. Due to the reliance on processed foods, adoption of sluggish lifestyle, the onslaught of pollutants, chemicals exposure, and drug abuse, these MetS health conditions have evolved as major morbidity and mortality factors. It is paradoxical that, while healthcare facilities have risen to unprecedented heights, human quality of life is not necessarily optimal. It is horrifying that the instances of terminal illnesses and premature deaths are soaring. The current situation calls for a scrutiny of lifestyle habits, which might be leading to the dismal picture of public health.

Among the conditions of MetS, obesity and diabetes are most common and often co-occurring, which has led to the terminology diabetes. Obesity is the metabolic disorder of high body mass index (BMI above 30), triggered by rapid adipocyte differentiation. It is a result of imbalance in energy and hormone homeostasis, and other important signaling components such as neurotransmitters. Diabetes is a metabolic, inflammatory, and autoimmune disease. In type 1 diabetes, ionic imbalance occurs due to the malfunction of hormone vasopressin. Perturbation of the renin-angiotensin-aldosterone system (RAAS) affects blood pressure, setting off type 1 diabetes. In type 2 diabetes, the malfunction of insulin raises fasting blood glucose concentration, which causes plasma hyper-osmolality. This hypertonic plasma is responsible for cell shrinkage, mitochondrial depolarization, loss of DNA integrity, cell cycle arrest, apoptosis, protein carbonylation, etc. The RAAS metabolite angiotensin II is a vasoactive peptide that promotes obesity by mediating hypertension, dyslipidemia, and insulin resistance. The adipose tissues secrete adipokines such as leptin (a 16-kDa peptide), and adiponectin, which fuel obesity. Once MetS starts to manifest, the vicious cycle continues, causing cancer, neuropathies, cardiovascular complications, and other inflammatory conditions. As the pathological mechanisms of diseases are being studied with advanced tools, their common origin is emerging. The links between obesity, diabetes, bipolar disorder, asthma, autism, endometriosis, polycystic ovarian syndrome (PCOS), and viral infections have emerged. All of them originate from the route of 'stressor-inflammation- acidosis – aberrant enzyme activation – disturbed immune-neuro-endocrine axis-pathologies'.

Drugs from different classes target different components of the human body, and evoke undesirable side effects as well. There are several anorectic anti-obesity drugs in the pharmaceutical market. Orlistat, an agonist at 5-HT<sub>2C</sub> (5-hydroxytryptamine or serotonin) receptors can also inhibit intestinal lipase to prevent fat absorption. The glucagon-like peptide-1 (GLP-1) analog liraglutide is another drug marketed for the management of obesity. However, liraglutide shows adverse effects like nausea, headache, and diarrhea. Other anti-obesity drugs like sibutramine (a serotonin and norepinephrine reuptake inhibitor) and rimonabant (a selective CB1 endocannabinoid receptor antagonist) cause cardiovascular, neuropsychiatric and congenital issues. The diabetes drug metformin has adverse effects like lactic acidosis, nausea, arrhythmia, etc. With the easy accessibility of drugs, multiple drugs are being taken by a patient, so adverse reactions are emerging. As public awareness towards the limitations and deleterious effects of drugs is rising, the interest towards alternative solutions is increasing. Western diet is replete with high glucose, saturated fats, and chemical additives. With the evidences of its association to multiple metabolic diseases, the deficiencies with Western diet are being recognized. Acidogenic attribute of dairy products, meat-based, and processed foods are coming forth. The acidosis caused by the high carbohydrate, high fat, highly-processed diet leads to aberrant posttranslational modifications of proteins and dysregulated enzyme activity (Patel, 2017). Epigenetic changes such as glycosylation, methylation, acetylation, phosphorylation, and sulfation of proteases such as serine proteases, metalloproteinases (MMP), and cysteine proteases (cathepsins) have been linked to diabetes, pancreatitis, cancer, neurodegenerative diseases, cardiac, and renal complications. The additives such as preservatives (e.g. potassium metabisulfite, benzoic acid, sorbic acid) in processed foods are known to



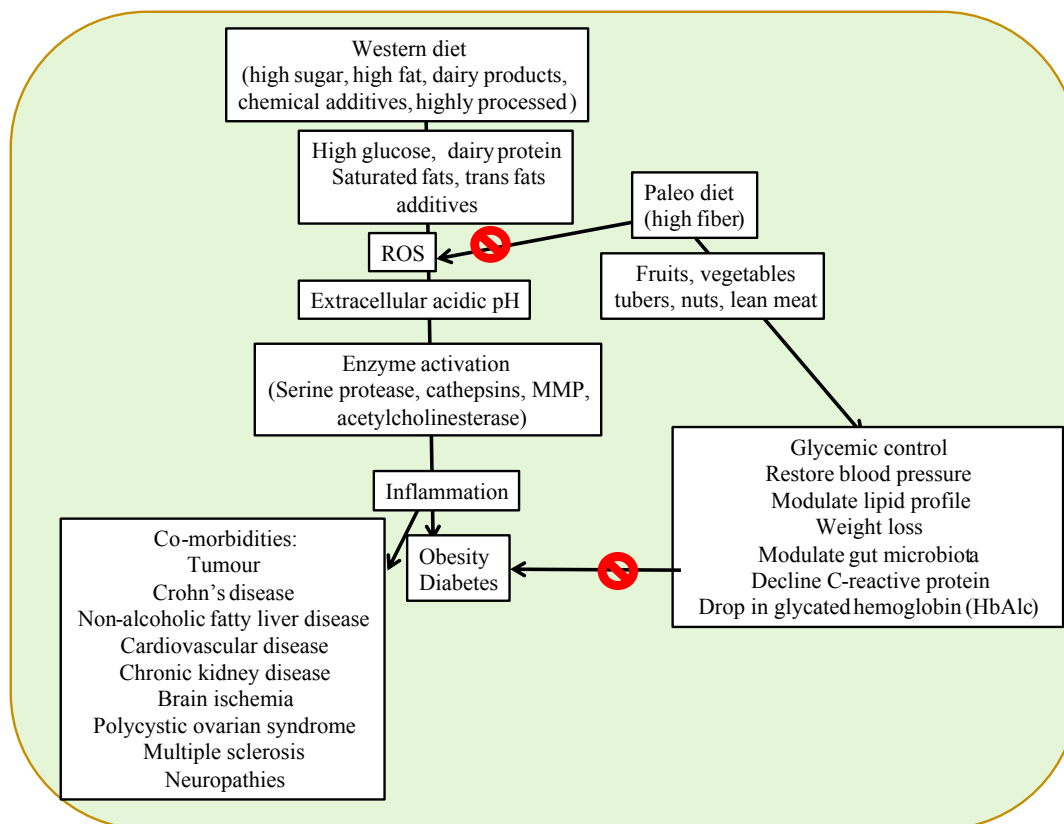
manipulate acetylcholinesterase. Some chemicals act as estrogen mimics, rendering the body susceptible to carcinogenesis. Cyclooxygenase-2 (COX-2), aromatase, kinases (MAPK, etc.), glycosidases, urease, and phospholipases are other crucial enzymes that are affected by the acidic milieu in the body. Acidic pH prevents the polymerization/depolymerization of actins, which are the ubiquitous proteins in the cytoskeletons, thus affecting vesicular trafficking, angiogenesis, autophagy, innate immunity, among a number of other crucial aspects. The hypoxic, acidic condition also favors the expression of efflux proteins such as p-glycoprotein (P-gp), and the cancer-associated G-protein-coupled receptor (GPCR) proteins such as OGR1 (ovarian cancer G-protein-coupled receptor 1), GPR4 (G-protein-coupled receptor 1), and TDAG8 (T-cell death-associated gene 8). P-gp, encoded by the ABCB1 gene, is overexpressed in cancers and infectious diseases, and it can eject drugs such as anticancer agents, antibiotics, antidepressants, reducing their efficacies. Due to the modified pH, sodium, potassium and calcium channels, which maintain ionic homeostasis, are manipulated, leading to pain, edema, and a range of other issues. Acidic pH is sensed as a stressful condition, and in retaliation, immune activation, neutrophil infiltration, platelet reactivity, platelet aggregation on collagen, etc. take place.

## Dietary Fibers

Incorporating functional ingredients in the diet to alleviate obesity and diabetes has spurred research in nutraceuticals. Dietary fiber encompasses polysaccharides (cellulose and hemicellulose), oligosaccharides, lignin, pectin, gum, wax, and mucilages and assorted phytochemicals. Fiber-rich foods are mostly from plant origin, though algae and fungi-based foods are fiber-rich as well. Seaweed polysaccharides (Patel, 2018) and mushroom beta glucan (Pillai and Uma Devi, 2013) have earned reputation as bioactive components. The inclusion of dietary fiber in food has been verified to ameliorate obesity and diabetes (Anderson et al., 2009). The total dietary fiber can be soluble or insoluble type. Soluble dietary fiber which encompasses oatmeal, beans, apples, nuts, and blueberries, can lower cholesterol level and blood pressure, can enhance mineral absorption, and can prevent gastrointestinal inflammations. Insoluble dietary fiber occur in cereal brans, whole grains, fruit peels etc. and on ingestion they prevent constipation, and hemorrhoids. The mucilage and oligosaccharides are substrates for gut bacteria that metabolize them into short-chain fatty acids (SCFA). Cereal brans that are rich in dietary fibers have been convincingly proven to confer satiety (Patel, 2015). Nutritional value of rice bran oil with tocotrienols, oat  $\beta$ -glucan fiber, anthocyanin in pigmented barley, the bran of wheat, sorghum, corn, barley etc. have been reported. Arabinoxylan, pectin, inulin, and resistant starch are other active components of dietary fibers with proven biological functions. Mediterranean diets dominated by olive oil, fruit, vegetables, and whole grain cereals are deemed beneficial for the patients with type 2 diabetes and high cholesterol. Fermented foods are well-regarded for their health attributes (Patel and Shukla, 2016). Kimchi, the fermented cabbage, was found to be low in calories, high in vitamins, minerals and phytochemicals, apart from dietary fiber. Red yeast rice, the mold *Monascus purpureus*-fermented product, owing to its polyketide monacolin K, apart from fiber content, has been shown to lower blood glucose level, by promoting insulin secretion (Patel, 2016). Fiber-rich oligosaccharides are important components of prebiotics, administered to promote the proliferation of probiotics in human gut (Patel and Goyal, 2010; Kothari et al., 2014). Paleo diet, the food habit of the human ancestors in the Paleolithic Era, which was dominated by plant products, has garnered much attention for their claimed and proven health benefits (Patel and Suleria, 2017). Several studies have confirmed their beneficial effects. The polyphenolic phytochemicals in the paleo diet lower acidity, maintain electrolyte balance and restore homeostasis. This diet improved glycemic control (glucose tolerance, insulin level) and cardiovascular risk factors (glucose tolerance, insulin level) in type 2 diabetes patients (Klonoff, 2009). Inflammation markers such as C-reactive protein (CRP) and glycated haemoglobin (HbA1c) were measured to be low in the paleo diet group. Paleo diet increased incretin (the hormone stimulating insulin secretion), anorectic gut hormone (glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), which confer satiety feeling. The levels of interleukin-6 (IL-6), IL-1 $\beta$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and inducible nitric oxide synthase (iNOS), known to be inflammation mediators, were reduced following paleo diet consumption. Fiber-rich foods attenuate inflammation and thus protect the insulin-secreting islets of Langerhans from oxidative stress. This diet also manipulates HMG-CoA reductase and sterol response element binding protein-2 (SREBP-2), reducing adiposity and tumor risks. Dietary fibers are fermented by gut bacteria, which generate SCFA such as acetate, and butyrate. The fibers improve microbiota composition, prevent constipation, reduce toxin generation (relieves the load on the liver and kidneys), and enhance vitamin B absorption from the gut. Also, they play role in modulating intestinal viscosity, passage frequency, and the production of gut hormones. Fig. 1 illustrates the variations in physiological effects of fiber-deficient Western diet and fiber-rich paleo diet.

The incorporation of dietary fibers of diverse sources such as algae, fungi, mushrooms, and underutilized plants have been endorsed by recent research findings. Fig. prunes, blueberry, barley, chicory root extract, flax seed, oat fiber, acacia fiber, apple pectin, and psyllium husks are increasingly used in diverse food products. *Amaranthus* flour has dietary fiber in a high amount (53.81 g/100 g), 79% of which is insoluble. The healthy property of cruciferous vegetables is attributed to the high content of fiber and phytochemicals such as glucosinolate. Phytochemicals are often associated with dietary fibers. Some of the phytochemicals such as berberine, catechins, curcumin have been consistently verified to be health promoters.

In recognition of the multiple health benefits of fiber-high diet, novel sources of dietary fibers are being investigated. Such research efforts has unveiled the potential of bamboo shoot, sugarcane, defatted strawberry seeds, soybean residue (okra), grape



**Figure 1** An illustration of the physiological effects of fiber-deficient Western diet and fiber-rich paleo diet. Western diet is acidogenic and inflammatory, which favors obesity and diabetes.

pomace, mango peel, date palm fruits, cassava root, agave bagasse, pumpkin seeds, coffee silverskin, grains (triticale), and pseudo grains (quinoa) to be included in food. The inclusion of phytochemicals such as quercetin with dietary fiber in nano-formulations has also been evaluated.

### Health Promotion Mechanisms of Dietary Fibers

Multiple studies have proven the mechanisms by which dietary fiber-rich plant diet lowers obesity and diabetes risk. Some prominent pathways have been mentioned below. Ghrelin hormone secreted by empty stomach induces hunger. Bulking agents quench hunger and provide satiety. Dietary fiber is difficult to digest, so its transit through the gut is slow, which gives a sensation of fullness. Satiety prevents further food intake, which helps in weight control. In studies, rice bran improved insulin resistance and wheat bran lowered glycemic index. Oat  $\beta$ -glucan fiber-based breakfast attenuated insulin secretion and improved the peptide hormone cholecystokinin. The metabolism of fiber-rich diet is likely to be generating metabolites, which favorably influence the anorexigenic pro-opiomelanocortin (POMC) neurons and the orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons of hypothalamus. Studies have shown that the blockade of CB1 cannabinoid receptor induced weight loss and improved metabolic parameters. The fiber metabolites might be able to block the above receptors. SCFA generated by dietary fiber metabolism by gut bacteria ameliorated inflammation in animal model of gout, by augmenting the production of anti-inflammatory mediators such as IL-10, TGF- $\beta$ , and annexin A1. The roles of dietary fibers in alleviating gastroesophageal reflux disease, ulcer, colon cancer, and hemorrhoids have also been observed. Therefore, the health effects of fibers have been proven to be multifarious.

### Food Processing and Its Impact on Dietary Fibers

Now that the health benefits of dietary fibers have been validated through different experimental models, they are aggressively marketed as panacea. A wide range of food products such as chocolate, dairy milk shakes, pastries, ice cream, pasta, pizza, yogurt, and cereals are now fortified with dietary fiber. Consumers are enticed by this labelling, but, there lies a lacuna, which gets underreported. Dietary fibers have no taste; therefore food manufacturers make them appetizing by adding sugar and other ingredients

like cheese, dairy products, egg, monosodium glutamate (MSG), etc. For aesthetic appeal and texture, leavening (sodium aluminium phosphate), vinegar, artificial color, and carrageenan etc. are added to the fibre-enriched products. For longer shelf-life of the products, preservatives are added. If a product has high amounts of dietary fiber but has sodium benzoate and antibiotics, the product is still proinflammatory. With these combinations, do the benefits of dietary fiber remain intact? Also, the effects of processing techniques on the dietary fiber are not well-investigated. Techniques like canning, grinding, frying, drying, freezing, microwave treatment, extrusion-cooking, and boiling, modify the physico-chemical properties of dietary fiber. If vegetable chips marketed as fiber-rich are fried in oil and are heavily salted, the benefits of fiber are questionable. Similarly, trading canned vegetables as fiber-rich is lost in its preservatives, and the use of catchy names is not going to make the product healthy. Modern consumers should be able to see through these gimmicks and ought to make appropriate dietary decisions.

### Adverse Effects of Dietary Fibers

Also, the anti-nutrient aspect of dietary fibers has not been investigated well. Oxalic acid associated with fibers might interfere with iron absorption. Also, other components such as phytic acid, nitrate, lectins, hemagglutinin, trypsin inhibitor,  $\alpha$ -amylase inhibitor, alkaloids, and saponin contents ought to be determined. Glucosinolates in cruciferous plants are goitrogenic glycosides, which meddle with thyroid function. So, high fiber diet and minimal processing entail these negative aspects. Though the relevance of dietary fiber in constipation is proven, depending on the fiber type, and the host metabolic status, fibers can promote constipation and related symptoms (bloating and abdominal discomfort). The causes have been explained to be due to the fermentation of dietary fiber by colonic bacteria, resulting in the production of carbon dioxide, hydrogen, methane, and other gases.

Food crops like the legumes have antinutrient oligosaccharides such as stachyose and raffinose, which are fermented by colon microflora to release gas and cause abdominal distension. Other plant products such as pumpkin and watermelon seed flours that are known to be high in dietary fiber can also cause bloating. Excess consumption of dietary fiber can obstruct the intestinal tract and cause bezoar, a gastrointestinal mass of hardened undigested materials. Pomegranate seeds, sunflowers seed, pumpkin seed hull, persimmon peel etc. have been frequently associated with bezoar formation. Phytobezoar formation is more likely in older individuals and patients with weak digestive system. So, in these groups, high-fiber diets might not be too beneficial. Depending on the severity, bezoar removal can require surgical intervention. In the existing science literature, often the negative attributes are overlooked and the positive effects are overemphasized. So, it is very important that the consumer is aware of the complete picture.

### Discussion

Drugs are not the solution for all diseases, not especially for chronic metabolic diseases. Every drug furnishes its therapeutic effects via enzymes such as cytochrome P450, which also synthesize steroids. So, any therapeutic intervention manipulates hormone as well, which is not risk-free. So, expecting medications to shed weight, or restore glucose or ionic balance, is naïve. General public should be aware that drugs are synthetic compounds, which save lives in serious health conditions, but they must not be used in a routine manner. Radical lifestyle modifications and ingestion of fiber-based food may be used to address the rising epidemic of obesity and diabetes, both of which are tied to the common cause of inflammations, originating due to different stressors, including acidogenic food. The inclusion of fruits, vegetables and whole grains in diet, while cutting down on acidogenic, and inflammatory agents is a recommendable antidote against chronic conditions like obesity and diabetes. The exploration of edible dietary fiber from underutilized sources, and developing technologies to incorporate them in diets can be an effective way to keep MetS away. The type of fiber, dosage ingested, and host genotype are the factors, which determine the safety and efficacy of the fibers in diet. Furthermore, in a pragmatic vision, it can be stated that the importance of fiber for alleviating metabolic diseases is only one approach, which can go null if lifestyle abuse in other forms continues. Adherence to a chemical-free lifestyle can improve the beneficial prospects of high-fiber diet.

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### Relevant Website

<https://www.hsph.harvard.edu/nutritionsource/carbohydrates/fiber/>.

# Protease Inhibitors

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## Introduction

Protease inhibitors (PIs) are natural or synthetic compounds that inhibit the activity of proteases. Most natural protease inhibitors are small proteins (Liener, 1989). They are ubiquitous in living organisms and play a significant role in many biological processes by regulating proteolytic functions of their target enzymes (Liener, 1980). PIs occur abundantly in many food plants such as legumes, cereals, tubers and oil seeds (Ryan, 1990; Liener, 1994). Plant PIs were investigated as early as 1938, when Read and Haas recognized the presence of an inhibitor in soybean (Liener, 1980). The fraction of the functional protein was then partially purified by Bowman, as well as Ham and Sandstedt, followed by its subsequent isolation in crystalline form by Kunitz (Kunitz, 1947; Liener, 1980). Because of their ability to inhibit proteases including those in the human digestive system and reduce protein bioavailability, PIs are long regarded as anti-nutrients in foods such as soybean. On the other hand, it is increasingly recognized that PIs may have a positive contribution to human nutrition and health. Studies show that plant PIs, especially Bowman-Birk type inhibitors (BBI), may have the capacity to prevent and mitigate a number of diseases such as cancer, multiple sclerosis, inflammation and Duchenne muscular dystrophy (Losso, 2008; Clemente et al., 2011; Kobayashi, 2013; Hernández-Ledesma and Hsieh, 2017).

## Classification of Protease Inhibitors

In the most recent release of the MEROPS database (Release 12.0, September 2017), 134011 PI sequences were identified (Rawlings et al., 2018). This is an increase of 59353 sequences over the July 2015 release (Release 9.13), which is a testimony to both the vast number of PIs in nature and rapid increase in their identification (Rawlings et al., 2016, 2018). These sequences are grouped into 82 families based on their sequence homology, which are further clustered into 39 clans based on similarity in their tertiary structure. Only about 725 of the PIs, a fraction of the total number, were experimentally characterized (Rawlings et al., 2018). These PI families are found to be specific in their inhibitory action against the five major mechanistic classes of proteolytic enzymes and are classified accordingly as serine, cysteine, aspartic, metalloprotease and threonine protease inhibitors (Lehninger et al., 1993; Whitaker, 1997; Shewry, 2000; Laskowski and Qasim, 2000; Lawrence and Koundal, 2002). Serine and cysteine PIs are the largest groups and also the most extensively studied, partly because of their reputation as major antinutritional factors in plant storage tissues such as seeds and tubers. In some legumes, such as soybean, PIs may account as much as 10% of the soluble proteins (Whitaker, 1997; Mandal et al., 2002).

Serine PIs are the most extensively studied protease inhibitors. They are strictly competitive inhibitors forming 1:1 complexes with the enzymes they inhibit (Batista et al., 1996; Mello et al., 2001; Bhattacharyya et al., 2006, 2007; Macedo et al., 2007). The major common elements in the structures of serine PIs are their reactive site peptides, which generally comprise 10 – 15 amino acid residues. Their specific nature strongly affects both the strength and the specificity of enzyme–inhibitor interaction (Bode and Huber, 1992; Laskowski and Qasim, 2000). These reactive site peptides adopt a conformation that is complementary to the surface of the target enzymes and resemble the conformation of a substrate bound to the active site. Therefore, a range of conformations are found in different inhibitor families. Plant serine protease inhibitors have been identified from various leguminous seeds (Batista et al., 1996; Mello et al., 2001; Garcia et al., 2004; Bhattacharyya et al., 2006, 2007; Macedo et al., 2007), which are able to inhibit serine proteases *in vitro* with the optimum inhibitory activity at pH 9 – 11 range (Lawrence and Koundal, 2002).

The Kunitz-type soybean trypsin inhibitor (SBTI) is a typical serine PI. It consists of a single polypeptide chain with molecular weight in the range of 18–24 kDa. The protein structure is cross linked by two disulfide bonds which are located at Cys 136–145 and Cys 39–86, and its reactive site is located at Arg 63 and Ile 64 (Liener, 1980, 1989; Norton, 1991; Liu, 1997). The isoelectric point (pH 4.5) and the dissociation constant ( $K_i = 3.2\text{--}6.2 \times 10^{-9}$ ) of SBTI at pH 8.0 were reported (Deshimaru et al., 2002). They are quite resistant to denaturing reagents such as urea and guanidinium chloride; however, the rupture of both disulfide bonds leads to inactivation (Liener, 1980). Apart from legumes, Kunitz-type PIs have been widely found in cereals and solanaceous species (Magdalena, 1996; Habib and Fazili, 2007).

BBIs are another major family of serine PIs that have a molecular weight of 8–10 kDa with high cysteine but lack glycine and tryptophan contents and consist of two homologous domains each bearing a separate reactive site. They are widely distributed in monocot and dicot species (Richardson, 1991). BBIs from monocotyledonous plants are of two types. One group (8 kDa) consists of a single polypeptide chain with a single reactive site and another group (16 kDa) has two reactive sites (Habib and Fazili, 2007). In soybean, BBI is a relatively small molecule with a single polypeptide chain of 71 amino acids with seven disulfide bonds (Liener, 1989). It exhibits marked stability toward heat, acid and alkali; even after the disulfide bonds are broken by heating it still has stable conformation (Liener, 1989; Liu, 1997). BBI binds to trypsin and chymotrypsin at independent sites – a trypsin-reactive site (Lys 16–Ser 17) and a chymotrypsin-reactive site (Leu 44–Ser 45) with reported dissociation constant ( $K_i$ ) of  $2.8 \times 10^{-10}$  and  $2.9 \times 10^{-8}$ , respectively (Liener, 1980; Liener and Seidl, 1972).

Cysteine proteases catalyze the hydrolysis of peptides through a thioester intermediate. Common proteases in this group include papain, calpain and lysosomal cathepsin. These proteases are widespread in nature and so are their inhibitors. Their optimum

inhibitory activity usually occurs in the pH 5 – 7 range (Lawrence and Koundal, 2002). Egg white cystatin and rice cystatin are classic examples of cysteine PI (Gomes et al., 1997; Lawrence and Koundal, 2002; Oliveira et al., 2007). Aspartic proteases include human immunodeficiency virus (HIV) proteases, angiotensin converting enzyme, carboxypeptidases, pepsin and renin. Aspartic PIs have been isolated from sunflower, barley, cardoon (*Cyanara cardunculus*) flowers and potato tubers (Lawrence and Koundal, 2002) as well as animal tissues. There are two families of metallo-protease inhibitors, namely metallo-carboxypeptidase inhibitor family in potato and tomato plants, and a cathepsin D inhibitor (27 kDa) family in potato tubers (Lawrence and Koundal, 2002). Compared with other proteases, threonine proteases are studied not as extensively and their inhibitors have not been widely reported in foods.

## Inhibition Mechanisms

An enzyme inhibitor is a molecule that decreases the measured rate of enzyme-catalyzed hydrolysis of a given substrate. Protease inhibition usually applies to the competition between two substrates in a reaction with an enzyme (protease) where one can decrease the apparent rate of hydrolysis of the other (Nagase and Salvesen, 2001). The mechanism of enzymatic inhibition can be considered both kinetically as how it affects the efficiency of enzyme–substrate interactions and molecularly to examine how the inhibitor protein, especially its active site, interacts with the substrate at molecular level. From the kinetic standpoint, there are two types of inhibition – reversible and irreversible. Reversible inhibitors fall into three categories – competitive, uncompetitive and non-competitive. Non-competitive inhibitors can be further divided into two additional categories which are pure and mixed inhibitors (Lehninger et al., 1993).

### Competitive Inhibition

In competitive inhibition, the substrate (S) and the inhibitor (I) compete for the same active site of the enzyme (E), but only the substrate molecule completes the catalytic reaction. The inhibitor binds to E to form E–I complex and thus blocks the active site, but no catalytic reaction will occur. Since the inhibitor and substrate compete for the same site, raising the substrate concentration can fully dislodge the inhibitor from the active site (Lehninger et al., 1993). All serine inhibitor families from plants are competitive inhibitors (Lawrence and Koundal, 2002) and most of the PIs interfere with their respective proteases through a similar mechanism, known as the conical (standard) mechanism (Rolka et al., 2013). These inhibitors are characterized by a number of common features: small molecular weight (1.5–2.1 kDa) with compact structures that are stabilized by several disulfide bridges and rather resistant to thermal and chemical denaturation. Also, they all have an exposed short primary binding segment of less than 10 amino acid residues, also known as the “binding loop”, which is responsible for direct interaction with the target enzyme (Bode and Huber, 2000). While most inhibitors have a single binding loop, some contain multiple such motifs, which allow them to interact with and inhibit more than one protease simultaneously. As studied extensively by Laskowski and co-workers (Laskowski and Kato, 1980; Laskowski and Qasim, 2000; Rolka et al., 2013), the PIs act by forming a stable complex with the target protease through a classic “lock and key” mechanism where the inhibitor binding loop inserts into the active site of the enzyme. Unlike the S–E interaction, however, the I–E complex dissociates very slowly, thus blocking access of the substrate to the site (Silverman et al., 2001). The enzyme cleaves the inhibitor in a similar fashion to that of the substrate, but at a much reduced speed, and leads to a modification of the inhibitor. As the incubation progresses, the amount of the modified inhibitor will continually accumulate until equilibrium is reached with the intact inhibitor (Rolka et al., 2013). Soybean Kunitz-type trypsin inhibitor (SBTI) is a classic example of competitive inhibitors.

### Other Types of Inhibition

**Uncompetitive inhibition:** Uncompetitive inhibitors do not compete for the active site with the substrate; they only bind to the E–S complex and form an enzyme-substrate-inhibitor complex (E–S–I). This can occur as a result of conformational changes in enzyme structure that accompanies substrate binding, opening up a previously occluded site. The binding of an inhibitor to the E–S complex leads to a decrease in the concentration of the E–S complex, thus accelerating the affinity of the enzyme for the substrate to form E–S complex. By Le Chatelier’s principle, the equilibrium of the enzyme and substrate will shift to form more E–S complex in the presence of an uncompetitive inhibitor (Lehninger et al., 1993). This type of inhibition is rarely found in single-substrate reactions, and usually occurs in two-substrate reactions (Belitz and Weder, 1990). It has not been reported for any leguminous protease inhibitors.

**Non-competitive inhibition:** Non-competitive inhibitors bind at the site distinct from the substrate active site and leave the active site unblocked. Therefore, the inhibitor can react equally with free enzyme or with the E–S complex. However, the E–S–I complex cannot convert the substrate into product. Furthermore, non-competitive inhibitors can be divided into two additional categories which are pure and mixed inhibitors. In pure non-competitive inhibition, the substrate has an identical affinity for both the free enzyme and the E–I complex. However, with mixed non-competitive inhibition, the affinity of the E–I complex for the substrate is not the same as the free enzyme (Lehninger et al., 1993). Therefore, non-competitive inhibition cannot be overcome by high concentrations of substrate (Belitz and Weder, 1990).

**Irreversible inhibition:** Irreversible inhibitors usually covalently modify an enzyme and, therefore, the inhibition cannot be reversed. Electrophilic groups of the inhibitor, such as aldehydes, haloalkanes or alkenes, react with the enzyme’s side chains



containing nucleophiles, such as hydroxyl or sulfhydryl groups, including serine, cysteine, threonine or tyrosine (Lehninger et al., 1993). The irreversible inhibitors form a reversible non-covalent complex as E-I or E-S-I complex and then produce a “dead-end complex”. The reaction cannot be reversed by diluting the reaction medium.

## Biological and Health Functions of Protease Inhibitors

Plant PIs can both positively and negatively impact human nutrition and health. Plant PIs are well documented antinutritional factors. They exhibit strong inhibitory activity against trypsin and chymotrypsin, by forming stable complexes in the digestive tract, thus causing a decrease in the ability of body to utilize proteins in foods (Liener, 1980, 1989; Norton, 1991; Vagadia et al., 2017). Prolonged consumption of food with high levels of active PIs may lead to protein deficiency, which can be especially serious in populations with insufficient supply of high protein foods. However, plant PIs are relatively heat labile (although they are usually more stable than most other proteins) and can be easily inactivated by either dry or moist heat treatment such as roasting, steaming and many of other methods of cooking (Deshpande, 1992; Liener, 1994; Vagadia et al., 2017). In most societies where legumes are consumed as a traditional staple, people have developed methods such as soaking and boiling of soy beans, usually by trial and error, to inactivate the PIs (Vagadia et al., 2017).

On the other hand, PIs can also play a number of significant positive roles in agriculture and human health. One of the early recognized beneficial functions of plant PIs is their effect on plant pathogens. As early as in 1947, Mickel and Standish observed that the larvae of certain insects were unable to develop normally on soybean products (Lawrence and Koundal, 2002). Since then, plant PIs have been investigated extensively for their potential use as bioinsecticides and fungicides (Macedo et al., 2003; Haq et al., 2004; Abdeen et al., 2005; Park et al., 2005; Habib and Fazili, 2007). Numerous PIs purified from legumes, cereal seeds and tubercles, especially those belonging to the Kunitz and Bowman-Birk families, have been found to play a significant role in plant defense against insect and phytopathogen attacks by interfering with the digestive enzymes of the pests (Richardson, 1991; Bode and Huber, 2000; Macedo et al., 2004; Bhattacharyya et al., 2007; Oliveira et al., 2007). Studies on the insecticidal activity of PIs from black-eye pea (Oliveira et al., 2002) and *Pithecellobium dumosum* seeds (Oliveira et al., 2007) suggest that the digestive system of Lepidopterans and Dipterans is mainly based on serine proteases whereas cysteine proteases are predominant in Coleopterans of the Bruchidae family. It has also been reported that cowpea vicilins (7S globulins) provide resistance to a range of invertebrate pests and the germination of fungal spores (Gomes et al., 1997).

Proteolysis is one of the most prevalent mechanisms of biological regulation and it is involved in many fundamental physiological processes. Aberrant functioning of certain proteases is well recognized as an underline cause of many pathological disorders such as cancer, angiogenesis, inflammation, neurodegenerative and cardiovascular diseases (Heutink et al., 2010; Clemente and Arques, 2014). As one of the regulating mechanisms of proteolysis, it not surprising that PIs may play a role in mitigating these disorders. Interest in the health functions of plants PIs gained special momentum following the discovery that certain PIs from legumes are effective in preventing or suppressing carcinogenic processes in a wide variety of *in vitro* and *in vivo* model systems and they might be used as chemopreventive agents for certain cancers (Kennedy, 1998a, 1998b). Of all the PIs, the health effects of BBI are the most intensely studied and the subject has been extensively reviewed (Kennedy, 1993a, 1993b, 1993c, 1994, 1995, 1998c, 2007, 2009, 2013; Kennedy and Wan, 2002; Morris et al., 2005). Numerous pharmacological effects and health functions have been reported for BBI, including antitumor and cancer preventive effects, anti-inflammatory activities, prevention of multiple sclerosis and Duchenne muscular dystrophy (DMD), as well as a number of other benefits such as protection against radiation-induced birth defect, hair and weight loss in cancer patients, improved efficacy of certain chemotherapeutic drugs and life-span extension (Armstrong et al., 2000, 2003; Morris et al., 2005, 2010; Gran et al., 2006; Arbogast et al., 2007; Touil et al., 2008; Dai et al., 2011; Leung et al., 2013; Clemente and Arques, 2014). Recent *in vivo* studies using animal models indicate that daily administration of BBI concentrate (BBIC) at 200 mg rat<sup>-1</sup> for 16 days can effectively inhibit and ameliorate clinical and pathological symptoms of experimental autoimmune neuritis (EAN) - an animal model of Guillain-Barré syndrome (GBS), suggesting that BBIC may have therapeutic effects in human GBS (Jin et al., 2016).

With regard to the antitumor properties, BBI has been reported to exhibit therapeutic effects against colorectal cancer, oral leukoplakia, prostate and breast cancers in a number of *in vitro* as well as *in vivo* studies using cancer cell lines and animal models (Kennedy, 1993a, 1993b, 1993c, 1994, 1995, 1998c, 2007, 2009, 2013; Kennedy and Wan, 2002; Morris et al., 2005; Chan et al., 2013). At the present the therapeutic targets of BBI have not been identified and the action mechanism is yet to be elucidated. Most of the exciting evidence suggests that BBIs exert their preventive effect through protease inhibition in carcinogenesis (Clemente and Arques, 2014). In one of the studies (St Clair et al., 1990), colon carcinogenesis in DMH-treated mice was suppressed when purified and semi purified soybean BBIC was administrated at 0.5% of the diet or less for 20 weeks. When the protease inhibitory activity of the BBIC was inactivated by high temperature treatment, such suppression on DMH-induced colon carcinogenesis was not observed, suggesting that the chemopreventive effect of BBI is associated with its ability to inhibit serine proteases. In another study, soybean BBI in the concentration range of 0–125 µM was found to cause a significant, dose- and time-dependent decrease in the proliferation of human colorectal adenocarcinoma cells, whereas BBI inactivated by alkaline and reducing agents was unable to exert such an effect (Clemente et al., 2010). Studies using BBI from mutant pea seeds that do not possess trypsin and chymotrypsin inhibitory activity, and BBI whose activity have been destroyed by chemical scission also demonstrate the linkage between the anti-proliferation activity of plant BBI and its protease inhibitory activity. These works suggest that trypsin- and chymotrypsin-

like proteases involved in the early stages of carcinogenesis are likely the targets of BBI for the therapeutic effects (Yavelow et al., 1985; Clemente et al., 2012).

A number of the studies have progressed to clinical trials. However, while the results of tissue culture and animal model studies have been generally promising, results of clinical trials are mixed. For example, in a Phase IIa clinical trial on the effect of BBIC on oral leukoplakia, it was found that about one-third of the subjects receiving BBIC at 200–1066 chymotrypsin inhibitor units (CIU) for one month showed a dose–response relationship in decreased lesion size (Armstrong et al., 2000). However, in the subsequent randomized, placebo controlled phase IIb trial, such effect was not observed (Armstrong et al., 2013).

Soybean BBI also exhibits potent anti-inflammatory effects. Both soybean BBI and BBIC were able to significantly suppress colon and anal gland inflammation of carcinogen-treated rodents (Billings et al., 1990; Armstrong et al., 2000). Soybean BBIC also reduced the severity of inflammatory conditions in mice with dextran sulfate sodium-induced ulcerative colitis (Ware et al., 1999). The mechanisms of BBI's anti-inflammatory action is not fully understood but could be related to their capacity to decrease the production and release of superoxide anion radicals and hydrogen peroxide, which are mediators of acute and chronic inflammation. The decrease in these highly oxidative species might reduce levels of oxidative damage to DNA, lipid peroxidation of cellular membranes and incidence of malignant transformation (Clemente and Arques, 2014).

A recent exciting development is the potential therapeutic role of soybean BBI in the prevention and treatment of HIV. It was shown that BBI could inhibit HIV reverse transcriptase (Ye et al., 2001; Ye and Ng, 2009; Prasad et al., 2010), inhibit HIV replication in macrophages through induction of the intracellular antiviral factors and block HIV entry into macrophages (Ma et al., 2016, 2018). Furthermore, BBI is also important in protecting lunasin from proteolysis in the gastrointestinal tract (GIT). Lunasin is a short peptide found in soybeans and cereals, which has been reported to confer a number of health benefits including anticancer effects (Jeong et al., 2002, 2003, 2007, 2009; Hernandez-Ledesma et al., 2013; Nakurte et al., 2013; Dinelli et al., 2014). Inhibition of gastric pepsin and pancreatic trypsin by BBI is crucial for the intact passage of lunasin through the GIT for absorption into the blood system of the body (Hsieh et al., 2010; Hernandez-Ledesma et al., 2013).

While most of the studies have focused on BBI of soybean, studies are beginning to explore BBI from other sources. Ng and co-workers isolated a BBI from brown kidney beans and trypsin inhibitor from the Faba beans (Fang et al., 2010a,b,c, 2011; Chan et al., 2013). The BBI, which has a MW of about 17 kDa, exhibited similar biochemical characteristics to soy BBI such as strong thermal and pH stability, and it inhibited the proliferation of human breast cancer MCF7 cells with an  $IC_{50}$  of 71.52  $\mu$ M (Chan et al., 2013). As BBIs are found widely in legumes and cereal seeds, their bioactivities remain a worthy subject for exploration. Other than BBI, Kunitz-type protease inhibitors (KTIs) have also been shown to exert anticancer effects. KTIs isolated from both Korean and Chinese mini-black soybean displayed potent anti-tumour effects by suppressing the proliferation of breast cancer MCF-7 cells, hepatoma HepG2 cells, nasopharyngeal carcinoma cells CNE-2 and HNE-2 cells (Fang et al., 2010a,b,c; Ye and Ng, 2011). They also showed strong inhibitory activities against HIV-1 reverse transcriptase, an important enzyme in the virus's replication in the host and entrance to host genome. Kunitz-type protease inhibitors isolated from other sources such as Bauhinia tree species also displayed antiproliferation activity against a number of cancer cell lines including human nasopharyngeal carcinoma cell CNE-1 and Hep G2 (Fang et al., 2010a,b,c, 2012). Moreover, a recent study has shown that legume seeds also contain matrix metalloproteinases (MMP) inhibitors which inhibited MMP-9 (an enzyme that is closely involved in colorectal cancer metastatic progression) and reduced colon cancer cell migration (Lima et al., 2016).

## Extraction of Protease Inhibitors

Extraction and purification of PIs from biological matrices involve a complex process, which usually involves multiple steps and requires careful consideration to select the most appropriate methods. Cell disruption is usually the first step to release the protein in a soluble form from its intracellular compartment. Several disruption techniques, both mechanical and chemical, are available for different materials. For plant seeds, grinding is found to be more efficient than alternatives such as enzymatic digestion, homogenization, pressing and sonication (Ahmed, 2005).

Many plant storage organs, such as seeds and tubers contain >10% of their soluble proteins as PIs (Mandal et al., 2002). Seed proteins are normally classified based on their solubility in a sequential series of solvents (Tanford, 1961). These are water soluble albumins, dilute salt solution soluble globulins, alcohol soluble prolamins and dilute acid or alkali soluble glutelins. However, proteins are extremely heterogeneous biological macromolecules. Their properties can be affected by small changes in hydrogen ion concentration, and thus a stable pH of the environment is necessary (Ahmed, 2005). Thus, appropriate buffer solution is usually used in the extraction of soluble proteins including PIs.

The purification of protein is an essential step for the study of its molecular and biological properties. On the basis of the properties of proteins, such as molecular weight, charge, hydrophobicity, binding affinity and isoelectric point (pI), several chromatographic and non-chromatographic (precipitation, electrophoretic and membrane filtration) separation techniques are available. These fractionation procedures need to be accompanied by bioactivity assays and possibly other characterization analyses. The purpose of these characterizations is to determine whether the specific activity of the PIs increases after fractionation. A failure to increase the specific activity suggests that the initial apparent inhibition might be due to endogenous substrates competing with the test substrate, rather than the presence of PIs (Nagase and Salvesen, 2001).

PIs may be purified by a single step using affinity chromatography, or by a combination of several steps, such as ammonium sulfate precipitation (salting out), organic solvent precipitation, ion-exchange and gel filtration chromatography (Bollag et al.,

1996). Among the commonly used salts, sulfites ( $\text{SO}_3^{2-}$ ) have the strongest effects in reducing solubility (Liu, 1997). Salting out process works best with divalent anions like sulfate, according to the Hofmeister series (Zhang and Cremer, 2006), especially ammonium sulfate  $((\text{NH}_4)_2\text{SO}_4)$ , which is highly soluble even at icy temperatures. It is useful for fractionating a mixture of proteins and purifying target PIs. Since large proteins tend to precipitate first, smaller ones will stay in solution (Tanford, 1961). The initial decrease in solubility is due to decreased electrostatic repulsion and enhanced hydrophobic interaction between protein molecules as a result of electrostatic shielding of charge groups in proteins by ions (Liu, 1997; Zhang and Cremer, 2006).

Column chromatography is a powerful method for fractionating proteins and the success of the technique is often determined by the selection of appropriate packing material for the column. In general, anion-exchange chromatography is employed for the purification of an acidic protein. Similarly, cation-exchange chromatography is suitable for the purification of a basic protein. Reverse-phase chromatography is suitable for a family of active proteins of similar charge (Ahmed, 2005).

Combination of anion-exchange chromatography, membrane ultrafiltration and reversed-phase or gel-filtration chromatography has been used successfully to obtain gram quantity of lunasin from a kilogram of defatted soy flour (Dia et al., 2009; Seber et al., 2012). However, these procedures are cumbersome, time-consuming and can be cost prohibitive. As a result, some innovative methods have been developed recently. For example, Krishnan and Wang (2015) developed a simple procedure to isolate and purify lunasin from soy flour by extraction with 30% ethanol followed by preferential precipitation of lunasin and protease inhibitors by calcium salt. The procedure was able to obtain 3.2 g of active lunasin concentrate from 100 g soy flour.

## Concluding Remarks

Protease inhibitors are a large and important group of molecules that are ubiquitously distributed in nature. They play a crucial role in many biological processes by regulating proteolytic functions of their target enzymes in living organisms. PIs are widely found in food plants with particular abundance in legumes but also in cereals and tubers, where they form part of the plant's defense against pest attack. Although PIs are traditionally regarded as anti-nutrients as they interfere with the digestion of protein in foods, studies over the last three decades have increasingly revealed their positive contribution to human health. There is a substantial and growing body of evidence demonstrating that plant PIs, especially soybean Bowman-Birk inhibitors, have preventive and therapeutic effects against several common cancers, as well as multiple sclerosis, inflammation and a number of other diseases. However, more research activities are needed to confirm these effects through clinical trials and to elucidate their mechanisms of action. Future research could also direct at exploring the health-benefitting properties of PIs from other legumes as well as cereals and other seeds.

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# Bioactive Carotenes and Xanthophylls in Plant Foods

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## Introduction

Carotenoids are lipophilic natural pigments responsible for the yellow, orange, or red color of many plant foods. They are known for their structural diversity and multiple functions and actions.

Plants are able to synthesize carotenoids *de novo*, thus carotenoids are widely distributed in plant-derived foods and the composition is variable and usually complex. Along with the principal carotenoids, low or trace levels of their biosynthetic precursors and derivatives are found (Rodriguez-Amaya, 1999a). The carotenoids are located in subcellular organelles (plastids), mainly associated with proteins in the chloroplasts and deposited in crystalline forms or as oily droplets in chromoplasts (Bartley and Scolnik, 1995).

Maturation/ripening of carotenogenic fruits, roots, and seeds is characterized by heightened carotenoid biosynthesis and oxidative cleavage of a part of these carotenoids to volatile compounds that contribute to the typical aroma/flavor (Rodriguez-Amaya, 2015).

Carotenoids have limited occurrence in foods of animal origin. Incapable of carrying out carotenogenesis, animals are limited to selectively or unselectively absorbing dietary carotenoids, which are accumulated unchanged or slightly altered into carotenoids typical of animal species.

## Chemical Structures and Physicochemical Properties

Food carotenoids are generally C<sub>40</sub> tetraterpenoids, having a centrally located conjugated double bond system (Fig. 1). The basic linear and symmetrical skeleton has lateral methyl groups separated by six C-atoms at the center and the others by five C-atoms. Modifications by cyclization, hydrogenation, dehydrogenation, introduction of oxygen-containing groups, migration of the double bonds, rearrangement, chain shortening or extension, or combinations thereof result in an impressive array of structures.

Carotenoids may be acyclic (e.g. lycopene) or may have a six-membered ring at one (e.g.  $\gamma$ -carotene) or both extremities (e.g.  $\beta$ -carotene,  $\alpha$ -carotene) of the molecule. Exceptionally, capsanthin and capsorubin have five-membered rings.

Hydrocarbon carotenoids are known as carotenes, and the oxygenated derivatives are called xanthophylls. Typical oxygen-containing substituents are hydroxyl (as in  $\beta$ -cryptoxanthin), epoxy (as in violaxanthin), and aldehyde (as in  $\beta$ -citaurin) groups.

Carotenols in ripe fruits are esterified with fatty acids (Breithaupt and Bamedi, 2001; Delgado-Pelayo and Hornero-Méndez, 2012; Etzbach et al., 2018; Inbaraj et al., 2008; Weller and Breithaupt, 2003), except in a few fruits, particularly those that remain green when ripe (e.g. kiwi) (Gross, 1987), in which limited or no esterification occurs. In green leaves (Kobori and Rodriguez-Amaya, 2008), hydroxycarotenoids are unesterified; those of corn (Rodriguez-Amaya and Kimura, 2004; De Oliveira and Rodriguez-Amaya, 2007) are mostly unesterified. Lutein, the principal carotenoid, occurs free or esterified in one (monoester) or both hydroxyl groups (diester) in nasturtium (Niizu and Rodriguez-Amaya, 2005) and marigold (Breithaupt et al., 2002) flowers, with the esters predominating. Esterification occurs progressively during maturation, increasing the lipophilic character of xanthophylls and facilitating their accumulation in the chromoplasts (Gross, 1987). Esterification was recently shown to facilitate accumulation of carotenoids and their deposition throughout tritordeum grains (Mellado-Ortega and Hornero-Méndez, 2018). Moreover, esterified carotenoids appear to have greater stability, as observed in red and hot chili peppers during processing (Schweiggert et al., 2007).

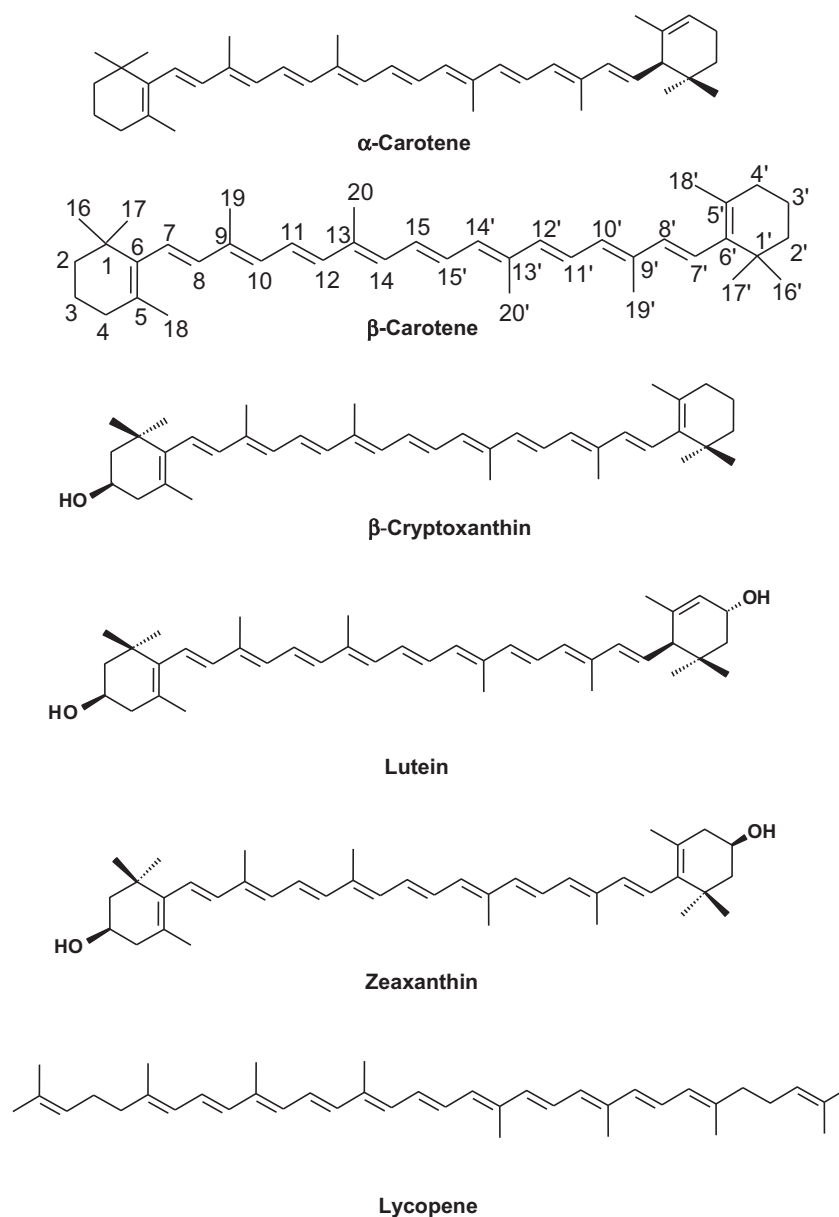
Carotenoids in which the carbon skeleton has been shortened by removal of fragments from one or both ends of the usual C<sub>40</sub> structure are called apocarotenoids. Natural examples are bixin and crocetin, the main coloring component of annatto and saffron, respectively.

The conjugated double bond system constitutes the light-absorbing chromophore that confers the carotenoid's attractive color and is mainly responsible for their special properties and many functions (Rodriguez-Amaya, 1999a). As the pale yellow  $\zeta$ -carotene, a carotenoid should have at least 7 conjugated double bonds in order to have perceptible color. Lycopene, with 11 conjugated double bonds in an acyclic structure, is red. Cyclization takes the  $\pi$  electrons of the ring double bond out of plane with those of the chain, thus the monocyclic  $\gamma$ -carotene and the dicyclic  $\beta$ -carotene, although possessing 11 conjugated double bonds, are red-orange and yellow-orange, respectively. The hydroxyl groups do not affect the chromophore, thus both  $\alpha$ -carotene and lutein are pale yellow, while  $\beta$ -cryptoxanthin and zeaxanthin, have the same color as  $\beta$ -carotene.

## Food Sources and Composition

The carotenoids most commonly encountered in foods are  $\beta$ -carotene,  $\alpha$ -carotene, lycopene,  $\beta$ -cryptoxanthin, lutein, and zeaxanthin (Fig. 1). Rich sources of  $\beta$ -carotene are some palm fruits, some squash cultivars, green vegetables, carrot, orange-fleshed sweet potato, cantaloupe, mango, and apricot.  $\alpha$ -carotene sometimes accompanies  $\beta$ -carotene, as in carrot and some varieties of squashes,





**Figure 1** Carotenoids commonly found in foods.

pumpkins, and palm fruits.  $\beta$ -Cryptoxanthin is the main pigment of many orange-fleshed fruits, such as peach, nectarine, orange-fleshed papaya, tangerine, persimmon, and tree tomato. Lycopene-rich foods are tomato, pitanga, pink-fleshed guava, red-fleshed papaya, and watermelon. The richest sources of lycopene, however, are the Asian gac fruit (Ishida et al., 2004; Vuong et al., 2006) and the Spanish sarsaparilla (Delgado-Pelayo and Hornero-Méndez, 2012). Corn and corn products are major dietary sources of zeaxanthin/lutein. Leafy and other green vegetables are the main sources of lutein.

The carotenoids of green vegetables have a defined pattern, with lutein,  $\beta$ -carotene, violaxanthin, and neoxanthin as the main carotenoids (Frazie et al., 2017; Kimura and Rodriguez-Amaya, 2002; Kobori and Rodriguez-Amaya, 2008). Lettuce, exceptionally, also has lactucaxanthin as a major carotenoid. Fruits, including those used as vegetables, have variable and often complex carotenoid compositions (Rodriguez-Amaya et al., 2008a; Rodriguez-Amaya, 2015).

The different factors affecting the carotenoid composition of plant foods have been extensively investigated, such as cultivar/variety, maturity, climate or season, geographic site of production, agronomic practices, part of the plant utilized, harvesting and post-harvest handling (Gross, 1987, 1991; Maiani et al., 2009; Rodriguez-Amaya, 1999a, 2015; Rodriguez-Amaya et al., 2008b).

## Stability and Alterations During Processing and Storage of Food

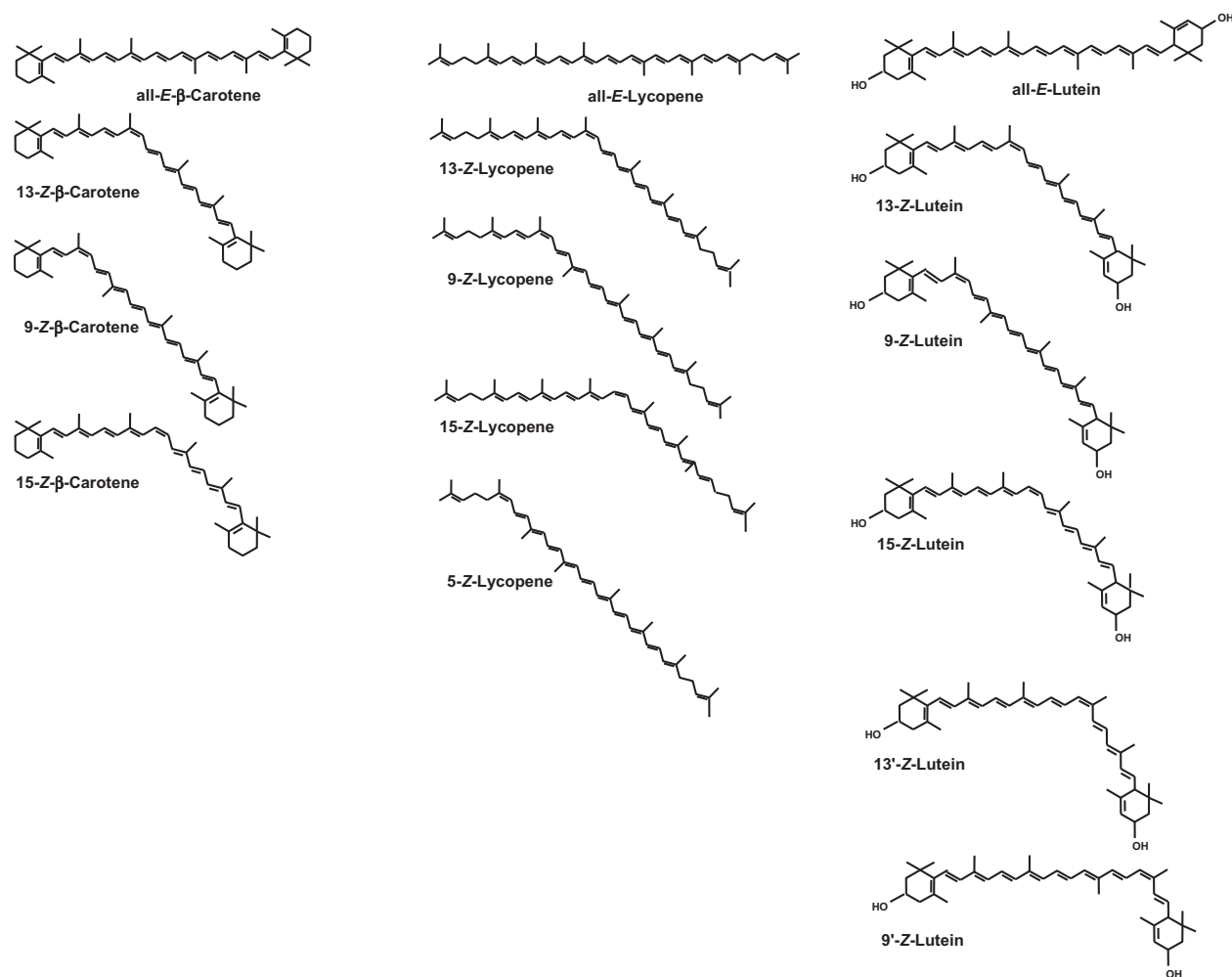
Carotenoid stability is influenced by the nature of the carotenoid (carotene or xanthophyll, *trans* (*E*-) or *cis* (*Z*-), esterified or unesterified) and the food matrix (fruit, root, leaf, juice, peeled, sliced, shredded), oxygen, light, water activity, atmosphere, oxidizing enzymes, antioxidants, metal catalysts, pro-oxidants, processing and storage conditions and duration.

Degradation increases with destruction of the food cellular structure, greater surface area or porosity, duration and severity of processing conditions, duration and inadequate conditions of storage, permeability of packaging material to O<sub>2</sub>, and exposure to light (Hager and Howard, 2006; Maiani et al., 2009; Pénicaud et al., 2011; Rodríguez-Amaya, 1999b, 2015; Shi and le Maguer, 2000; Xianquan et al., 2005).

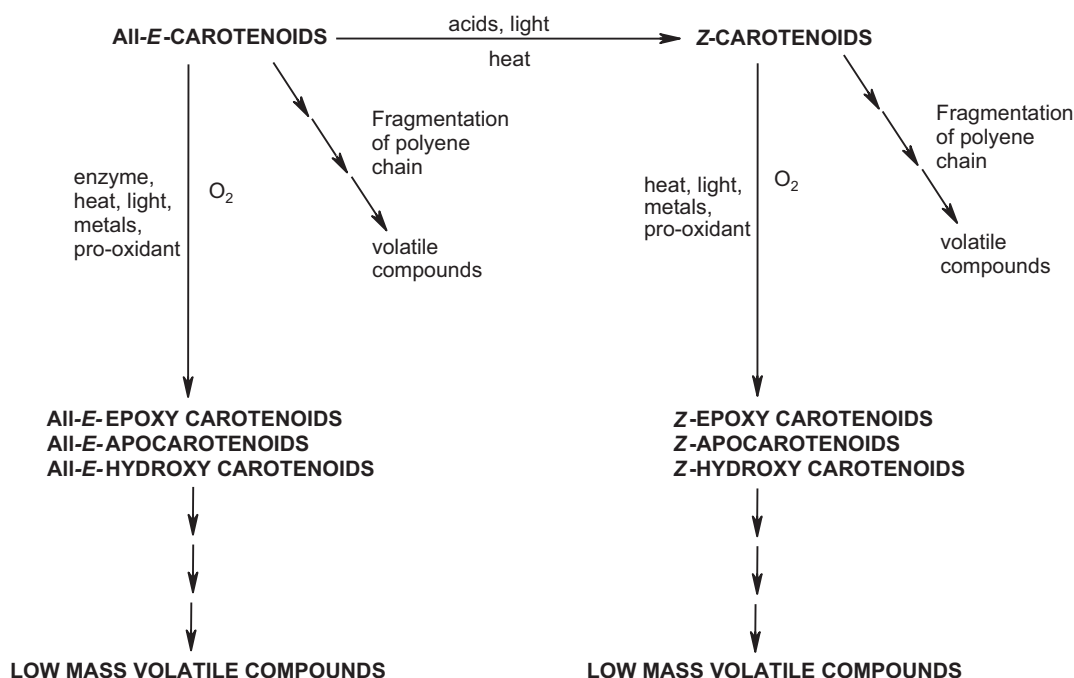
Alteration or loss of carotenoids during processing and storage of foods takes place through physical removal, geometric isomerization, and enzymatic or nonenzymatic oxidation (Rodríguez-Amaya, 1999b, 2015). Carotenoids are more concentrated in the peel than in the pulp, thus peeling and juicing result in substantial losses of carotenoids. Enzymatic oxidation of carotenoids can occur to a greater extent than thermal decomposition in many foods. Blanching may provoke some losses of carotenoids, but the inactivation of oxidative enzymes will prevent further and greater losses during processing and storage.

In nature, carotenoids occur primarily in the all-*E*-configuration. *E-Z* isomerization is promoted by acids, heat, and light. The release of organic acids during slicing, pulping or juicing of fruits can provoke this isomerization, but it occurs to a greater extent during thermal processing (Cervantes-Paz et al., 2014; Imsic et al., 2010; Knockaert et al., 2012; Lessin et al., 1997; Marx et al., 2003; Mayer-Miebach et al., 2005; Nguyen and Schwartz, 1998; Seybold et al., 2004; Shi et al., 2003; Updike and Schwartz, 2003; Vásquez-Cañedo et al., 2007).

Some double bonds are prevented from isomerizing because the *Z*-configuration is sterically hindered. Thus, the *Z*-isomers of symmetrical  $\beta$ -carotene commonly found in foods are 9-*Z*-, 13-*Z*-, and 15-*Z*-  $\beta$ -carotene (Fig. 2). The acyclic lycopene is isomerized to 5-*Z*-lycopene, along with the 9-*Z*-, 13-*Z*-, and 15-*Z*-isomers (Li et al., 2012; Schierle et al., 1997; Stinco et al., 2013; Tiziani et al.,



**Figure 2** Geometric isomers of  $\beta$ -carotene, lycopene, and lutein. Taken from Rodríguez-Amaya (2015).



**Figure 3** General scheme for the oxidative degradation of carotenoids. Taken from [Rodríguez-Amaya \(2015\)](#).

2006). The unsymmetrical  $\alpha$ -carotene,  $\beta$ -cryptoxanthin, and lutein ([Achir et al., 2010](#); [Aman et al., 2005](#); [Dachtler et al., 2001](#); [Humphries and Khachick, 2003](#); [Lessin et al., 1997](#); [Updike and Schwartz, 2003](#)) give rise to 13'-Z- and 9'-Z-isomers in addition to 13-Z-, 9-Z-, 15-Z-isomers.

E-Z isomerization decreases color intensity and the ability to quench singlet oxygen ([Conn et al., 1991](#); [Stahl and Sies, 1993](#)). It also results in loss of provitamin A activity and alteration of bioavailability and metabolism.

Enzyme-catalyzed oxidation takes place prior to heat treatment, during peeling, slicing, and pulping, but can also occur in minimally processed foods and in unblanched frozen foods during thawing ([Rodríguez-Amaya, 1999a, 2015](#)).

Nonenzymatic oxidation (also called autooxidation) is accompanied by isomerization, and both the Z- and E-isomers are oxidized ([Fig. 3](#)) ([Rodríguez and Rodríguez-Amaya, 2007, 2009](#)). Oxidation initially involves epoxidation, cleavage to apocarotenals, and hydroxylation ([Marty and Berset, 1988, 1990](#); [Rodríguez and Rodríguez-Amaya, 2007, 2009](#); [Zepka and Mercadante, 2009](#)). Subsequent fragmentations result in a series of compounds of low molecular masses (volatiles).

Epoxidation of  $\beta$ -carotene begins with oxygen attack at the terminal double bond of the conjugated double bond system on one side of the molecule, and then on the other side, forming  $\beta$ -carotene-5,6-epoxide and  $\beta$ -carotene-5,6,5',6'-diepoxide, respectively. Rearrangement of the 5,6- to the 5,8-epoxide yields  $\beta$ -carotene-5,8-epoxide and  $\beta$ -carotene-5,8,5',8'-diepoxide ([Henry et al., 2000](#); [Kanasawud and Crouzet, 1990](#); [Marty and Berset, 1986, 1988, 1990](#); [Rodríguez and Rodríguez-Amaya, 2007](#)).

Epoxidation of lycopene occurs at both the terminal conjugated double bonds and the isolated double bonds with the formation of lycopene-1,2-epoxide and lycopene-1,2,1',2'-diepoxide, along with lycopene-5,6-epoxide and lycopene-5,6,5',6'-diepoxide ([Khachik et al., 1998a,b](#); [Rodríguez and Rodríguez-Amaya, 2009](#)). Combinations of these epoxides and cyclization result in a greater number of products and a more complicated epoxidation scheme.

Cleavage of  $\beta$ -carotene results in  $\beta$ -apo-carotenals ( $\beta$ -apo-15-carotenal,  $\beta$ -apo-14'-carotenal,  $\beta$ -apo-12'-carotenal,  $\beta$ -apo-10'-carotenal, and  $\beta$ -apo-8'-carotenal). Similarly, lycopene is cleaved to apo-15-lycopenal, apo-14'-lycopenal, apo-12'-lycopenal, apo-10'-lycopenal, apo-8'-lycopenal, and apo-6'-lycopenal ([Kopeck et al., 2010](#); [Rodríguez and Rodríguez-Amaya, 2009](#)).

Volatile compounds are generated from carotenoids by direct cleavage of the polyene chain, sequential cleavage, and transformation of the initial volatiles ([Rodríguez-Amaya, 2015](#)). These are mostly aldehydes, ketones, alcohols, hydrocarbons, furans, and pyrans ([Caris-Veyrat et al., 2003](#); [Kanasawud and Crouzet, 1990](#); [Kobori et al., 2014](#); [Rios et al., 2008](#)). Now devoid of the color and the biological activities of carotenoids, they contribute to the desirable flavor of foods and beverages, as in wine and tea, or to off-flavor, as in dehydrated carrot.

## Carotenoids as Food Colorants

Carotenoids are produced commercially as food and feed color additives and supplements. Commercial carotenoids (e.g.,  $\beta$ -carotene, astaxanthin, and canthaxanthin) are mostly products of chemical synthesis, but they are also produced by extraction from

a small number of rich natural sources (annatto, paprika, saffron, tomato lycopene extract or concentrate, marigold lutein) and by microbial fermentation ( $\beta$ -carotene by the microalga *Dunaliella* sp., astaxanthin by the microalga *Haematococcus pluvialis*, and  $\beta$ -carotene by the fungus *Blakeslea trispora*).

Stimulated by consumers' concern about the safety of artificial food dyes and the possible health-promoting effects of natural colorants, the replacement of the former by the latter is a current trend. Natural colorants, however, are usually less stable, more costly, not as easily utilized as synthetic dyes, besides having weaker tinctoral strength, interaction with food components, and limited range of hues (Sigurdson et al., 2017; Wrolstad and Culver, 2012). The utilization of carotenoids in particular is hampered by their insolubility in water, instability, and low bioavailability. The first two problems have been addressed by the formulation of water-dispersible market products, as colloidal suspensions, emulsions, or dispersions in suitable colloids. In recent years, attention has centered on encapsulation and nanoencapsulation (Rodriguez-Amaya, 2015).

## Health Effects

The long established function of carotenoids in terms of human health is the provitamin A activity. To serve as a precursor of vitamin A, a carotenoid should have an unsubstituted  $\beta$ -ring with an 11-carbon polyene chain.  $\beta$ -carotene can potentially yield two molecules of vitamin A.  $\alpha$ -Carotene and  $\beta$ -cryptoxanthin would have about half the bioactivity of  $\beta$ -carotene. The acyclic carotenoids (i.e., lycopene) and xanthophylls, in which both  $\beta$ -rings have substituents (i.e., lutein and zeaxanthin), are not provitamins A.

In more recent years, other health benefits have been attributed to carotenoids, provitamins A or not, such as immunoenhancement and reduction of the risk of developing chronic diseases such as cancer, cardiovascular diseases, cataract, and macular degeneration (Krinsky and Johnson, 2005; Rao and Rao, 2007; Tapiero et al., 2004).

The action of carotenoids against diseases has been widely attributed to their antioxidant activity (Müller et al., 2016; Kiokias and Gordon, 2004; Krinsky, 2001; Rodriguez-Amaya, 2015; Stahl and Sies, 2003). However, non-antioxidant mechanisms are increasingly cited, such as retinoid-dependent signaling, modulation of carcinogen metabolism, regulation of cell growth, inhibition of cell proliferation, enhancement of cell differentiation, stimulation of intercellular gap junction communication, gene regulation, modulation of DNA repair mechanisms, induction of detoxifying enzymes, hormonal and immune system modulation, and filtering of blue light (Krinsky and Johnson, 2005; Rao and Rao, 2007; Stahl et al., 2002; Tapiero et al., 2004).

Lycopene's possible role in human health has drawn considerable attention, especially in relation to prostate cancer (Hadley et al., 2002; Miller et al., 2002; Stacewicz-Sapuntzakis and Bowen, 2005; Wertz et al., 2004). Lutein and zeaxanthin selectively accumulate in the macula of the human retina (Bone et al., 2001; Landrum and Bone, 2001) and are associated with reduced risk of age-related macular degeneration, the major cause of irreversible blindness in the elderly (Bone et al., 2001; Carpentier et al., 2009; Gale et al., 2003; Moeller et al., 2000), and cataract (Delcourt et al., 2006; Gale et al., 2001; Moeller et al., 2000).

The literature on carotenoids and human health is now voluminous, but inconsistent results persist, the carotenoid intake having significant or no association/effect on disease incidence. These discrepancies have been mainly attributed to differences in the experimental protocols applied in the different studies (Costa-Rodrigues et al., 2018). Mordente et al. (2011), for example, in his review of 54 clinical trials of lycopene supplementation and cardiovascular diseases, noted that the number of subjects varied from 12 to 100, the dose from 4 to 80 mg/day and the duration from 1 to 60 days. There are, however, at least three major findings that make studies on this topic especially challenging, raising the possibility that consistent results may not be achieved even when the protocols are optimized and standardized (Rodriguez-Amaya, 2015). First, the effect of bioactive compounds on the human body may be very small over relatively short periods (months or a few years), but can contribute significantly throughout life as part of the daily diet. The health effect may not be observed during the study period, perhaps explaining studies reporting no significant effect. Doses above the physiological levels have been used in an attempt to observe the outcome better, but the results cannot be extrapolated to actual human situations. Second, it is now known that reduction of the risk of chronic diseases is not due to a single constituent or a single class of compounds but to the mixture of bioactive compounds contained in foods. Thus, while the evidence for the beneficial effects of plant foods is compelling, that for individual phytochemicals is often inconclusive. On the other hand, when food is used, attributing the effects to specific bioactive compound is presumptive. Third, there is considerable variation in inter-individual responses to the administered bioactive compound, further masking the health effects and making it difficult to draw conclusions. Genetic variation appears to be a key factor in this huge inter-individual variability.

The consensus is that there is ample evidence to support and recommend increased consumption of carotenoid-rich foods, but caution and more investigations are needed to evaluate the benefits and risks of supplements. High-dose  $\beta$ -carotene supplement was shown to be harmful to smokers and asbestos-exposed workers, increasing rather than lowering lung cancer incidence (ATBC, 1994; Omenn et al., 1996). The applied doses of  $\beta$ -carotene in the intervention studies were 20 mg per day in the ATBC study and 30 mg per day plus 25,000 IU vitamin A in the CARET study. In the epidemiological studies in which dietary  $\beta$ -carotene was inversely associated with decreased risk of lung cancer, the daily intake of  $\beta$ -carotene was about 4 mg (CARIG, 1996).

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## Bioactive Gums

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### Introduction

Gums and mucilages are widely distributed in plants, animals, seaweeds, fungi as well as in microorganisms. They play an important role in structure and metabolism. Since plants are the major sources of gums and mucilages, this section will focus on seed polysaccharide gums including gum Arabic, guar gum, locust bean gum, flaxseed gum, psyllium and fenugreek. These gums, composed mainly of polysaccharides, are used by the food industry to enhance viscosity, improve texture, stabilize foams and emulsions, and for film formation and coating purposes (Dabestani et al., 2018). Over the years, however, increasing evidence has accrued regarding the bioactive properties of many of these natural gums. Besides being economical, they are readily available and generally non-toxic (Prajapati et al., 2013).

### Gum Arabic

Gum Arabic (GA), also known as Acacia gum, is an edible gummy exudate obtained from the stems and branches of *Acacia senegal* and *Acacia seyal* trees growing in abundance in central Sudan, central Africa and in West Africa (Dauqan and Abdullah, 2013; Sander et al., 2018). GA is very rich in low viscosity soluble fibers and provides the trees with a protective layer that prevents invasion by insects and molds when wounded. It is the oldest and probably the best known of the plant natural gums (Verbenken et al., 2003). Guar gum is composed of a complex polysaccharide varying in composition, depending on the tree, and occurring as either neutral or slightly acidic. The major backbone consists of 1,3-linked  $\beta$ -D galactopyranosyl units joined with linked branches of arabinose and rhamnose by  $\alpha$  1,6 linkages found in nature as a magnesium, potassium and calcium salt. The gum is used extensively in the food industry as a stabilizer, emulsifier, flavoring agent or thickener in confectionery, baking, dairy and beverages as well as a microencapsulating agent (Mariana et al., 2012).

GA has been used in folk medicine internally to treat inflammation of the intestinal mucosa and externally to cover inflamed surfaces (Gamal el-din et al., 2003). Animal studies demonstrated the protective antioxidant properties of GA against experimental gentamicin and cisplatin nephrotoxicity (Al-Majed et al., 2002, 2003) and dioxorubin cardiotoxicity (Abd-Allah et al., 2002) in rats and against acetaminophen-induced hepatotoxicity (Gamal el-din et al., 2003) in mice. Ali et al. (2004), however, were unable to confirm the work by Al-Majed et al. (2003) as they observed only a slight palliation of gentamicin in fed GA. Using eight different polysaccharides including GA, Trommer and Neubert (2005) showed GA protected against lipid peroxidation. However, Cindoruk et al. (2007) found GA ineffective in reducing hepatocellular damage in cholestasis induced by fenofibrate in rats. Subsequent work by Ali et al. (2004) further suggested that GA did not exhibit strong antioxidant properties.

### Guar Gum

Guar gum (GG) is a galactomannan gum found in the large endosperm of guar beans (*Cyamopsis tetragonoloba*). It is used extensively as a food additive in the food industry because of its thickening and binding properties (Butt et al., 2007). It is composed of high molecular weight polysaccharides with a molecular weight ranging from 50000–8000000 Da (Kawamura, 2008). Structurally it is a galactomannan composed of a linear chain of  $\beta$  1  $\rightarrow$  4 D-mannose residues with D-galactose linked 1,6 at every second mannose and the ratio of galactose to mannose being approximately 1:2.

The beneficial effects of GG were demonstrated by Hartesmink et al. (1999) who showed that it is completely degraded by *Chloridium butyricum*. While a high concentration of GG above 1% interferes with the nutritional, physicochemical and sensory properties of food, partial hydrolysis lowers reduced chain length and molecular weight and reduces the viscosity. Under these conditions, the hydrolyzed guar gum increases dietary fiber ability, which reduces diarrhea and symptoms of irritable bowel syndrome (Greenberg and Sellman, 1998; Slavin and Greenberg, 2003). The water-solubility and non-gelling properties of partially hydrolyzed guar gum proved ideal for treating both constipation and/or diarrhea predominant in either forms of irritable bowel syndrome (Gianni et al., 2006).

The beneficial effects of the gel forming properties of GG include its ability to lower cholesterol and glucose. The hypocholesterolemic effect of GG is attributed to the increase in bile acids in feces and decrease in enterohepatic bile acid. This may enhance bile acid production thereby reducing the amount of hepatic free cholesterol (Rideout et al., 2012). With respect to GG's ability to lower glucose, it acts as a barrier between starch and the starch-digesting enzymes (Dartois et al., 2010). The gel-forming properties of GG also increases satiation due to slow gastric emptying thereby increasing weight loss and preventing obesity by decreasing appetite, hunger and the desire to eat (Butt et al., 2007).

## Locust Bean Gum

Locus bean gum is obtained from the seeds of the carob tree (*Ceratonia siliqua*) grown in abundance in the Mediterranean region, particularly Spain, Italy and Cyprus. The gum is a high molecular weight polysaccharide composed of galactose and mannose units referred to as a galactomannan. It is a linear chain of 1→4-linked β-D-mannopyranosyl units with (1→6)-linked α-D-galactopyranosyl residues as side chains (Barak and Mudgil, 2014). Using gel-permeation chromatography, the average molecular weight of locust bean gum ranged from 0.3–2.0 million, depending on seed source, plant growing conditions and manufacturing conditions. It has an extended ribbon-like structure at the solid state and a semi flexible coil-like conformation in solution. The galactose-mannose ratio in locust bean gum ranges from 1:3.1 to 1:3.9 (Gaisford et al., 1985; Kok, 2007; McCleary et al., 1985).

Locust bean gum is considered a dietary fiber suitable as a supplement for weight control and for the treatment of diabetes and hyperlipidemia. As a viscous soluble fiber, it slows down the degradation of carbohydrates during digestion to regulate postprandial blood glucose and insulin levels. The latter are important events in the prevention and treatment of obesity and diabetes (Ferguson and Harris, 2005). The hypocholesterolemic effects of locust bean gum were reported by Zavoral et al. (1987) in familial hypercholesterolemic adults and children and shown to be a safe and effective approach to controlling hyperlipidemia.

## Flaxseed

Flaxseed is obtained from the flax plant (*Linum usitatissimum*). The seed coat or testa is rich in a thick mucilage (epidermis) layer that is soluble in cold water (Cui et al., 2007). The mucilage is composed of high-molecular weight water soluble heteropolysaccharides which represent 3%–9% of the total seed (Kaur et al., 2018). Depending on the different varieties and growing conditions, flaxseed gum contains 50%–80% carbohydrates, 4% to 20% protein and 3%–9% ash. Two major polysaccharide fractions are found in flaxseed gum, a neutral polysaccharide (composed of xylose, arabinose, and galactose), and an acidic polysaccharide (composed of D-galactose, L-rhamnose, and D-galacturonic acid). In the neutral polysaccharide, arabinoxylan forms a (1→4)-β-D-xylosyl backbone with arabinose and galactose residues on the 2 and 3 positions. The backbone in the acidic polysaccharide is a (1→2)-linked α-L-rhamnopyranosyl (1→4) linked D-galactopyranosyluronic acid with fucose and galactose side chains.

*In vitro* studies strongly suggested that flaxseed gum was capable of lowering cholesterol (Fodje et al., 2009; Denis et al., 2007; Theuvsissen and Mensink, 2008). This was attributed to its high bile acid-binding capacity with the generation of acetate and propionate. The prebiotic nature of flaxseed gum was also demonstrated by its stimulation of *Lactobacilli* growth in vivo (Alzueta et al., 2003). A recent study by Lou et al. (2018) reported that flaxseed gum reduced body weight by regulating the gut microbiota. Using male Sprague Dawley rats, flaxseed gum decreased Firmicutes/Bacteroidetes ratio and altered specific bacteria.

## Psyllium

The seed coats, including both the husk and hull, from the plants of the *Plantago* genus are rich in mucilage (dietary fibre). Commercially it is produced in European countries, Pakistan and India from the husks of *Plantago ovato* and *Plantago psyllium*. The largest importer of psyllium husks remains the United States where the pharmaceutical industry uses it for the production of fiber-based laxative products (Cui et al., 2007). Psyllium mucilage is a heteroxylan composed of (1→3) and (1→4) linked β-D-xylopyranosyl backbone chains with 1,4-linked β-D-xylopyranosyl residues attached at the 2- and 3-positions in the chain. The side chains consist of β-D-xylopyranosyl and α-D-arabinofuranosyl residues. Pectic acid is also found containing 1→4 linked α-D-GalpA residues with small amounts of 1,2,4-linked Rha and 1,3-, 1,6- and 1,3,6-linked Galp (Samuelson et al., 1999).

Psyllium has a long history of medicinal use. Using rats, many studies showed psyllium was far more effective in lowering lipids compared to other dietary fibers. A meta-analysis of 12 studies involving 404 adults, suffering from mild to moderate hypercholesterolemia, found a respective reduction in total and LDL cholesterol of 5% and 9%, respectively when fed a psyllium-enhanced cereal product (Olson et al., 1997). An earlier study by Davidson et al. (1996) reported a 7% reduction in LDL cholesterol in hypercholesterolemic children on a low-fat diet were fed psyllium-enriched cereal. Similar results were reported by Anderson et al. (2000) who reported 4.7% and 6.7% reductions in serum total and LDL cholesterol, respectively in hypercholesterolemic subjects given 5.1 g psyllium twice a day over 26 days. The overall evidence from these and other studies clearly confirmed the ability of psyllium to lower serum cholesterol, particularly LDL-cholesterol levels.

The ability of water-soluble fibers, such as psyllium, to lower postprandial glucose and insulin levels in non-insulin dependent diabetic patients when taken with meals, was confirmed in several studies (Hannary et al., 2006; Pastoro et al., 1991). Overall studies suggest that including psyllium with a conventional diet provides a safe approach for improving the glycemic index (Anderson et al., 1999; Ziai et al., 2005).

Other health benefits associated with psyllium is its extensive use as a laxative (Marlett et al., 2000) and its ability to prevent colon and breast cancers (Morita et al., 1999; Nakamura et al., 2004, 2005). Brum et al. (2018) recently studied the satiety effects of psyllium supplementation on the diets of healthy male and female volunteers prior to breakfast and lunch over a 3 day period. In addition to being well tolerated, psyllium supplementation significantly affected satiety by decreasing hunger, increasing fullness, and reducing the desire for eating between meals.

## Fenugreek

Fenugreek (*Trigonella foenum graecum*) is an annual leguminous plant grown in northern Africa, the Mediterranean, western Asia and northern India. It produces a pod containing 10–20 seeds that are rich in mucilage or gum (Cui et al., 2007). Fenugreek gum, a galactomannan, was shown by Brummer et al. (2003) to be highly substituted with the ratio of galactose to mannose ratio being from 1.00:1.02 to 1.00:1.14. While the gum had a higher molecular weight than locust bean gum and guar gum, it exhibited a reduced intrinsic viscosity and rheological behavior. It possessed substantial surface activity, which is normally associated with the presence of small amounts of protein. However, the gum was still shown to possess substantial surface activity even after separation of protein residues (Garti et al., 1997).

Seed powders from fenugreek were reported to exert antidiabetic and hypocholesterolemic effects in humans (Al-Habori and Raman, 1998). The main components of fenugreek gum, galactomannans, are associated with reduced cholesterolemia (Roberts, 2011). Rats treated with fenugreek extract were shown by Xue et al. (2007) to have lower triglycerides, total cholesterol and higher HDL cholesterol in a dose dependent manner. These researchers also reported an increase in body weight and decrease in kidney/body ratio weight in rats treated with fenugreek extract compared to the corresponding diabetic group. It was apparent that rats treated with fenugreek extract had lower blood glucose and glycated hemoglobin than in the diabetic group.

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# Prebiotics in Food and Health: Properties, Functionalities, Production, and Overcoming Limitations With Second-Generation Levan-Type Fructooligosaccharides

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## Introduction

With the emergence of enhanced technologies to assess and quantitate their therapeutic and practical applications, functional foods are receiving increased recognition. Among these are dietary fibers, which in recent decades, have been attributed with properties promoting cardiovascular, gastrointestinal, and overall health (Brown, 2004; Marshall, 2008; Roberfroid, 2007; Walker and Painter, 2015). However, frequent modifications to dietary guidelines and nutritional recommendations have instilled public skepticism. Currently, a highly controversial topic is the potential of prebiotic dietary fibers in modulating and mitigating disease risks and outcomes. Owing to advances in this field of research, governmental public health agencies are increasingly recommending a suitable intake of prebiotics with the aim of maintaining and improving health and well-being (Slavin, 2013). Historically, prebiotics have been an integral component of the human diet; however, it is only recently that their therapeutic properties and key mechanistic actions have been elucidated, allowing for the identification of limitations to the current commercial generation, as well as the development of innovative strategies to produce well-defined prebiotics with targeted health benefits (Marshall, 2008).

The term 'prebiotic' was first coined in the mid-1990s by Roberfroid (2007), which subsequently led to its success as a therapeutic approach that specifically targets indigenous colonic bacteria that are regarded as beneficial (Marshall, 2008; Roberfroid, 2007) for promoting gastrointestinal health (Brown, 2004; Kaczmarczyk et al., 2012; Slavin, 2013). As a class of dietary fiber, prebiotics also confer a variety of systemic health benefits (e.g., protection against atherosclerosis and cardiovascular diseases, anti-tumor/cancer activity, antioxidant properties, etc.) (Guinane and Cotter, 2013; Huebner et al., 2007; Kaczmarczyk et al., 2012) and are hence finding increasing applications in functional foods, owing to their high stability and resistance to heat (Marshall, 2008; Padma Ishwarya and Prabhasankar, 2013). Certain prebiotics can also improve food quality characteristics such as mouthfeel by serving as low-calorie fat mimetics (Laguna et al., 2014; O'Brien et al., 2003), while others are exploited for their use as sweetening agents (Gallagher et al., 2003; Padma Ishwarya and Prabhasankar, 2013). Within the context of food and health, our review of prebiotics examines: the criteria required to fulfill their designation; sources in a standard human diet; the range of physiological benefits they confer; functional deficits of commercial preparations; enzymatic approaches for synthesizing novel, well-defined compounds with enhanced prebiotic potential; and their application as multi-functional food ingredients.

## Modulation of Health and Well-Being by the Intestinal Microbiota

The human alimentary tract is becoming more prominently recognized as a hidden metabolic 'organ' owing to its integral impacts on overall health (including metabolism, physiology, nutrition and immune function) that are the result of the colonization and metabolic activities of diverse microbial communities occurring along its entire length (Guinane and Cotter, 2013; Marshall, 2008; Vrieze et al., 2010). This environment promotes bacterial growth due to its slow transit time, readily available nutrients, and favorable pH (Slavin, 2013). On a quantitative basis, 10–20 genera dominantly reside in the colon, including *Bacteroides*, *Lactobacillus*, *Clostridium*, *Fusobacterium*, *Bifidobacterium*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, *Escherichia* and *Veillonella* (He and Shi, 2017; Kolida and Gibson, 2007). The large intestine can harbor pathogens either belonging to the resident flora, or existing as transient members (Marshall, 2008; Roberfroid, 2007). Attachment and subsequent overgrowth of these undesirable microorganisms generally results in acute diarrheal infections, or can be manifested in the form of chronic intestinal diseases, including inflammatory bowel diseases (ulcerative colitis and Crohn's disease) and colon cancer (Campieri and Gionchetti, 2001; Guinane and Cotter, 2013; Rastall and Gibson, 2015). To varying extents, microflora composition and metabolic activity, and thereby the structure of the human diet since it provides the nutrients for their growth, have been implicated in the incidence of these disorders (Kaczmarczyk et al., 2012; Rastall and Gibson, 2015; Walker and Painter, 2015). In fact, it has been established for several decades that numerous disease states associated with modern Western civilization are related to the transit time of digesta and to the bulk and consistency of stools, which are factors heavily influenced by dietary fiber consumption (Walker and Painter, 2015). Further, an increasing body of evidence suggests that the stimulated growth of beneficial bacteria within the intestinal microbiome can reduce the risk of disease through pathogen inhibition and the production of benign and favorable metabolites (Kolida and Gibson, 2007; Roberfroid, 2007).

According to Gibson and Collins (1999), the diverse availability of fermentation substrates is arguably the most crucial determinant of the dynamics and stability of species existing in the human colon (Marshall, 2008). Human colonic bacteria consume primarily complex carbohydrates (i.e., food fibre, resistant starch, oligosaccharides, etc.) that are resistant to degradation by gastric acid and the host's digestive enzymes (Mardo et al., 2017; Sonnenburg et al., 2010). This distinct group of anaerobic beneficial bacteria include *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Roseburia*, *Faecalibacterium*, *Anaerostipes*, and *Coproccoccus* (Fernández



et al., 2016; Guinane and Cotter, 2013a). Fermentation in the colon primarily occurs by saccharolytic and proteolytic activity of resident bacteria (Marshall, 2008; Slavin, 2013). The proximal colon is a saccharolytic environment in which the majority of non-digestible carbohydrates are fermented by *Bacteroides*, *Bifidobacterium*, *Ruminococcus*, *Eubacterium*, *Lactobacillus* and *Clostridium* (the main saccharolytic species among colonic microflora) (Guinane and Cotter, 2013; Marshall, 2008). Saccharolytic fermentation is more advantageous to the host due to the range of metabolic end-products generated (Kolida and Gibson, 2007). These include short-chain fatty acids (predominantly butyrate, acetate, and propionate) and other metabolites of microbial origin that confer an array of beneficial functions for the host, including the production of vitamins, modulation of the immune system, enhanced digestion and absorption, inhibition of harmful species, as well as the removal of carcinogens and other toxins (Adhikari and Kim, 2017; Kolida and Gibson, 2007; Nyman, 2002; Phillips, 2013). SCFAs are rapidly absorbed by the colonic mucosa and contribute towards the energy requirements of the host (Fernández et al., 2016; Roberfroid, 2007; Slavin, 2013). Furthermore, propionate is a gluconeogenesis factor that has been implicated in the suppression of cholesterol synthesis, and is recognized by several authors as an anti-tumor agent, owing to its ability to down-regulate cell multiplication pathways and promote pro-apoptotic routes (Fernández et al., 2016; Nyman, 2002). Butyrate is preferentially metabolized by colonocytes that are simultaneously regulated in terms of cell growth and differentiation, and serves as the primary energy source for beneficial bacteria residing in the colon (i.e., bifidobacteria) (Cummings et al., 2001; Fernández et al., 2016; Valcheva and Dieleman, 2016). Certain oligosaccharides, particularly fructooligosaccharides, galactooligosaccharides and lactulose, may be preferentially fermented by bifidobacteria, an effect that initially gave rise to the concept of prebiotics. Mechanisms regarding unfavorable alterations in gastrointestinal behavior have previously been postulated in explaining the occurrence of common disorders such as ischemic heart disease, diverticular disease, as well as appendicitis, and varicose veins (Walker and Painter, 2015). To varying extents, each has been linked to microflora composition and activities, thus underlining the instrumental role of diet in gastrointestinal health, since it is the major source of their growth.

### Approaches for Managing Colonic Microflora: Prebiotics, Probiotics, and Synbiotics

Generally, there are two concepts involved in the modulation of colonic microflora for beneficial health outcomes: 1) prebiotics; and 2) probiotics (Marshall, 2008; Rastall and Maitin, 2002; Roberfroid, 2007; Slavin, 2013). According to the scientific consensus, a prebiotic is defined as a 'non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health' (Fernández et al., 2016; Marshall, 2008; Roberfroid, 2007). A crucial mechanism of action for prebiotics is fermentation in the colon, the products of which confer numerous local and systemic health benefits (Marshall, 2008; Rastall and Gibson, 2015; Slavin, 2013). In this context, the stimulated bacteria should mainly constitute bifidobacteria and lactobacilli, considered to be probiotic strains (Fernández et al., 2016; Guinane and Cotter, 2013; Sonnenburg et al., 2010). According to Marshall (2008), human breast milk can be considered the original prebiotic with specific roles in gut microflora management due to its potent bifidogenic effects. The majority of prebiotics, however, are of plant origin, demonstrating chemical stability across wide temperature and pH ranges (Rastall and Gibson, 2015; Singh et al., 2017). Table 1 summarizes the range of natural prebiotic sources in a standard human diet, with the most frequently occurring ones being inulin, FOSs, and galactooligosaccharides (Marshall, 2008; Singh et al., 2017; Valcheva and Dieleman, 2016). The concept of probiotics was introduced to select for beneficial colonic taxa (Adhikari and Kim, 2017; Chow, 2002).

While the definition for probiotics is continuously evolving, this class of microorganisms is officially designated as 'non-pathogenic, live microbial, mono- or mixed-culture preparations, which, when administered in sufficient doses, beneficially affect the host by improving the intestinal microbial balance and its properties' (Adhikari and Kim, 2017; Marshall, 2008; Rastall and Maitin, 2002). The presence of prebiotic carbohydrates can be instrumental to the sustained survival of these populations, especially if they have been evidenced in the selection for useful species of *Bifidobacterium* and *Lactobacillus* (Chow, 2002; Kailasapathy and Chin, 2000; Reid, 2008). Among probiotic species, different genetic mechanisms are employed for the metabolism of

**Table 1** Natural sources of dietary prebiotics

Prebiotic compound	Sources	Type of glycosidic linkage	References
Inulin (fructan)	Jerusalem artichoke, chicory root	$\beta$ -(2,1)	Adhikari and Kim, 2017; Marshall, 2008; Reid, 2008; Roberfroid, 2007; Singh et al., 2017; Slavin, 2013; Subin et al., 2017; Valcheva and Dieleman, 2016
Fructooligosaccharides	Asparagus, sugar beet, garlic, chicory, wheat, rye, honey, banana, barley, tomato	$\beta$ -(2,1) and/or $\beta$ -(2,6)	
Galactooligosaccharides	Milk	$\beta$ -(1,3) and $\beta$ -(1,4)	
Maltooligosaccharides <sup>1</sup> , Isomaltooligosaccharides <sup>2</sup>	Honey, sugarcane juice, food-grade molasses	<sup>1</sup> $\alpha$ -(1,4) <sup>2</sup> $\alpha$ -(1,6) and $\alpha$ -(1,2) or $\alpha$ -(1,3) or $\alpha$ -(1,4)	
Lactulose <sup>1</sup> , lactosucrose <sup>2</sup> , lactulosucrose <sup>3</sup>	Milk (whey)	<sup>1</sup> $\beta$ -(1,4) <sup>2,3</sup> $\beta$ -(1,4) and $\alpha$ -(1,2)	
Raffinose, stachyose	Soybean and other pulses	$\alpha$ -(1,6)	



non-digestible oligosaccharides, hence the physiological benefits conferred by a given prebiotic are markedly determined by their structure (Rastall, 2010).

Mixtures of probiotics and prebiotics that function synergistically to improve the survival and implantation rate of viable microbial supplements in the gastrointestinal tract, referred to as synbiotics, have the capacity to improve therapeutic potential in the gastrointestinal tract (Roberfroid, 2007; Marshall, 2008). This may occur by multiple mechanisms: 1) increased survival of probiotic bacteria owing to the supplementation of prebiotics in consumer products, resulting in increased shelf-life and thereby ingestion of viable cells (Shin et al., 2000); 2) a higher count of probiotics reaching the colon due to simultaneous consumption of a prebiotic, which can competitively serve as an energy substrate and promote survival (Holzapfel and Schillinger, 2002); and 3) stimulated growth and/or activity of not only the supplemented probiotic strain(s), but also that of beneficial indigenous bacteria (Roberfroid et al., 2010).

### Criteria for Prebiotic Classification and Evaluation of Their Efficacy

Since it was first introduced by Roberfroid in 2007, the term 'prebiotic' has been mis-used on frequent accounts. While all prebiotics can be classified as dietary fibers, not all types of dietary fiber fulfill the criteria necessary to demonstrate the full spectrum of characteristics exhibited by prebiotics (Marshall, 2008; Roberfroid et al., 2010). Officially, prebiotics must: 1) resist gastric acidity, hydrolysis by mammalian digestive enzymes, and absorption in the upper gastrointestinal tract; 2) remain intact as it reaches the distal colon, where it is fermented by indigenous intestinal microflora; and 3) selectively stimulate the growth and/or activity of intestinal bacteria, which are associated with health and well-being (i.e., bifidobacteria and lactobacilli) (Marshall, 2008; Roberfroid, 2007; Slavin, 2013). Considering these criteria, non-digestible oligosaccharides (highlighted in Table 1) have demonstrated the highest efficacy, through their ability to confer selectivity to the target bacterial species (Marshall, 2008). In particular, fructooligosaccharides (FOSs) have been one of the most frequently explored sub-classes in this context. These comprise a glucose moiety followed by fructose moieties ranging from 2 to 60 linked by  $\beta$ -(2,1) or  $\beta$ -(2,6) glycosidic bonds (Singh et al., 2017). FOSs remain relatively intact during transit through the alimentary tract until they reach the colon, where the products of their fermentation constitute primarily SCFAs, and other bioactive molecules beneficial to human health (Hutkins et al., 2016). On the basis of chemical structure, FOSs are categorized as being either of the inulin or the levan type, the distinguishing coordinate being the nature of the glycosidic bond joining the fructose monomers (Rastall, 2010). In inulin and oligosaccharides derived from such, fructose units are linked in a linear fashion by  $\beta$ -(2,1) bonds, while in levan and associated derivatives,  $\beta$ -(2,6) bonds join the fructose moieties in the fructan main chain, and  $\beta$ -(2,1) bonds occur at branching points (Slavin, 2013). These crucial structural features will be discussed later in comparing the functional capacities of various FOSs.

The majority of early studies on prebiotics were performed on pure cultures, which typically involves the selection of a representative range of *Bifidobacterium* and *Lactobacillus* strains, as well as other bacteria (i.e., *Bacteroides* spp., *Clostridium* spp. and *Escherichia coli*) (Eckburg et al., 2005; Marshall, 2008). In these cases, a carbohydrate was classified as a prebiotic if bifidobacteria metabolized oligosaccharides more efficiently than other bacteria (Bouhnik et al., 2004; Gopal et al., 2001; Kilian et al., 2002; Marshall, 2008). The paramount limitation of this methodology; however, is that the selected strains cannot truly simulate human colonic microbiota and thus should solely be used for initial screening purposes (Goodman et al., 2011). Alternatively, *in vitro* methods, entailing the use of fecal inocula (mixed cultures), for investigating prebiotic oligosaccharides have also been explored. In these cases, changes observed in populations of selected genera or species can establish whether fermentation is selective (Guinane and Cotter, 2013; Marshall, 2008). However, while the use of fecal inocula may be representative of events in the distal colon, it is in the proximal regions that saccharolytic activity is more likely to occur, and both the composition and metabolic behavior of the microbiota indigenous to the colon are variable depending on the region sampled (Marshall, 2008; Rastall and Gibson, 2015; Slavin, 2013). In addition, identification of the genera and species present in fecal inocula is highly challenging, making this approach insufficient for accurately simulating changes in the demographics of the intestinal microbiome. Emerging molecular-based methods of identifying bacteria are significantly more reliable, as evidenced by the recently implemented rapid, cost-effective DNA sequencing methods. In this context, the 16S rRNA gene has been most frequently targeted for compositional analysis of changes in colonic microflora, owing to its ubiquity in all prokaryotic cells, and the presence of variable sequence domains that allow for different taxa to be distinguished (Guinane and Cotter, 2013). While this approach provides data regarding the demographics of the intestinal microbiome, it lacks the ability to detect microbial viability or the functional potential of the populations present, a deficit that has recently been overcome by the application of shot-gun sequencing in metagenomic studies (Kurokawa et al., 2007; Qin et al., 2010).

### Emerging Prebiotic Candidates

Recently, numerous non-digestible carbohydrates exhibiting prebiotic potential have been identified; however, further human/animal trial data need to be acquired in order to determine whether these compounds fulfill the designation criteria proposed by Gibson and Roberfroid. Current scientific efforts are towards the implication of the compounds in minimizing health risks, such as those associated with diabetes, cardiovascular disorders, cancer, acute infection, inflammation, and obesity (Marshall, 2008; Singh et al., 2017; Slavin, 2013; Subin et al., 2017). Emerging prebiotic candidates include gentiooligosaccharides, a derivative of lichen, which has not only exhibited anti-cancer properties, but also stimulatory effects on *Bifidobacterium infantis* and *Lactobacillus*

*acidophilus* (Kothari and Goyal, 2015). Similarly, chitosan oligosaccharides have demonstrated stimulatory effects on *Bifidobacterium bifidum* and *Lactobacillus* sp., and are currently the subject of study for anti-tumor, anti-inflammatory, antioxidant, and immune-modulatory properties (Liu et al., 2008; Subin et al., 2017). Other prebiotic candidates include the complex heteropolysaccharide, pectin, as well as oligosaccharides derived from such (e.g., rhamnogalacturonooligosaccharides, arabinooligosaccharides, oligogalacturonides, etc.), as they have been observed to exert potent bifidogenic effects (Sulek et al., 2014). In addition to possessing prebiotic potential, these compounds have previously been found to confer anti-ulcer, anti-cancer, anti-obesity, and anti-inflammatory properties (Gomez et al., 2016). Xylooligosaccharides, constituting xylose monomeric units, also possess prebiotic potential, and are emerging in the agricultural, nutraceutical, and pharmaceutical industries as functional ingredients (Nabarlatz et al., 2007). Most recently, fucoidans (sulfated polysaccharides) naturally occurring in the cell wall of brown algae are being investigated to confirm their anti-tumor, immuno-modulatory, and anti-inflammatory bioactivities (Ale et al., 2011; Fitton et al., 2015). As the use of more refined and reliable technologies are applied to prebiotics research, the range of candidates is likely to expand. In designating non-digestible carbohydrates as prebiotics, it is necessary to take into consideration that these compounds are metabolized differently among probiotic bacteria, and that therefore, the extent of therapeutic efficacy is highly dependent on the type of prebiotic biomolecule consumed.

### Physiological Health Benefits of Consuming Prebiotics

Prebiotics are frequently studied for their roles in promoting overall consumer well-being, as they have been widely reported to have positive effects on gastrointestinal physiology (i.e., improved colonization resistance, reduced toxigenic microbial metabolism, increased mineral absorption, fecal bulking), lipid metabolism, glucose homeostasis, and general health (Adamberg et al., 2015; Dahech et al., 2013; Marshall, 2008), as evidenced in Table 2. Physiological effects of prebiotics (specifically non-digestible oligosaccharides) are primarily determined by their chemical structure, in particular the nature of the glycosidic bonds, level of solubility, viscosity, fermentability, and degree of polymerization (Marshall, 2008; Rastall and Gibson, 2015; Singh et al., 2017). However, the degree of efficacy also strongly hinges upon the composition of products from their utilization by colonic bacteria. For instance, it has been reported that high proportions of propionate to acetate from the fermentation of non-digestible oligosaccharides has

**Table 2** Health benefits of prebiotic consumption and/or supplementation

Scope of physiological effect	Prebiotic activity	Functional compounds	Models	References
Gastrointestinal	Bifidogenic	GOSs, FOSs, inulin, soybean oligosaccharides, neokestose	Human studies: infants and adults; animal studies: rodents; <i>in vitro</i> fermentation	Bouhnik et al., 2004; Kaczmarczyk et al., 2012; Kilian et al., 2002; Marshall, 2008
	Fecal bulking, contributing to constipation relief and protection against colorectal cancer	Inulin, oligofructose	Human studies: healthy adults; animal studies: rats	Nyman, 2002
	Inhibition of enteropathogens	Inulin, FOSs, GOSs, lactulose, raffinose	<i>In vitro</i> fermentation	Shoaf et al., 2006
	Prevention and treatment of acute diarrheal infections		Human studies: infants	Tuohy et al., 2003
Systemic	Modulation of lipid metabolism resulting in decreased serum TAGs and phospholipids	Inulin, oligofructose	Animal studies: male Wistar rats	Delzenne et al., 2002
	Reduced risk factors for cardiovascular disease and atherosclerosis; hypolipidemic effects; antioxidant activity	Levan polysaccharide	Animal studies: male rats	Belghith et al., 2012; Dahech et al., 2013
	Anti-inflammation (by decrease of inflammation-associated biomarkers)	GOSs, FOSs	Human studies: infants and adults; animal studies: rodents	Kaczmarczyk et al., 2012; McLoughlin et al., 2017
	Enhanced immune function in GALT resulting from increased antibody levels	FOSs, MOSs, inulin	Animal studies: chickens	Adhikari and Kim, 2017

FOS: fructooligosaccharide; MOS: maltooligosaccharides; GOS: galactooligosaccharide; TAG: triacylglycerol; GALT: gut-associated lymphoid tissue.

pronounced benefits on glucose and lipid metabolism (Nyman, 2002). Additionally, production of SCFAs contributes to a lower luminal pH, which has been implicated in the enhanced bioavailability and absorption of essential mineral nutrients (e.g., iron, magnesium, and calcium), as well as increased colonic mobility (Chen et al., 2016; Fernández et al., 2016).

Table 2 highlights the bifidogenic properties of most types of prebiotics characterized to date. Stimulation of bifidobacteria is beneficial for the host as these species operate several mechanisms, which inhibit the growth of common pathogens and infectious agents in the alimentary tract (Marshall, 2008; Rastall and Gibson, 2015; Roberfroid, 2007; Slavin, 2013; Sonnenburg et al., 2010). For instance, the intestinal microbiome of breast-fed infants, high in bifidobacteria, has demonstrated preventative effects against gastrointestinal conditions in comparison to those who were bottle-fed (Marshall, 2008). As research methodologies advance and human clinical trials and meta-analyses continue to reach scientifically-substantiated conclusions regarding the health benefits of consuming prebiotics (whether in the form of dietary supplements or from natural sources) their implications in overall consumer well-being are likely to expand beyond those summarized in Table 2.

## Enzymatic Approaches for Prebiotic Synthesis

Until recently, methods of obtaining prebiotic compounds primarily relied on extraction from natural sources and chemical synthesis/hydrolysis. These techniques are progressively receiving less scientific effort as, in general, extraction yields are low to exceptionally low, making them impractical for industrial applications (Itaya et al., 2007; Sangeetha et al., 2005), and chemical synthesis is an inefficient, laborious, multi-step endeavor due to the use of toxic polluting reagents and the challenge of manipulating the stereospecificity of glycosidic bonds (Michaud and Clermont-ferra, 2005; Palcic, 1999). In addition, due to the inevitable formation of brown contaminants from conventional thermal processes, chemical hydrolysis of polysaccharides to defined-size oligosaccharides is difficult to manipulate (Warrand and Janssen, 2007).

In comparison to the aforementioned methods, enzymatic approaches to the production of FOSs confer regiospecificity and stereospecificity to glycosidic linkages present in the end-products, and hence are more promising for the synthesis of commercial oligosaccharides with enhanced prebiotic potential (Plou et al., 2007). FOSs can be synthesized by  $\beta$ -fructofuranosidases (EC 3.2.1.26) or fructosyltransferases (EC 2.4.1), also referred to as fructansucrases (Antosova and Polakovic, 2002 (Miasnikov, 1997), which have been reported in plants, bacteria, and fungi. Fructansucrases comprise inulosucrase (EC 2.4.1.9) and levansucrase (EC 2.4.1.10), and are fructosyl-transferring enzymes that employ sucrose for the synthesis of inulin (Tungland, 2003) and levan (Li et al., 2016), respectively. Previous studies have mainly focused on levansucrases rather than on inulosucrases due to the broader range of acceptors, and hence wider range of expected end-products (Cote and Tao, 1990; Li et al., 2015; Seibel et al., 2006). It has been observed that certain fructosyltransferases exclusively catalyze the synthesis of FOSs, and not polymers (Antosova and Polakovic, 2002; Linde et al., 2012; Trujillo et al., 2001). Levan-degrading enzymes, referred to as levanses (2,6- $\beta$ -D-fructanohydrolase, EC 3.2.1.65), characteristically hydrolyze  $\beta$ -(2,6)-linked fructans consisting of more than 3 fructose units to fructose or various levanoligosaccharides (Lim et al., 1998; Mardo et al., 2017). They can further be classified according to two types: 1) exo-levanase (EC 3.2.1.64), and 2) endo-levanase (EC 3.2.1.65). Fructose is essentially the only product of levan hydrolysis by exo-levanases, while endo-levanases degrade levan into various fructooligosaccharides as the final hydrolysis products (Miasnikov, 1997). Typically, in addition to levan, these enzymes are also capable of hydrolyzing sucrose and inulin (Lim et al., 1998; Mardo et al., 2017; Miasnikov, 1997); however there are exceptions with regards to substrate specificity, as evidenced by the *Bacillus subtilis* 168 endolevanase characterized by Jensen et al. (2016), which does not possess the ability to hydrolyze inulin. Increased interest in levanses is primarily attributed with the prebiotic properties demonstrated by the products of their biocatalysis. Table 3 summarizes sources of the aforementioned prebiotic-synthesizing enzymes according to their functional classification, as well as their corresponding end-products.

**Table 3** Prebiotic-synthesizing microbial enzymes and their corresponding end-products

Enzyme	Sources	Prebiotic products	References
$\beta$ -fructofuranosidases (EC 3.2.1.26)	<i>Aureobasidium pullulans</i> , <i>Aspergillus niger</i> and <i>A. oryzae</i>	Short-chain FOSs	Fernandez-Arroyo et al., 2007; Shin et al., 2004; Sangeetha et al., 2005
Inulosucrase (EC 2.4.1.9)	<i>Lactobacillus johnsonii</i> NCC 533, <i>L. gasseri</i> DSM 20604, <i>Leuconostoc citreum</i>	Inulin-type short-chain FOSs	Anwar et al., 2008; Díez-Municio et al., 2016; Olivares-Illana et al., 2002
Levansucrase (EC 2.4.1.10)	<i>Pseudomonas syringae</i> , <i>Gluconobacter diazotrophicus</i> , <i>Zymomonas mobilis</i> , <i>Erwinia amylovora</i> , <i>Bacillus</i> spp., <i>Lactobacillus</i> spp., <i>Leuconostoc mesenteroides</i> )	Polymeric levan; levan-type FOSs	Beckers et al., 2003; Dahech et al., 2013; Mardo et al., 2017; Mendez-Lorenzo et al., 2015; Li et al., 2015; Vigants et al., 2003
Endolevanase (EC 3.2.1.65)	<i>Arthrobacter</i> spp., <i>Bacillus</i> spp., <i>Gluconobacter diazotrophicus</i> SRT4	Levan-type FOSs	Avigad and Bauer, 1966; Jensen et al., 2016; Mendez-Lorenzo et al., 2015; Miasnikov, 1997; Porras-Domínguez et al., 2014

To date, there has been limited investigation of the combined use of complementary/synergistic biocatalysts for the production of prebiotics. High concentration/yield of FOSs (i.e., up to 90%) have previously been obtained from the combined use of *Aureobasidium pullulans* KFCC 10524 fructosyltransferase and *Aspergillus niger* glucose oxidase (Yun and Song, 1993). A subsequent study on FOS production employing *Aspergillus japonicus* CCRC 93007  $\beta$ -fructofuranosidase and a commercial glucose oxidase achieved more than 90% (w/w) yield (Sheu et al., 2001). With the emergence of levan-type prebiotics, Tian et al. (2014) investigated and optimized the combined use of *B. amyloliquefaciens* levansucrase and a commercial endo-inulinase in one-step and two-step bi-enzymatic systems, achieving maximal FOS yields of 65% (w/w). Most recently, Porras-Domínguez et al. (2017) reported the production of levan-type FOSs (6-kestose, levanbiose, and blastose) up to 40% (w/w) from a fusion enzyme constituting *B. subtilis* levansucrase and *Bacillus licheniformis* endolevanase.

### Limitations of Commercial Prebiotic FOSs and the Shift Towards Levan-Type FOSs

Currently, the prebiotics industry is dominated by galactooligosaccharides and inulin-type FOSs: 1-kestose, nystose and fructofuranosylnystose (Rastall, 2010; Subin et al., 2017), which are hampered by major deficits, namely the lack of targeted functions, and most importantly selective fermentation by beneficial colonic bacteria at the species level (Manning and Gibson, 2004). This can be attributed to their relatively low molecular weights, which prevent them from reaching the distal colon, and limit their physiological effects exclusively to the proximal region (Rastall and Maitin, 2002; Van de Wiele et al., 2007). During digestion, as nutrients are transited to the distal colon, carbohydrate availability decreases, and proteins and amino acids become the dominant metabolic energy sources (Marshall, 2008). In this context, the primary products of fermentation are phenolic compounds, amines and ammonium (Manning and Gibson, 2004). These metabolites, which can be detrimental to host well-being, underline the health significance of tailored prebiotic preparations that can be competitively and selectively fermented by beneficial bacteria in the distal colon. In this regard, the functional properties of commercial prebiotic FOSs may be improved by employing sucrose analogues (Seibel et al., 2006), for instance, galactose (Baciu et al., 2005) as acceptors. This promotes the synthesis of FOSs with different terminal residues (i.e., galactose), which may possess anti-adhesive properties similar to those of galactooligosaccharides (Shoaf et al., 2006). The production of levan-type FOSs is becoming an increasingly attractive method of enhancing commercial prebiotic preparations. Recently, neokestose, a  $\beta$ -(2,6)-linked FOS, revealed higher bifidogenic effects than commercial FOSs (Marx et al., 2000; Kilian et al., 2002; Omori et al., 2010). Furthermore, Adamberg et al. (2015) observed increased growth of levan-degrading (e.g., *Bacteroides*) and butyrate-producing (e.g., *Faecalibacterium*) taxa following levan supplementation. These authors not only reported modulation of colonic microbiota in the presence of levan, but also that the principal products of its fermentation were acetate, lactate, butyrate, propionate, and succinate, which confer numerous targeted physiological benefits as previously discussed. Long-chain, levan-type FOSs have also been demonstrated to resist digestive degradation to a greater extent than their short-chain counterparts (de Paula et al., 2008; Marx et al., 2000; Rastall and Maitin, 2002). The production of longer-chain FOSs would facilitate prebiotic activity in the distal colon, which is more susceptible to colon cancer (Guinane and Cotter, 2013; Rastall and Maitin, 2002). Furthermore, highly polymerized FOSs are less likely to provoke intestinal discomfort (Fanaro et al., 2005). In general, current efforts in the development of prebiotic preparations are towards those which are structurally well-defined and demonstrate higher selectivity and colonic persistence than their first-generation, inulin-type predecessors (Rastall and Maitin, 2002).

### Applications of Prebiotic FOSs: Past, Present, and Future

Since the introduction of the prebiotic approach, one of the first and most prevalently available applications is fortified infant formulas (Marshall, 2008). Currently regarded as a class of GRAS (Generally Recognized As Safe) additives, prebiotic FOSs are being increasingly exploited in the food, nutraceutical, and pharmaceutical industries (Adhikari and Kim, 2017; Huebner et al., 2007; Reid, 2008; Singh et al., 2017). Research advances within this scope have allowed for the identification of key functional attributes, namely the low glycemic load and caloric density of prebiotic FOSs, which make them highly attractive for the food industry as functional ingredients in reduced-calorie products (Fernández et al., 2016; Roberfroid and Slavin, 2000). Furthermore, inulin and other FOSs possess the ability to extend shelf-life and aid in moisture retention (e.g., of bakery products) (Franck, 2002; Karimi et al., 2015; Padma Ishwarya and Prabhasankar, 2013). Generally, FOSs are more soluble than inulin and also impart a higher degree of sweetness, making them more desirable as sweetening agents and sugar substitutes (Fernández et al., 2016). Other food applications of prebiotics, either already commercially available or which are under formulation trials include: fortified dairy products; beverages and health drinks; confectionery products (e.g., chocolate); savory products (e.g., soups); sauces and dressings; meat products; non-perishable foods (e.g., canned and dried instant products); and dietary supplements (Celligoi et al., 2015; Fernández et al., 2016; Marshall, 2008; Slavin, 2013). Regarding the future use of prebiotics as therapeutic agents, additional applications will likely be elucidated as clinical trials and meta-analyses continue to reach concrete findings. For instance, several authors have conducted preliminary trials to determine the ability of prebiotics to prevent allergies in infants (Cuello-Garcia et al., 2017), and (Notay et al., 2017) recently reported the successful administration of prebiotics in the prevention of adult dermatological diseases, with promise for the combined probiotic and prebiotic treatments they will continue to investigate.

## Concluding Remarks

As the scope of knowledge regarding prebiotics continues to expand, methodologies for investigating their functionality, particularly in colonic microflora management, are becoming increasingly advanced and stringent. Clinical trials to determine the value of prebiotics in the mitigation of gastrointestinal and systemic health disorders are ongoing, as is the development of a quantifiable index to evaluate and compare therapeutic efficacy. The numerous drawbacks of natural extraction and chemical methods for producing commercial inulin-type prebiotics underline the demand for novel technologies, namely enzymatic synthesis of structurally-defined, controlled-size levan-type compounds. However, knowledge regarding the structural and functional attributes of enzymes exploitable for prebiotic synthesis remains limited to date, as are studies divulging the structure-function relationships of different prebiotics. These are undoubtedly the most commercially relevant barriers to the production of ideal prebiotic preparations, and will need to be overcome in order to characterize their complete range of physiological benefits and functional properties for industrial applications.

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# Bioactives From Seafood Processing By-Products

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## Introduction

Interest in full utilization of marine resources and processing by-products, especially their bioactive components, has increased considerably in recent years. According to Food and Agricultural Organization (FAO, 2016), the total global capture fishery production reached 93.4 million tonnes in 2014 including those from both marine (81.5 million tonnes) and inland (11.9 million tonnes) waters. It is predicted that fisheries could contribute to food security and adequate nutrition for world population. China is the major contributor of marine fisheries followed by Indonesia, United States and the Russian Federation (FAO, 2016). Fish is a rich source of proteins that contain essential amino acids, long chain omega-3 polyunsaturated fatty acids (PUFAs), vitamins (A, B and D) and minerals (calcium, iodine, zinc, iron, potassium, phosphorus and selenium). Average edible portion of fish is approximately 49%, on a dry weight basis, while by-products contributes up to 51%: these include head (18%), backbone/frames (10%), off-cut (10%), viscera (7%) and skin (6%) (Rustad, 2007). The utilization of world fisheries catch for human consumption has significantly increased, reaching 146 million tonnes in 2014 and the remaining 21 million tonnes were utilized for non-food applications such as fish meal and fish oil for use in different applications including aquaculture feed (FAO, 2016). Hence utilization of seafood processing by-products is most important for reducing waste in a safe and hygienic manner. For example, by-products such as heads, frames and fillet cut-offs are directly used as food or processed into fish sausage, fish cake, gelatin, snack and sauce. Moreover, other by-products could be used to produce biodiesel/biogas, enzymes (proteolytic enzymes, lipase), dietetic products (chitosan, glucosamine), pharmaceuticals (oils), natural pigments (astaxanthin and other carotenoids), minerals, cosmetics (collagen), silage and fertilizers, handicrafts, and leather (FAO, 2016). Several research reports have appeared on bioactives from seafood processing by-products (Shahidi and Synowiecki, 1991; Shahidi et al., 1994a, b, c, 1995, 1996, 2002; Shahidi, 1997, 2007a, b; Shahidi and Kim, 2002; Shahidi and Kamil, 2001; Rustad, 2003; Shahidi and Zhong, 2007; Ferraro et al., 2010; Roslan et al., 2014; Sila and Bougatef, 2016; Ambigaipalan and Shahidi, 2017).

## Proteins From By-Products

Generally, fish is a good source of protein. The amount of crude protein in fish flesh varies between 17% and 22% (w/w), while it is present at 8%–35% in fish by-products (Roslan et al., 2014; Sila and Bougatef, 2016). Fish proteins are a rich source of essential amino acids such as lysine and leucine as well as non-essential ones like aspartic and glutamic acids (Nunes et al., 2011). Protein-rich by-products include cut-offs, backbone, head, skin, milt, stomach, viscera and blood that could be used to produce collagen/gelatin and proteoglycan, protein hydrolysate, bioactive peptides, surimi, protein concentrate and fish silage, among others.

## Protein Hydrolysate and Bioactive Peptides

Protein hydrolysates are produced chemically or biologically by breaking down the proteins to peptides of varying sizes with bioactive peptides so produced generally containing 2–20 amino acid units but this range may vary in certain cases. Fish protein hydrolysates have desirable functional properties such as good solubility, foaming and gelling ability, emulsifying property and water holding capacity, hence contribute to the texture of food (Skanderby, 1994; Kristinsson, 2007). Bioactive peptides have been shown to exert antihypertensive, antagonists, immunomodulatory, anti-thrombotic, antioxidant, anti-cancer and anti-microbial activities (Clare and Swaisgood, 2000; Elias et al., 2008; Shahidi and Zhong, 2008). In addition, di- and tripeptides are absorbed more rapidly than free amino acids and even more rapidly than intact protein (Di Pasquale, 1997). Several studies have shown the utilization of marine by-products to produce protein hydrolysates and bioactive peptides (Table 1).

## Collagen and Gelatin

Seafood processing by-products are valuable sources of biomolecules such as collagen and gelatin. Utilization of fish collagen and gelatin in fish processing could produce value-added products and reduce any adverse impact on the environment (Kittiphattanabawon et al., 2005, 2013; Wang et al., 2014). Several marine by-products have been used as a source of collagen and gelatin such as fish skin, scales, bones and fins, mantle of scallops, the muscle layer of ascidians and the adductor of pearl oysters (Nomura et al., 1995; Mizuta et al., 2002; Kim and Mendis, 2006; Shen et al., 2007; Hernández-Briones et al., 2009). Fish collagen and gelatin have been widely used in food, pharmaceutical and cosmetic industries. A summary of marine by-products used for the production of collagen and gelatin is shown in Table 2.

**Table 1** Marine by-products used to produce protein hydrolysates/bioactive peptides

Marine by-products	Product	Activity	References
Shrimp shell	Protein hydrolysate		Synowiecki and Al-Khateeb, 2000
Alaska pollack skin	Gelatin hydrolysate	Antioxidant	Kim et al. (2001)
Yellowfin sole frame	Protein hydrolysate/ bioactive peptides	Antioxidant	Jun et al. (2004)
Alaska pollack frame	Protein hydrolysate/ bioactive peptides	Antioxidant	Je et al. (2005b)
Jumbo squid skin	Gelatin peptides	Antioxidant	Mendis et al. (2005)
Hoki frame	Protein hydrolysate/ bioactive peptides	Antioxidant, decreased t-butylhydroperoxide-induced cytotoxicity on human embryonic lung fibroblasts and protected free-radical-induced DNA damage	Je et al. (2005a), Kim et al. (2007)
Tuna backbone	Protein hydrolysate/ bioactive peptides	Antioxidant	Je et al. (2007)
Cod backbone	Protein hydrolysate	Antioxidant	Šližytė et al. (2009)
Sardinelle by-products (heads and viscera)	Protein hydrolysate/ bioactive peptides	Antioxidant	Bougatef et al. (2010)
Salmon skin	Collagen peptide	Improved glucose and lipid metabolism in diabetic and hypertensive patients	Cui-Feng et al. (2010)
Tuna frame	Bioactive peptide	Antihypertensive	Lee et al. (2010)
Chum salmon skin	Collagen peptide	reduced oxidative damage in the brain in mice	Pei et al. (2010)
Salmon by-product (pectoral fin)	Protein hydrolysate/ bioactive peptides	Antihypertensive	Ahn et al. (2012)
Atlantic salmon skin	Collagen hydrolysates/ Peptides	Antihypertensive	Gu et al. (2011)
Skate skin	Protein hydrolysate/ bioactive peptides	Antihypertensive	Lee et al. (2011)
Black pomfret viscera	Protein hydrolysate/ bioactive peptides	Antioxidant	Nazeer and Kumar (2011)
Bluefin tuna head	Protein hydrolysate	Antioxidant	Bougatef et al. (2012)
Pacific cod skin	Bioactive peptide	Antihypertensive and protected the cellular macromolecules from oxidative damage	Himaya et al. (2012)
Horse mackerel ( <i>Magalaspis cordyla</i> ) and croaker ( <i>Otolithes ruber</i> ) skin	Protein hydrolysate/ bioactive peptides	Antioxidant	Kumar et al. (2012)
Blacktop shark skin	Gelatin hydrolysate	Antioxidant	Kittiphattanabawon et al. (2012, 2013)
Cuttlefish skin	Gelatin hydrolysate	Antioxidant	Jridi et al. (2014)
Salmon pectoral fin	Protein hydrolysate/ bioactive peptides	Anti-inflammatory	Ahn et al. (2015)
Bluefin leatherjacket skin	Protein hydrolysate/ bioactive peptides	Antioxidant	Chi et al. (2015)
Seabass skin	Gelatin hydrolysate	Antioxidant, immunomodulatory, antiproliferative	Sae-leaw et al. (2016)
Shrimp shell discards	Protein hydrolysate/ bioactive peptides	Antioxidant, Antihypertensive	Ambigaipalan and Shahidi (2017)

## Proteoglycan

Proteoglycans are cartilaginous tissues produced from marine by-products that include glycosaminoglycans, namely chondroitin-4-sulphate and chondroitin-6-sulphate (Garnjanagoonchorn et al., 2007; Iozzo and Schaefer, 2015). Kato (2016) identified a method for extracting proteoglycans from fish cartilage using water to heat small particles of frozen fish. This method that uses only water for extraction was found to be efficient, and safe compared with using organic solvents, alkali or acid for extraction. Proteoglycan was extracted from salmon nasal cartilage using magnesium chloride and then isolated using anion-exchange chromatography. When prepared, it was found that the protein core was intact (Tatara et al., 2015). A new proteoglycan material was also made from shrimp waste and different binders. Testing showed that this method can be used to produce proteoglycans as feed supplement for monogastrics (Widjastuti and Haetami, 2015). Salmon nasal cartilage was examined and found to contain 14 types of proteoglycans, one of which, epiphykan (EPY) is a type of leucine-rich proteoglycan (Tatara et al., 2013).

**Table 2** Marine by-products used to produce collagen/gelatin

Marine source	By-product	References
Cod ( <i>Gadus morhua</i> )	Backbone	Gildberg et al. (2002)
Flounder ( <i>Platichthys flesus</i> )	Skin	Fernández-Díaz et al. (2003)
Black drum ( <i>Pogonia cromis</i> ) and sheepshead seabream ( <i>Archosargus probatocephalus</i> )	Bones and scales	Ogawa et al. (2004)
Baltic cod ( <i>Gadus morhua</i> ), salmon ( <i>Salmo salar</i> ), herrings ( <i>Clupea harengus</i> )	Skin	Kołodziejaska et al. (2008)
Channel catfish ( <i>Ictalurus punctatus</i> )	Skin	Liu et al. (2008)
Deep-sea redfish ( <i>Sebastes mentella</i> )	Skin, bone and scale	Wang et al. (2008)
Usky spinefoot ( <i>Siganus fuscescens</i> ), sea chub ( <i>Kyphosus bigibbus</i> ), eagle ray ( <i>Myliobatis tobijei</i> ), red stingray ( <i>Dasyatis akajei</i> ) and yantai stingray ( <i>Dasyatis laevis</i> )	Skin	Bae et al. (2008)
<i>Catla catla</i> and <i>Cirrhinus mrigala</i>	Skin, scales and fins	Mahboob (2015)

Proteoglycans have been shown to exert therapeutic benefits such as anti-angiogenic and anti-metalloproteinase effects as well as improving joint function and help in pain reduction in osteoarthritis patients (Morreale and Manopulo, 1996; Ronca and Palmieri, 1998; Iozzo and Schaefer, 2015).

## Marine Lipids

Marine lipids include marine fish oil and mammal oil as well as squalene, mainly from shark liver oil.

### Marine Fish and Mammal Oils

Marine by-products have been utilized for the production of fish oils which contain omega-3 PUFAs that provide a myriad of health benefits in humans (Shahidi and Miraliakbari, 2004, 2005; Shahidi and Ambigaipalan, 2015, 2018). Fish by-products from tuna (heads), herring, salmon (head and viscera) and walleye pollock (head, skin, frame and viscera) have been utilized for production of fish oil (Shahidi, 1994a,b; Chantachum et al., 2000; Aidos et al., 2002; Wu and Bechtel, 2008, 2009; Shahidi, 2007a,b,c). Rubio-Rodríguez et al. (2012) extracted fish oil using supercritical fluid extraction from cut offs of hake orange roughly and salmon as well as livers of jumbo squid. Chantachum et al. (2000) reported that tuna by-products obtained during canning process serve as a good source of docosahexaenoic acid (DHA)-rich oil.

On the other hand, marine mammals are a unique source of omega-3 PUFAs, where the lipids are mainly stored subcutaneously, known as blubber (Shahidi, 1998; Shahidi, 2007a,b,c). Wanasundara et al. (1998) reported that seal blubber oil is the only significant source of docosapentaenoic acid (DPA) in addition to eicosapentaenoic acid (EPA) and DHA. Shahidi (1998) reported that the distribution pattern of fatty acids in triacylglycerols (TAG) differs in fish and marine mammal oils, which greatly influence the metabolism, deposition, and potential health benefits. Long-chain  $\omega$ 3 PUFAs are mainly located in the sn-2 position of TAG in fish oils, whereas in marine mammal lipids they are predominantly in the sn-1 and sn-3 positions.

### Squalene

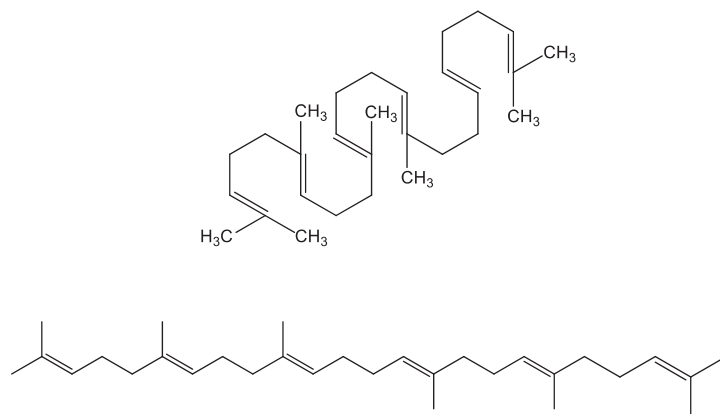
Squalene ( $C_{30}H_{50}$ ) is a highly unsaturated triterpenoid, an aliphatic hydrocarbon with six unconjugated double bonds. It is a thermally unstable and light-sensitive molecule (Fig. 1). Livers of deep sea sharks provide an excellent source of squalene (~89%) (Summers, 1987; Spanova and Daum, 2011).

Squalene is a metabolic precursor of cholesterol and other sterols. Squalene has been shown to exert antioxidant activity and prevent oxidation of lipids (Boskou, 1999; Warleta et al., 2010). It also acts as a moisturizer, wrinkle remover, protect skin from sun and UV damage and wound healer (Kaiya, 1990), chemopreventive and improves the immune system (Rao et al., 1998; Kim and Karadeniz, 2012).

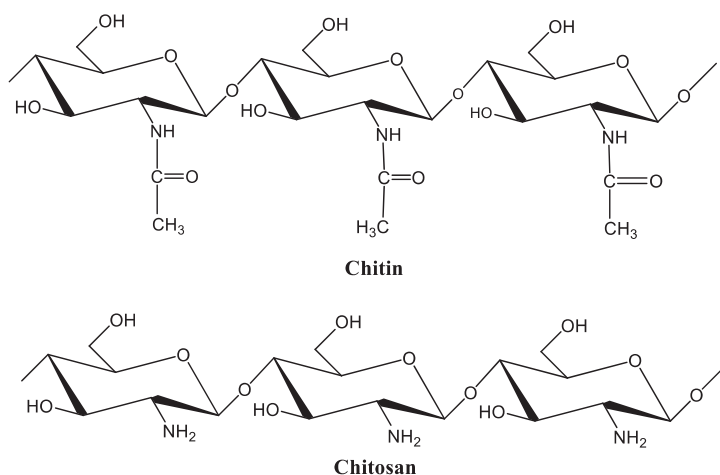
## Chitin, Chitosan and Their Oligomers and Monomers

### Chitin, Chitosan and Chitosan Oligomers

Chitin and chitosan polymers are natural amino-polysaccharides (Fig. 2). Chitin is made up of *N*-acetyl- $\beta$ -D-glucosamine units, while chitosan is derived from varying degrees of deacetylation of chitin (Shahidi, 2007a,b,c). Chitosan is a copolymer of chitin consisting of  $\beta$ -(1 $\rightarrow$ 4)-2-acetamido-D-glucose and  $\beta$ -(1 $\rightarrow$ 4)-2-amino-D-glucose units as the deacetylation of chitin is usually incomplete (Shahidi et al., 1999, Shahidi, 2007a,b,c).



**Figure 1** Chemical Structure of squalene.



**Figure 2** Chemical structure of chitin and chitosan.

Chitosan has been widely used in medicine and pharmacology, agriculture, food, biotechnology, nutraceuticals, bioremediation, gene therapy and cosmetics due to their biodegradability, non-toxicity, biocompatibility, physiological inertness and hydrophilicity (Shahidi et al., 1999; Jeon et al., 2000; Shahidi, 2007a,b,c; Hayes and McKeon, 2014).

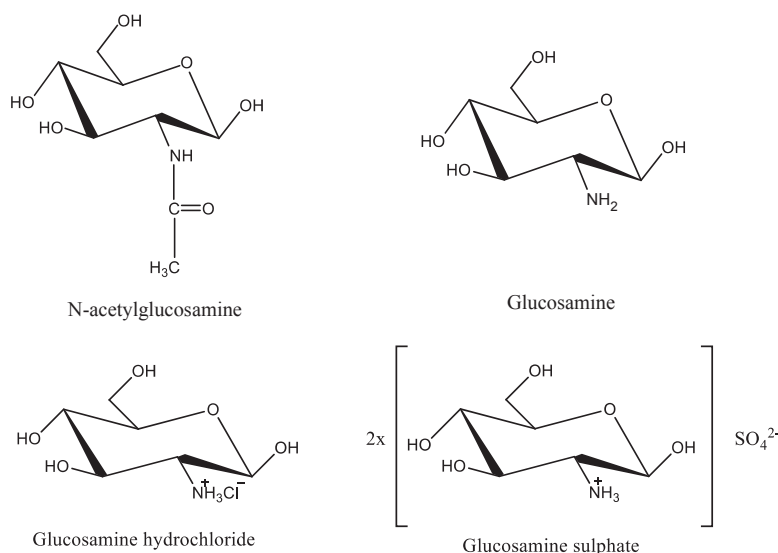
Chitosan oligosaccharides (COS) and low-molecular-weight chitosan (LMC) are partially depolymerized products of chitosan or chitin, using enzyme or acid hydrolysis. Kim et al. (2008) reported that the degree of polymerization (DP) and degree of acetylation influence the biological activities of COS and LMC. By-products from shrimp and different parts of crab have been shown to contain 17%–32% of chitin on a dry weight basis (Shahidi and Synowiecki, 1991). Recently, Hamed et al. (2016) reported that crustacean by-products are excellent sources of chitin, and chitosan, which is well known, but also of chitoooligosaccharides which may improve their industrial application.

### Glucosamine and N-Acetylglucosamine

Glucosamine (GlcN) and N-acetylglucosamine (GlcNAc) are the monomers of chitosan and chitin, respectively (Fig. 3). Glucosamine is most widely used to treat osteoarthritis due to its anti-inflammatory properties (Shikhman et al., 2001). Glucosamine used as pharmaceutical products is generally available in the form of hydrochloride salt or glucosamine sulphate (Fig. 3) (Shikhman et al., 2001). Chitin, chitosan, their oligomers and monomers are used as orthopedic/periodontal and wound-dressing materials, as well as for tissue engineering or controlled drug/gene delivery (Patrulea et al., 2015).

### Fish Bone Minerals (Calcium and Phosphorus)

Fish bone is a potential source for minerals, especially calcium and phosphorus (Kim and Mendis, 2006; Shahidi, 2007a,b,c). Few studies in the literature have shown the use of fish bone as a functional material and a source of minerals (Larsen et al., 2000; Kim



**Figure 3** Chemical structures of glucosamine and its derivatives.

and Jung, 2007). Jung et al. (2005) reported that fish meal prevents Ca deficiency in rat models due to the increased Ca bioavailability by the intake of fishbone phosphopeptides.

## Carotenoids

Crustacean processing discards are the major potential sources of carotenoids, mainly astaxanthin and its esters that occur as carotenoprotein and could be economically and feasibly recovered and used (Shahidi et al., 1998). Carotenoids are red, yellow, and orange pigments that include highly unsaturated hydrocarbon carotenes (C<sub>40</sub>H<sub>56</sub>; lycopene,  $\alpha$ -carotene,  $\beta$ -carotene, and  $\epsilon$ -carotene) and xanthophylls (cryptoxanthin, lutein, zeaxanthin, rhodovibrin, capsanthin, rhodoxanthin, violaxanthin, flavoxanthin, luteochrome, bixin and crocetin) (Fig. 4). Xanthophylls are oxygenated while carotenes are devoid of oxygen and are usually orange and red in colour and generally soluble in petroleum ether. Shahidi and Synowiecki (1991) reported that 3.4–14.7 mg/100 g of carotenoid pigments, mostly astaxanthin and its esters, were recovered from by-products of shrimp and different parts of snow crab. Charest et al. (2001) extracted astaxanthin from crawfish shells using supercritical CO<sub>2</sub>.

## Seafood Flavour

Seafood flavours may be extracted from finfish and shellfish processing by-products using aqueous extraction, fermentation and enzymatic hydrolysis (Lee, 2007). Free amino acids and peptides act as precursors of the volatile compounds, which are often formed through Maillard reaction (Izzo and Ho, 1992). Hayashi et al. (1981) reported that glycine and glutamic acid are the most important taste-active components of lobster and prawn. Seafood flavours are widely used in seafood sauces, chowders, soups, bisques, instant noodles, snacks and surimi products (Lee, 2007). It was shown that shrimp by-products (heads, shells, and tails) could serve as a potential source of flavorants in shrimp sauce (Kim et al., 2003). Several other studies have also shown the utilization of fish by-products for the development of seafood flavours (Peinado et al., 2016).

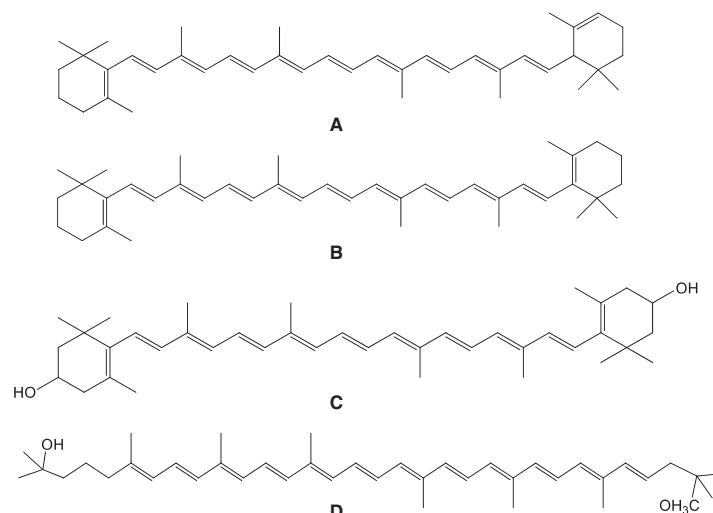
## Enzymes

Marine processing by-products, especially the glands and digestive organs such as stomach, pyloric cecum, pancreas, and intestines are major sources of enzymes (Nayak et al., 2003; Kurtovic et al., 2009). Several enzymes have been isolated from fish and shellfish processing discards such as alkaline phosphatase, hyluronidase, acetylglucosaminidase, chitinase and protease (Table 3, Venugopal, 1995a). Raa (1990) prepared and commercialized production of alkaline phosphatase from thaw water of shrimp.

## Marine Carbohydrates

Marine algae contain a large number of polysaccharides such as carrageenan, alginate, fucoidan and agar-agar that have been of industrial importance, especially in the food industry (Senanayake et al., 2011). Fucoidan, a sulphated polysaccharide derived





**Figure 4** The chemical structures of  $\alpha$ -carotene (a),  $\beta$ -carotene (b), zeaxanthin (c) and rhodovibrin (d).

**Table 3** Marine derived enzymes

Marine source	Enzyme	References
Lobster and shrimp processing by-products	Polyphenolase	Ferrer et al. (1989); Gallas-Galvan et al. (1999); Simpson et al. (1988)
Fish liver	Urease	Kinsella et al. (1985)
Atlantic cod viscera	Pepsin, trypsin, chymotrypsin, elastase, collagenase and alkaline phosphatase	Simpson and Haard (1984a, 1984b) Gildberg et al. (1990)
Carp and harp seal stomach	Chymosin	Cohen and Gertler (1981); Han and Shahidi (1995)
Hake stomach	Gastricsin	Sanchez-Chiang and Ponce (1981)
Fish intestinal viscera	Collagenases, elastases and carboxypeptidases	Shahidi and Kamil (2001)
Pyloric ceca/pancreas of Atlantic cod	carboxyl ester lipase	Gjellesvik et al. (1992)
Viscera (pyloric ceca, intestines, and associated mesenteries) of Grey Mullet	Lipase	Aryee et al. (2007)
Squid liver, shrimp waste silage and shrimp by-products	Chitinase	Matsumoto et al. (2004); Olsen et al. (1991)
Spleen of skipjack tuna, yellowfin tuna and tongol tuna	Trypsin-like serine proteinases	Klomklao et al. (2006)
Tilapia intestine	Aminopeptidase and carboxypeptidase	Taniguchi and Takano (2004)
Walley Pollock liver	Transglutaminase	Kumazawa et al. (1996)

from marine algae are effective coagulation modulators and have the potential to be used as alternatives to heparin, a conventional anticoagulant drug (Collic et al., 1994).

## Summary

Seafood by-products (cut-offs, backbones, heads, skin, milt, stomachs and viscera blood) are potential sources of bioactives (protein hydrolysates, bioactive peptides, collagen and gelatin, marine mammal and fish oils, squalene, chitin, chitosan and chitosan oligomers, glucosamine, fish bone minerals, carotenoids and various enzymes). These bioactives have been shown to possess a myriad of health benefits. More work is needed to explore high-value products for food, medicinal and cosmoceutical applications.

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# Phytosterols and Phytostanols

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## Glossary

**Hypercholesterolemia** a condition in which a patient's blood cholesterol levels are elevated, as indicated by a lipid blood panel.

**Coronary heart disease (CHD)** a disease where plaque accumulates in the arteries responsible for supplying oxygen-rich blood to the tissues of the heart.

**Hypocholesterolemic effects** effects which lower the amount of cholesterol in the blood.

**Thermally labile** susceptible to destruction/degradation by heat treatment.

**Atherogenesis** a dysfunction that occurs in artery walls characterized by a multi-step process whereby atherosclerotic plaques are formed.

**Atherosclerosis** a disease of the arteries characterized by the deposition of plaques on the inner walls.

**Micelles** aggregates of molecules in a colloidal solution, formed by differences in the polarities and charges of the substances in the solution.

**Micellar solubilization** the process of incorporating a substance into a micelle. This process facilitates the absorption of sterols and cholesterol.

**Myocardial ischemia** reduced blood flow to the tissues of the heart via a coronary heart disease. A consequence of CHD.

**Microencapsulation** the process of creating very small droplets or capsules with a coating around them, meant to protect the inner contents. Frequently used in food applications to protect capsule contents from degradation due to heat, oxidation, etc.

**Lyophilic colloids** a type of colloid which is "solvent-loving." Adding these to a solution will result in a stable solution due to the affinity of the colloid for the solvent.

**Colloidal/jet milling** a process that results in the reduction of the particle size of either a solid or a liquid of a suspension or emulsion. This process works on the rotor-shear force.

**Micronization** the process of reducing the average diameter of solid particles to obtain very fine micro-sized particles via mechanical means such as grinding or milling.

## Introduction

The term phytosterols (plant sterols) encompasses both phytosterols and phytostanols. These compounds are the most-studied group of nutraceuticals, with health benefits that have been recognized for more than seven decades. The earliest studies on phytosterols were conducted in the twenties and thirties, on various animal species including rabbits, mice, rats, cats and dogs (Pollak, 1952). These studies showed that simultaneous feeding animals with cholesterol and phytosterols resulted in inhibition of hypercholesterolemia induced by consumption of cholesterol. Consequently, researchers focused on phytosterols due their health benefit potential related to the risk reduction of coronary heart disease (CHD). Between 1950 and 1980, a pharmaceutical company, Eli Lilly, marketed phytosterols under the drug name Cytelin™. This product contained phytosterols derived from tall oil, and was supported by one of the first human clinical studies, where subjects consumed 9 to 50 g/day of Cytelin™ (Pollak and Kritchevsky, 1981). Over the years, phytosterols have been proven to be very effective in attenuating the risk of coronary heart disease, via their hypocholesterolemic effects, which are a function of their chemical structure. Furthermore, phytosterols have antioxidant and anti-inflammatory capabilities, which may also contribute to the risk reduction of CHD, albeit in the lesser extent (Jenkins et al., 2005; Van Rensburg et al., 2000). Over 200 clinical studies conducted globally support the health benefits of phytosterols and this has led to worldwide regulatory approval of these compounds for use in foods, dietary supplements and natural health products (Table 1). Furthermore, foods containing phytosterols are eligible to bear health claims in many regulatory jurisdictions (Zawistowski and Jones, 2015). There are many functional food categories enriched with phytosterols available in global markets. This chapter will provide an overview of phytosterols including their sources, chemical structure and properties, and the health benefits of consuming sterols. Additionally, the practical challenges encountered when formulating sterol-enriched food products are discussed in addition to the regulatory aspects of marketing phytosterol-containing foods in different countries.

## Occurrence and Manufacturing of Phytosterols

As the name suggests, phytosterols can be derived from many plant sources including fruits and vegetables, nuts, seeds, cereals, vegetable oils and coniferous trees. They occur in relatively small amounts – vegetable oils such as olive, and sesame oils contain between 2 and 10 mg/kg (Moreau et al., 1999). Other oils such as soybean, rapeseed and corn may contain 2 to 8 g/kg (Phillips

**Table 1** Examples of regulatory venues in various countries for food products enriched with phytosterols and/or phytostanols<sup>a</sup>

<i>Country</i>	<i>Regulatory venue</i>	<i>Type of health claim</i>	<i>Examples of health claim wording</i>	<i>Foods enriched with phytosterols that may bear the CHD health claim</i>
USA	GRAS Notification Dietary Supplement Health and Education Act (DSHA)	Disease risk reduction (the Nutrition Labelling and Education Act - Sterol/stanols and coronary heart disease claim)	“Foods containing at least 0.5 g per serving of plant sterols/stanols eaten with meals or snacks for a daily total intake of 2 g as a part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease. A serving of [name of the food] supplies X g of plant sterols/stanols.”	Conventional foods, which complied with requirements of the disqualifying nutrient levels (CFR 21.101.14 a 4), low saturated fat (21CFR 101.62 c 2), and low cholesterol (21CFR 101.62 d 2) rules and, the minimum nutrient contribution requirement (CFR 21.101.14 e 6) Dietary Supplements
European Union	Novel Food Regulations	Disease risk reduction (Regulation EC, 1924/2006) on Nutrition and Health Claims Made on Foods, Article 14)	“Plant sterols (stanols) have been shown to lower/reduce blood cholesterol. High cholesterol is a risk factor in the development of coronary disease”.	Yellow fat spreads; milk-type products with the addition of fruits or cereals or vegetable fat or protein; yogurt-type products; and other fermented milk-type drinks, soy and rice drinks, fat spreads, salad dressings, spicy sauces, and cheese-type products with fat $\leq$ 12% fat; rye bread ( $\geq$ 50% rye flour, $\leq$ 4% sugar, no added fat); edible oils, mayonnaise Unstandardized spreads, margarine, calorie-reduced margarine, mayonnaise, salad dressing and unstandardized salad dressings, yogurt, yogurt drinks, vegetable and fruit juices
Canada	Novel Foods Regulations (Food and Drug Regulations, Part B, Division 28	Therapeutic Claim	“Plant sterols help reduce/lower cholesterol. High cholesterol is a risk factor for heart disease”.	Edible oil spreads, breakfast cereals (excluding breakfast cereal bars), milk, and yogurt. Cheese and processes cheese (tall oil phytosterols only)
Australia and New Zealand	Novel Food Standard, Standard 1.5.1	Standard 1.2.7., Schedule 2 – High level health claims. Requires pre-approval by FSANZ	“With natural plant sterols which reduce cholesterol uptake”	Edible oil spreads, breakfast cereals (excluding breakfast cereal bars), milk, and yogurt. Cheese and processes cheese (tall oil phytosterols only)
Japan	Food of Specified Health Uses (FOSHU)	Function Claim	“Good for those concerned about serum cholesterol”, “Good for those having relatively high serum cholesterol and triglycerides with mild obesity” “Assisting blood lipids reductions”	Edible Oils, fat spreads, other food products and dietary supplements as long as approved under standardized FOSHU
China	Decree of State Food and Drug Administration No. 19 (Health Foods)	General Biological Function	“Assisting blood lipids reductions”	Milk drink, other food products as long as approved under the Resource Food Regulations
South Korea	Health Functional Food Act	Disease risk reduction, Structure function claim	“Phytosterols may reduce risk of coronary heart disease”	Green tea, coffee and milk drinks, cooking oils, dietary supplements
Taiwan	Health Food Control Act	Maintenance Claims	“Consumption of the product may help lower blood total cholesterol”	Milk enriched with tall oil phytosterols



Malaysia	Food Act and Regulation, Food Supplement	Nutrient Function Claims	“Plant sterol or stanol helps lower or reduce cholesterol”	Milk, and soy drinks
Indonesia	Drug and Food Control Regulations	Disease Risk Reduction	“May reduce risk of coronary heart disease”	Milk, smoothies, fat spreads, and foods with phytosterols approved in the US
Singapore	Food Regulation Act, Approval on case-by-case bases by Food Control Department of Singapore	Disease Risk Reduction	“Plant sterols/stanols have been shown to lower/reduce blood cholesterol. High blood cholesterol is a risk factor in the development of coronary heart disease.”	Fat spreads, salad dressing, mayonnaise, and low fat dairy products
Thailand	The notification for foods for special dietary uses	Nutrient Function Claims	“Plant sterol may help lower cholesterol”	Yogurt drink
Philippines	Food Fortification Act	Structure/function claim	“This product contains natural plant sterols that help lower cholesterol”	Milk, milk and yogurt drinks
Brazil	Technical regulation on procedures for registration of foods and or new ingredients	Structure/function claims	“Phytosterols help in reducing the absorption of cholesterol.” “helps to maintain healthy level of cholesterol when associated with a healthy diet and life style”	Fat spreads, yogurt, and milk

Note: the term of phytosterols/phytostanols includes free sterols and stanols, as well as sterol and stanols esters.

<sup>a</sup>Adapted from [Zawistowski and Jones \(2015\)](#).

**Table 2** Phytosterol composition of selected edible oils<sup>a</sup>, crude tall oil<sup>b</sup>, cereals, vegetables and fruits<sup>c</sup> (mg/100g)

Sample	Sitosterol	Campesterol	Stigmasterol	Avenasterol	Stanols	Total
Soybean oil	123.9–158.2	34.2–55.1	36.9–60.2	–	55.6.7	203–285
Rapeseed oil	363.8–395.0	202.6–237.6	2.5–3.5	17.2–36.0	2.0–2.9	715–736
Corn oil	463.0–509.4	124.1–138.9	52.3–58.5	35.1–41.3	22.6–26.5	699–766
Crude tall oil	3523–8089	386–1320	8–66	–	535.5–1618	5000–10,000
Barley	43.7–48.4	15.0–19.2	2.4–3.6	5.6–6.9	1.7–1.9	72.0–80.1
Millet	37.1	11.2	1.8	8.7	–	77
Rice	37.5	14.6	10.4	2	3.2	72.3
Broccoli	28.5–31.0	6.7–6.9	0.8–1.1	0.2	1.8	36.7–39.0
Carrot	7.0	1.0	3.0	–	–	12
Lettuce	5.0	1.0	4.0	–	–	10
Tomato	3.0	1.0	3.0–3.5	–	–	7
Apple	13.0–15.7	0.4–0.9	0.1	0.7	0.8	13.0–18.5
Banana	11	2	3	–	–	16
Raspberry	23.3	0.9	–	1	0.2	27.4

<sup>a</sup>Adapted from Phillips et al. (2002).<sup>b</sup>Author's unpublished data.<sup>c</sup>Adapted from Piironen and Lampi (2004).

et al., 2002). Some specialized oils such as those extracted from corn fiber may contain up to 10 g of sterols per 100g of oil (Moreau, 2005). Cereals, nuts, vegetables, berries and other fruits contain sterols in the range of 0.05–0.22 g/100 g (Piironen et al., 2000). Table 2 shows a list of common phytosterol sources and their respective phytosterol contents. Approximately 200 plant sterols exist but the most predominant are:  $\beta$ -sitosterol, campesterol, stigmasterol, and in a lower degree, their saturated counterparts - stanols (Piironen et al., 2000).

The most common sources of plant sterols for commercial production are tall, soybean and rapeseed oils. Sterols from these sources occur as by-products of the manufacturing of other products. Tall oil is produced from the processing of coniferous trees; sterols from soybeans and other oilseeds are obtained from the production of vitamin E (Quilez et al., 2003). Phytosterols may also be obtained during the production of biodiesel fuel, which in turn, uses a variety of vegetable oils. Since the sterols are produced as a by-product of these processes, they are not produced in very large quantities; 2500 tonnes of vegetable oil or the same volume of coniferous trees are required to yield just one tonne of phytosterols. The exception to this is when sterols are manufactured directly from the deacidification of rapeseed oil, or from deodoriser distillates that are created during fatty acid methyl ester (FAME) production (Verhe et al., 2007). In these cases, about 54 kg can be produced from only one tonne of distillate (Verhe et al., 2007). Despite these small yields, manufacturers who already process these materials can profit from extracting the sterols from their post-production materials. For example, breweries and distilleries can derive 100 tonnes of sterols from 18 000 to 65 000 tonnes of grain residues (Wong, 2008).

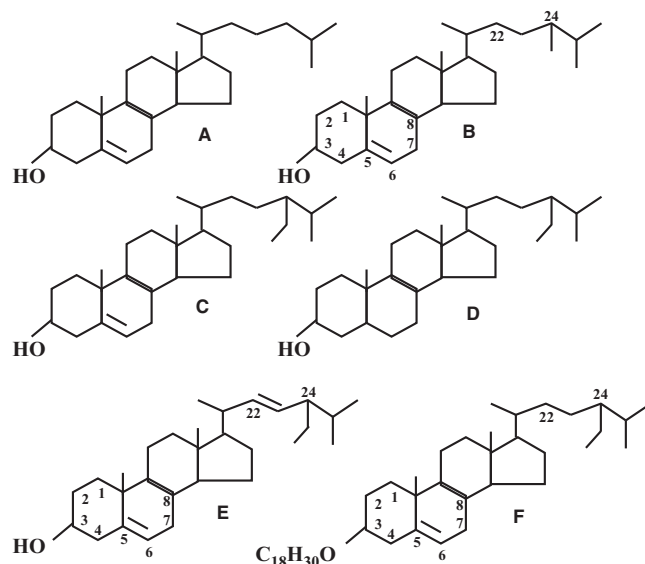
## Chemistry and Properties of Phytosterols

Phytosterols are triterpenes, which are lipid-like compounds that are closely related to cholesterol, except sterols are more hydrophobic. A comparison of the structures of various phytosterols and cholesterol can be found in Fig. 1. This is attributable to the presence of a side chain substitution of a methyl or an ethyl group on carbon 24. The methyl-substituted variation is campesterol, and the ethyl-substituted configuration is called sitosterol. Stigmasterol has an additional double bond at position 22 on a side chain. The similar chemical structures of cholesterol and sterols are reflected in their closely-related functions in the organisms from which they originate; cholesterol stabilizes and regulates the cell membranes of animals and phytosterols perform a similar function in plants. In nature, phytosterols exist as free alcohols; steryl esters with the fatty acid moiety located at C12–C18, steryl glucosides and acetylated steryl glucosides (Moreau et al., 2002; Ferrer et al., 2017). In terms of manufacturing functional foods, sterols and steryl esters are the most important.

Stanols are a type of phytosterol formed from the hydrogenation of plant sterols. Thus, campestanol is the saturated counterpart of campesterol while sitostanol is the saturated counterpart of sitosterol, and both are formed through hydrogenation of the 5 $\alpha$ -position double-bond. Moreover, hydrogenation of stigmasterol yields stigmastanol, which is the same as sitostanol, since both double bonds at the 5 and 22 positions are saturated.

## Solubility

Due to their lipophilic nature, phytosterols exhibit minimal solubility in aqueous solutions. Their solubility in fats and oils will depend on the properties of the fat/oil as well as the temperature. Generally, phytosterols are better solubilized in more polar oils such as diacetyl glyceride oil and this can be attributed to the partially polar nature of phytosterols, which is imparted by the hydroxyl group. It is possible to incorporate 10%–20% of phytosterols into vegetable oil at 50–80 °C but upon cooling, the



**Figure 1** Chemical structure of the main phytosterols and cholesterol. a – cholesterol, b–campesterol, c –  $\beta$ -sitosterol, d –  $\beta$ -sitostanol, e – stigmasterol, f –  $\beta$ -sitosteryl ester.

phytosterols will crystallize (Engel and Schubert, 2005). Below 50 °C, the solubility of phytosterols decreases even further; at these temperatures in corn oil, solubility is limited to 2%–6%. However, it has been shown that sterols and mixtures of sterols and stanols exhibit improved solubility when compared to stanols alone. Different stanols have varying levels of solubility; wood stanols tend to solubilize better than those from soybeans (Vaikousi et al., 2007). When water is added to oil/lipids, this decreases the solubility of phytosterols by two-fold due to the formation of monohydrate molecules (Christiansen, 2002).

Another factor which contributes to the generally poor solubility of phytosterols is their melting point, which is in the range of 138–145 °C (Vaikousi et al., 2007). There is an inverse relationship between the log solubility and melting points for many aromatic compounds (Yalkowsky, 1981).

In order to improve fat solubility, esterification of phytosterols with long-chain polyunsaturated fatty acids was introduced in the late 1980's (Mattson et al., 1977). Ester is formed via the hydroxyl group at the position 3 (Fig. 1). This technique increased the solubility of phytosterols in fat by ten-fold and enabled the incorporation of sterols into fat-based food products such as vegetable fat spreads (Ostlund, 2002). Among phytosterol esters, those with a larger number of double bonds and a longer fatty acid chain length are more soluble (Noakes et al., 2005). These features are also associated with a decrease in the melting point, which makes these phytosterols easier to incorporate into the food matrix. Commercial sterol esters with long chain polyunsaturated fatty acids have melting points in the range of 25–45 °C (ADM, 2007; Cognis, 2007). Even esterification with medium-chain fatty acids can reduce the melting point of phytosterol: the esterification of stanols with C8–C12 fatty acids was able to reduce the melting point of the stanols by about 40 °C (Vaikousi et al., 2007).

Some phytosterols, including avenasterol and fucosterol, feature an ethylidene side chain on their structures (Boskou, 1998; Guillen and Manzanos, 1998). This is significant because it gives the sterols antioxidant capabilities and they may inhibit the thermal oxidation of various oils; for example, avenasterol is able to prevent the deterioration of soybean oil when heated to 180 °C (White and Armstrong, 1986). This is most likely because free lipid radicals react with phytosterols at allylic carbon atoms, forming stable isomers and slowing down further oxidation by interrupting the chain of auto-oxidation. Not all sterols have the same antioxidant capacity, however; in the example above, avenasterol is able to exert antioxidant effects, but  $\beta$ -sitosterol is not because it lacks a double bond on a side chain. It is also possible that some phytosterols actually have pro-oxidant effects, such as stigmasterol (Yoshida and Niki, 2003).

### Thermostability

Phytosterols, as antioxidants, undergo oxidation to spare more thermally labile compounds such as polyunsaturated fatty acids. However, heating may cause reduction of phytosterol content, and lower or even eliminate their efficacy. In addition, oxidized phytosterols may be harmful to health in the same way as oxidized cholesterol (Dutta, 2002).

Sterols are relatively thermoresistant as has been demonstrated by several studies. Continuous frying in various oils for 9 h caused phytosterol losses in the range of 4%–6% (Winkler et al., 2007). Similarly, commercial frying for two days only reduced phytosterol content slightly, in the range of 6%–8% (Dutta and Appelqvist, 1996). When subjected to extreme heat treatment, changes in phytosterol content depend on the type of oil. For example, when various oils were continuously fried for 35 h, phytosterol content in corn, soybean, and expeller-pressed soybean oils decreased by 15%, 9.4% and 4%, respectively (Winkler et al., 2007). This is likely due to the differences in saturation of the fatty acids that these oils are composed of. Unsaturated fatty

acids are oxidized faster, providing some thermal protection to phytosterols (Ghavami and Morton, 1984; Soupas et al., 2004). For this reason, stanols, the saturated counterparts of sterols, are more thermoresistant and losses of stanols upon thermal treatment were significantly lower than those of sterols.

## Health Benefits of Phytosterols

The blood lipid profile is a screening tool which is used to assess a patient's risk of developing cardiovascular disease. This profile examines the amount of triacylglycerides (TAGs) as well as the amounts of various lipoproteins in circulation (NHLBI, 2018). A high level of TAGs in the blood is a risk factor for cardiovascular disease because they promote atherogenesis. Lipoproteins are significant because the levels of cholesterol in the blood depend on their cholesterol-transporting activity. Since cells are not able to destroy excess cholesterol, they must transport it instead (NHLBI, 2018). Low-density lipoprotein (LDL) is a lipoprotein, which is known to be harmful because it transports cholesterol from the liver to the bloodstream and then to peripheral tissues. Furthermore, it is susceptible to oxidation which also promotes atherogenesis. Meanwhile, high-density lipoprotein (HDL) transports excess cholesterol in the opposite direction to reduce the amount of cholesterol in the blood, and bring it to the liver, where it is converted to bile salts (NHLBI, 2018). Therefore, it is desirable to have lower levels of TAGs and LDL and a higher level of HDL in the blood.

Cholesterol in the intestine originates from three sources: diet (200–400 mg/day), bile (up to 1 g/day) and exfoliated enterocytes (300–400 mg/day) (Northfield and Hoffman, 1975; Vuoristo and Miettinen, 1985). An estimated 50% of this amount is absorbed into the intestinal lumen, re-esterified by intestinal cells, formed into chylomicrons and secreted into the bloodstream (Turley and Dietschy, 2003). This amount may be modified by genotype or by diet (Sehayek, 2004). The consequence of a high level of cholesterol in the blood, or hypercholesterolemia, is increased susceptibility to atherosclerosis (Law et al., 1994). Data from epidemiological studies suggest that lowering LDL (LDL-C) cholesterol by 10% to 19% reduces the risk of CHD by about 41% (WSCP, 1998). It has been estimated that lowering blood cholesterol by only 10% may prevent approximately 100,000 deaths annually (Havel and Rapaport, 1995). This can be achieved by a dietary intervention using phytosterols. In fact, phytosterols are recommended as a part of adjunctive lipid-lowering therapy by the US National Cholesterol Education Program (NCEP, 2001).

## Hypocholesterolemic Effects

### Effect on LDL-Cholesterol

The most notable health benefit of consuming phytosterols is the reduction in risk of CHD achieved through decreased blood cholesterol. This effect has been studied and recognized since the early 1950's. The optimal efficacious dose is 2 g of phytosterols consumed daily over three to four weeks. Initial research on the efficacy of stanols when compared to sterols revealed that stanols are more potent, and practically unabsorbable, which make them better hypocholesterolemic nutraceutical than stanols (Heinemann et al., 1986). However, later research showed that sterols were equally as effective as stanols, if they were a component of a food (Hallikainen et al., 2000; Heinemann et al., 1986; Judd et al., 2002; Vanhanen and Miettinen, 1992; Vuorio et al., 2000).

As mentioned earlier, the amounts of sterols, which naturally occur in plant-based foods such as vegetables, vegetable oils, nuts and seeds, are negligible in comparison to the 2 g/day required to observe an optimal hypercholesteremic effect. A wide variety of products have been formulated to be a better phytosterol delivery system and these are a more effective way to take advantage of the hypocholesterolemic effects of these bioactives. Functional food products have been studied extensively as the subjects of clinical trials, which often examine the effects of phytosterols on total and LDL cholesterol. Dairy drinks such as single-shot yogurt and milk have shown the highest efficacy among clinically-tested sterol-containing beverages. The cholesterol-lowering effect of sterol-containing foods depends on the matrix. For example, milk enriched with phytosterols is about three times more efficacious than bread or cereals containing the same amount of sterols (Clifton et al., 2004). Other factors which may impact the efficacy of phytosterols include the dose of sterols, the genotype of the clinical subject and if the drink is consumed with food. Generally, consuming the enriched food product with meals will result in a greater reduction in LDL-C than when consumed alone (Seppo et al., 2007). It has been conjectured that this is due to the increased bile flow that occurs with the energy and fat intake from meals, which increases the efficacy of sterols (Seppo et al., 2007). It had previously been believed that for sterol-enriched foods to be effective, they needed to have a high level of fat but this was disproven by more recent studies, and it is now known that low-fat products such as low-fat fermented milk can be just as effective as other, high-fat matrices (Clifton et al., 2004; Hansel et al., 2007; Plana et al., 2008). Frequency of consumption of sterol-containing dairy drinks has not been shown to make a significant difference in hypocholesterolemic effects (Niittynen et al., 2008). It has been shown, however, that the method of incorporating sterols into no- or low-fat foods and beverages can affect efficacy; when emulsifiers were used, the efficacy of the sterols was improved compared to sterols incorporated into foods without emulsifiers (Noakes et al., 2005). Commonly used for this purpose is lecithin, which prevents the crystallization of sterols and makes them more soluble in the mixed micelles, thereby improving efficacy (Engel and Schubert, 2005).

### Effect on Other Blood Lipids

It was previously thought that the consumption of functional foods with phytosterols did not impact the levels of TAGs and HDL in blood and rather, that the sterols exert their effect only through the modulation of LDL-C. However, recent clinical studies have shown that sterols consumed in low-fat yogurt drinks and fermented milk (1.6 g of free sterol equivalents) may reduce the levels

of circulating TAG by 14% as compared to control (Plana et al., 2008). It does appear that the magnitude of this effect depends on the initial baseline concentration of TAG, with normolipidemic subjects exhibiting almost no change in their circulating TAGs when treated with sterol-containing beverages. Significant reduction of plasma TAG was shown in patients with dyslipidemic metabolic syndrome. It was suggested that this was associated with lowering of hepatic large TAG-rich very low-density lipoprotein particles, which are present in blood of metabolic syndrome patients (Plat and Mensink, 2009). Meta-analysis of twelve clinical studies by Demonty and co-workers (2013) revealed that consumption of phytosterols by subjects with normal to borderline high TAG levels modestly lowered plasma TAG by 6%.

Recently, a few studies have also shown that phytosterols can cause modest increases in HDL-C, potentially via an increase in a specific sub-fraction of HDL, the HDL3-C (Ruiu et al., 2009). The plasma level of HDL-C is also increased by 7.5% when consumption of sterol-containing margarine (1.8 g of free sterol equivalents) is combined with physical activity in previously sedentary hypercholesterolaemic patients (Varady et al., 2004). The effects of phytosterols on HDL-C and TAG are not as extensively studied as their effects on LDL-C and thus, the factors that modulate their efficacy are not clear. These may include food matrix, baseline blood lipid profile of the clinical subjects, among others, and these should be further studied to optimize the health benefits of sterols.

### **Mechanism for Hypocholesterolemic Action**

As mentioned before, phytosterols have similar chemical structures to cholesterol and this is the basis for the hypercholesteremic effects of sterols. They are similar enough that they both must undergo the same process of micellar solubilization in order to be absorbed, which allows phytosterols to compete with cholesterol for these micelles (Gylling and Miettinen, 1999; Hendriks et al., 1999). However, a key difference between the structures of phytosterols and cholesterol afford plant sterols an advantage whereby they are preferentially incorporated into micelles (Gylling and Miettinen, 1999; Hendriks et al., 1999). Sterols have a higher affinity for micelles than cholesterol due to a bulkier hydrophobic side chain (Fig. 1). This results in the displacement of cholesterol from micelles, reducing its absorption and promoting its excretion from the body (Gylling and Miettinen, 1999; Hendriks et al., 1999). It should be noted that this mechanism applies to both free sterols and sterol esters because the latter are hydrolyzed by pancreatic cholesterol esterase in the intestine.

Furthermore, phytosterols are minimally soluble in the intestinal chyme, which interferes with the micellar solubility of cholesterol. This results in the co-precipitation of cholesterol and phytosterols in the intestinal lumen, which reduces the bioavailability of cholesterol.

Similar to how sterols compete with cholesterol for micelles, they also compete with cholesterol for esterification in enterocytes which is a prerequisite for absorption into the bloodstream (Moghadasian, 2000). They also compete for various transporters, present in the apical surface of enterocytes: Niemann-Pick C1-like 1 protein (NPC1L1), and ATP cassette protein binding transporters (ABCG5/G8), which regulate the influx and efflux of cholesterol and sterols between the intestinal lumen and the intestinal mucosa (Calpe-Berdiel et al., 2006). These mechanisms are less significant than the previously mentioned ones but they do contribute to the reduction of cholesterol absorption into the blood stream.

The decrease in cholesterol absorption caused by these mechanisms induces compensatory cholesterol synthesis in the liver but this is not sufficient to offset the decrease in cholesterol caused by sterols. The overall effect will still be a decrease in circulating cholesterol levels.

### **Antioxidant and Anti-inflammatory Effects**

Sterols also produce antioxidant and anti-inflammatory effects, which help to decelerate the processes of atherosclerosis and CHD.

#### **Antioxidant**

Oxidative stress is caused by free radicals generated by molecules with unpaired electrons. Free radicals may lead to membrane structural damage and dysfunction as well as increasing the risk of myocardial ischemia due to decreased membrane fluidity and permeability. Free radical scavengers are those compounds, which can accept electrons and act as free radical scavengers that demonstrate antioxidant properties. Sterols such as  $\beta$ -sitosterol have been reported to have protective effects against free radical damage due to its ring structure that includes an acetyl group and the presence of oxygen that accepts electrons (Van Rensburg et al., 2000). The antioxidant effect of  $\beta$ -sitosterol is even more pronounced in  $\beta$ -sitosterol glycoside due to the presence of glucose (Van Rensburg et al., 2000). Other studies have noted that  $\beta$ -sitosterol also inhibits the oxidation of LDL-C in vitro.  $\Delta^5$ -avenasterol, a substantial sterol in oats; and fucosterol, from algae, also have antioxidant properties due to the presence of an ethylidene group (Boskou, 1998; Moreau et al., 2002). In a study done on mice, a mixture of phytosterols was able to increase the activity of various antioxidant enzymes (Moghadasian et al., 1999). The same study found that sterols were more effective at preventing atherosclerotic lesions than probucol (Moghadasian et al., 1999). The authors postulated that the increased antioxidant enzyme activity caused by the sterols may have contributed to the athero-protective effect observed (Moghadasian et al., 1999). The antioxidant properties of phytosterols do not only positively contribute to health, but also prevent oxidative damage in various food matrices. Sterols inhibited the thermal oxidation of olive oil, soybean oil and medium-chain triglyceride oils, which were heated at 180 °C (Du and Zawistowski, 2002; Gordon and Magos, 1983; White and Armstrong, 1986; Winkler et al., 2007). Finally, the antioxidant properties of sterols may contribute to antimicrobial activities, as is the case with other antioxidants. This was demonstrated in milk, as the incorporation of a mixture of coniferous sterols was found to extend the shelf life (Monu et al., 2008). There also exists

preliminary evidence to suggest that  $\beta$ -sitosterol obtained from several plants possesses antimicrobial activity against *Bacillus subtilis* and *Candida albicans* (Beltrame et al., 2002; Moshi et al., 2004).

### Anti-Inflammatory

Inflammation is believed to contribute to atherogenic plaque formation and consequently, the risk of heart disease and stroke. Therefore, current clinical studies of sterols, in addition to investigating their hypocholesterolemic efficacy, are also focused on their anti-inflammatory properties. The anti-inflammatory effect is studied by monitoring the levels of C-reactive protein (CRP), a recognized inflammation bio-marker (Devaraj et al., 2006). It appears that the consumption of foods containing phytosterols alone is not sufficient to cause a reduction in CRP but sterols as part of the portfolio diet were found to reduce CRP levels (Jenkins et al., 2005; Jenkins et al., 2008). Clinical studies have found that the anti-inflammatory effect is even more pronounced when the phytosterols are consumed with omega-3 fatty acids and aside from the reduction in CRP, other markers of inflammation such as interleukin-6, tumor necrosis factor- $\alpha$  and leukotriene B4 were also reduced (Micallef and Garg, 2009).

## Challenges in Food Product Formulation With Phytosterols

Free phytosterols are available commercially in crystalline form. These can present several challenges for food product formulation. The main challenge is the insolubility of sterols in water and their sub-optimal solubility in lipids. In addition, sterols may negatively impact the sensory attributes of products because of their waxiness, grittiness and powder-like qualities.

The poor solubility of phytosterols can be overcome by using phytosterol esters, which are in the form of a semi-liquid paste. The process of stanol esterification was patented by Finnish food manufacturer, Raisio Oy., in 1989 and this led to the development of the very first stanol ester-containing functional foods. The first products to be marketed which contained phytosterols were margarines and vegetable fat spreads. In 1995, Raisio Oy. developed a margarine called Benecol for the EU market and this initiated the widespread marketing and popularity of functional foods in the EU and subsequently, the US. Sterol esters are more soluble in fat and have a lower melting point so they are more easily incorporated into foods. However, sterol esters present unique challenges not encountered with free phytosterols. They are more thermally labile when compared to free phytosterols. Sterol esters are also more likely to produce oxidative products when subject to extreme thermal treatments (Zawistowski, 2010). In terms of the nutritional quality of the final product, sterol esters may pose a problem because they contain added fat, so more fat must be added to a product to achieve the same levels of sterols. This is a challenge in low-fat products especially, where the formulator must keep the fat content below a certain threshold such as in a low-fat vegetable oil spread.

## Challenges and Solutions Pertaining to Beverage Formulation

Beverages are increasing in popularity as they are a more convenient source of phytosterols and are more easily incorporated into the daily diet when compared to more traditional sources such as fat spreads. Due to their liquid form, they are also associated with unique formulation challenges, which are discussed in this section.

Uniform distribution of the sterols throughout the liquid product requires a stabilization system because without one, the free sterols will sediment or float within the matrix. They may also aggregate and form non-dispersible clusters. For this reason, it is necessary to use sterols with the smallest particle size possible to improve solubility and stability, preferably between 15–20  $\mu\text{m}$ . Smaller particles are advantageous because they have more surface area per unit of volume and therefore, they are more likely to stay in solution. They also contribute to improved mouthfeel, as larger particles are perceived as having a gritty texture.

As most issues surrounding the formulation of sterol-enriched beverages are related to the particle size of the sterols, most solutions present various methods of minimizing particle size. This can be achieved by mechanical grinding (including dry milling and micronisation) as well as wet milling (including colloidal/jet milling and microfluidization). Microfluidization, also known as particle collision technology, is a method in which phytosterols are processed in high concentration slurries to create uniform dispersions comprising particles of less than 10  $\mu\text{m}$  (Zawistowski, 2003). Nanotechnology is a novel approach to provide solutions for creating sterol-fortified beverages and can make phytosterols dispersible even in clear beverages (Yoon et al., 2003). In this method, sterol micelles of a small size (preferable below 300 nm) are formed (Yoon et al., 2003). Phytosterol nanoparticles are not currently available on the market due to safety concerns regarding the enhanced absorbability of sterols in this form. Some authors suggest that absorbed phytosterols can actually increase the risk of CHD (Weingartner et al., 2009) and while these findings are not conclusive, increased sterol absorption should be avoided, especially as this does not increase their efficacy.

Most other methods for formulating beverages with phytosterols involve the addition of lyophilic colloids to stabilize the suspension. These may include carrageenan, alginate, guar gum, and xanthan gum. Other stabilizers include microcrystalline cellulose and emulsifiers such as polysorbates, sucrose-fatty acid esters, lecithin and protein (Zawistowski, 2003; Moreau, 2004). One technique was developed where lecithin micelles were used to formulate stanol-enriched fat-free beverages (Ostlund et al., 1999). The presence of lecithin allowed for the use of only 300 mg of stanols to achieve comparable efficacy to the optimal 2 g/day (Ostlund et al., 1999). However, lecithin is expensive, which limits its commercial applications. Another novel approach used to increase the aqueous solubility and stability of sterols was recently developed, and it involved the use of a sterol-egg yolk lipoprotein complex (PSY) (Matsuoka et al., 2008). Due to the emulsifying capacity of egg phospholipids, the PSY is readily dispersible in water (Matsuoka et al., 2008). Other techniques for the formulation of dispersible phytosterols for beverage



formulation include the use of various emulsifiers, and subjecting the mixture to homogenization. A characteristic of emulsifiers called the hydrophobicity-lipophilicity balance (HLB) may be helpful when choosing an emulsifier (Auriou and Ferreres, 2002). An emulsifier with a HLB than that of the sterol is desired to promote solubility (Auriou and Ferreres, 2002). Microencapsulation followed by spray-drying is also a feasible approach to sterol-fortified beverage formulation.

## Regulatory Aspects

Review of the global regulatory frameworks for functional foods containing phytosterols and phytostanols was recently published (Zawistowski and Jones, 2015). Table 1 contains a summary of the regulatory framework and health claims on foods with phytosterols in various jurisdictions. This is important when designing a sterol-enriched product for the market because it will have a significant impact on the choice of food product, the serving size, the ingredient list and nutritional composition. It will also be important when marketing products to ensure that all advertisements are truthful, well-substantiated and do not mislead consumers.

## Conclusion

There are many clinical studies which support the health benefits of consuming phytosterols and thus, regulatory agencies have acknowledged their benefits by approving these compounds for use in foods. Phytosterol-containing food products and beverages are already quite popular and as consumers continue to demand health benefits from their food beyond just nutrition, the demand for these products is growing. It is therefore important to find ways of overcoming the challenges associated with adding phytosterols to food products.

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# Caseinophosphopeptides

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## Nomenclature

ACP amorphous calcium phosphate

ACFP amorphous calcium fluorophosphate

CPPs caseinophosphopeptides

DF diafiltration

DH degree of hydrolysis

E:S enzyme to substrate ratio

EtOH ethanol

FOS fructo-oligosaccharides

ITF inulin-type fructans

nd not determined

o/n overnight

RCT randomized controlled trial

RO reverse osmosis

UF ultrafiltration

## Introduction

The basic nutritional role of milk proteins is in the provision of nitrogen and essential amino acids. Additionally, milk proteins have the potential to beneficially modulate a range of different physiological systems within the human body. Intact milk proteins possess many bioactive properties such as antimicrobial, antidiabetic, antihypertensive, anticancer and anti-inflammatory activities (Nongonierma and FitzGerald, 2015a). Milk proteins, in addition, contain an extensive range of bioactive peptide sequences which are encrypted within their primary structures (FitzGerald and Meisel, 2003; Korhonen and Pihlanto, 2006; Miralles et al., 2018; Nongonierma, O'Keeffe and FitzGerald, 2016). Milk protein-derived bioactive peptides, with a diverse range of physiological targets in the immune, nervous, skeletal, endocrine and vascular systems, have been identified following different *in vitro* and *in vivo* studies (Korhonen, 2009; Miralles et al., 2018; Nongonierma and FitzGerald, 2015b; Nongonierma et al., 2016; Sultan et al., 2018). Therefore, milk protein-derived bioactive peptides have been proposed as health promoting and disease risk reducing agents.

Casein-derived phosphorylated peptides are known as caseinophosphopeptides (CPPs). Numerous CPP sequences have been identified within heat-processed, hydrolyzed and fermented bovine milk products (Pinto et al., 2012b). Additionally, milks from other mammals, i.e., human, equine, goat, donkey and buffalo have also been shown to be a source of CPPs (Caira et al., 2016; Cunsolo et al., 2009; Ferranti et al., 1998; Matéos et al., 2010; Smialowska et al., 2017). Due to their high content of carboxylate (Glu and Asp residues) and phosphate groups esterified to Ser and Thr residues, CPPs are negatively charged and therefore have the ability to bind and solubilize bivalent cations such as calcium, iron, copper and zinc (Bouhallab and Bouglé, 2004; FitzGerald, 1998; Mekmene and Gaucheron, 2011). These properties have been explored with the aim of developing CPPs as natural ingredients having the ability to enhance mineral bioavailability. The interest in increasing calcium and iron solubility and bioavailability is relevant due to the increasing prevalence of public health issues related to conditions such as osteoporosis, iron deficiency anemia and dental caries (Bonjour et al., 2009; Nongonierma and FitzGerald, 2012). Therefore, the main aim of this chapter is to review current scientific information with respect to CPPs as remineralizing agents. The structure of CPPs will be outlined followed by details of the production and enrichment techniques applied to the preparation of CPPs. The scientific data on the biofunctional properties of CPPs will be discussed with a specific focus on human studies. Finally, the applications of CPPs as ingredients in food products will be outlined.

## Structure of CPPs

CPPs are casein-derived peptides rich in clusters of phosphorylated Ser and occasionally Thr residues. Caseins are phosphorylated during milk biosynthesis via the activity of specific kinases in the mammary gland (FitzGerald, 1998). As shown in Table 1, different

levels of phosphorylation have been observed in the individual caseins depending on the mammalian species. In addition, the extent of phosphorylation may depend on the number of Ser/Thr phosphate groups present in individual protein molecules, which is influenced by genetic polymorphism in the caseins (Caroli et al., 2009; Selvaggi et al., 2014; Uniacke-Lowe et al., 2010).

A number of different phosphorylated sequences occur in  $\alpha_{s1}$ -,  $\alpha_{s2}$ - and  $\beta$ -casein. This is linked with the higher level of phosphorylation observed in these three individual caseins in comparison to  $\kappa$ -casein, which generally contains a lower number or, in some instances, no CPP sequences (Table 1). A specific sequence consisting of 3 serine phosphate groups followed by two glutamic acid residues (i.e., Ser(P)-Ser(P)-Ser(P)-Glu-Glu, with Ser(P) a phosphorylated Ser residue), also known as the “acidic motif”, has been identified in several CPPs (Bouhallab and Bouglé, 2004). The “acidic motif” is found in the sequence of  $\alpha_{s1}$ -casein (f66–70),  $\alpha_{s2}$ -casein (f8–12) and (f56–60) and  $\beta$ -casein (f17–21) (Cruz-Huerta et al., 2015). The “acidic motif” plays a central role in the binding of bivalent minerals to CPPs (Fig. 1).

## Production and Enrichment of CPPs From Milk Proteins

CPPs are encrypted within the primary sequence of caseins. Therefore, they need to be released in order to display their bioactive properties. CPPs have been reported as naturally occurring at low levels in bovine milk as a result of milk protein hydrolysis by plasmin and somatic cell proteinases (Pinto et al., 2012a). CPPs are also released during other processes such as microbial fermentation (in cheese and yoghurt) (Ardö et al., 2007; Ferranti et al., 1997; Ferrazzano et al., 2008; Kawahara et al., 2005; Roudot-Algaron et al., 1994). Additionally, CPPs have been produced using *in vitro* enzymatic hydrolysis of milk proteins (McDonagh and FitzGerald, 1998; Reynolds et al., 1994). Finally, CPPs have been identified within digestates of human subjects following milk ingestion, indicating their *in vivo* release in the digestive tract (Boutrou et al., 2013; Chabance et al., 1998; Meisel et al., 2003).

The main method employed to produce CPPs at laboratory and industrial scale is through enzymatic hydrolysis of milk proteins. A summary of the different processes used during the generation of CPPs is outlined in Fig. 2. The milk protein substrates used during hydrolysis generally consist of commercially available casein-rich preparations (e.g., sodium caseinate) which possess protein contents as high as 90% (w/w). Several commercial enzyme preparations have been described during the generation of CPPs (Table 2). Porcine trypsin and the *Bacillus licheniformis*-derived preparation, Alcalase™ (Novozymes, Bagsvaerd, Denmark),

**Table 1** Level of phosphorylation of individual caseins in different mammalian species

Casein	Human	Bovine	Ovine	Equine
$\alpha_{s1}$	9	9	11	not available
$\alpha_{s2}$	absent	10	8	not available
$\beta$	5	5	5	9
$\kappa$	0	3	3	2

Adapted from Uniprot.

### ■ $\alpha_{s1}$ -casein

f(59-79)5P Gln-Met-Glu-Ala-Glu-Ser(P)-Ile-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ile-Val-Pro-Asn-Ser(P)-Val-Glu-Gln-Lys

### ■ $\alpha_{s2}$ -casein

f(1-21)4P Lys-Asn-Thr-Met-Glu-His-Val-Ser(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser-Ile-Ile-Ser(P)-Gln-Glu-Thr-Tyr-Lys

f(46-70)4P Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser(P)-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys

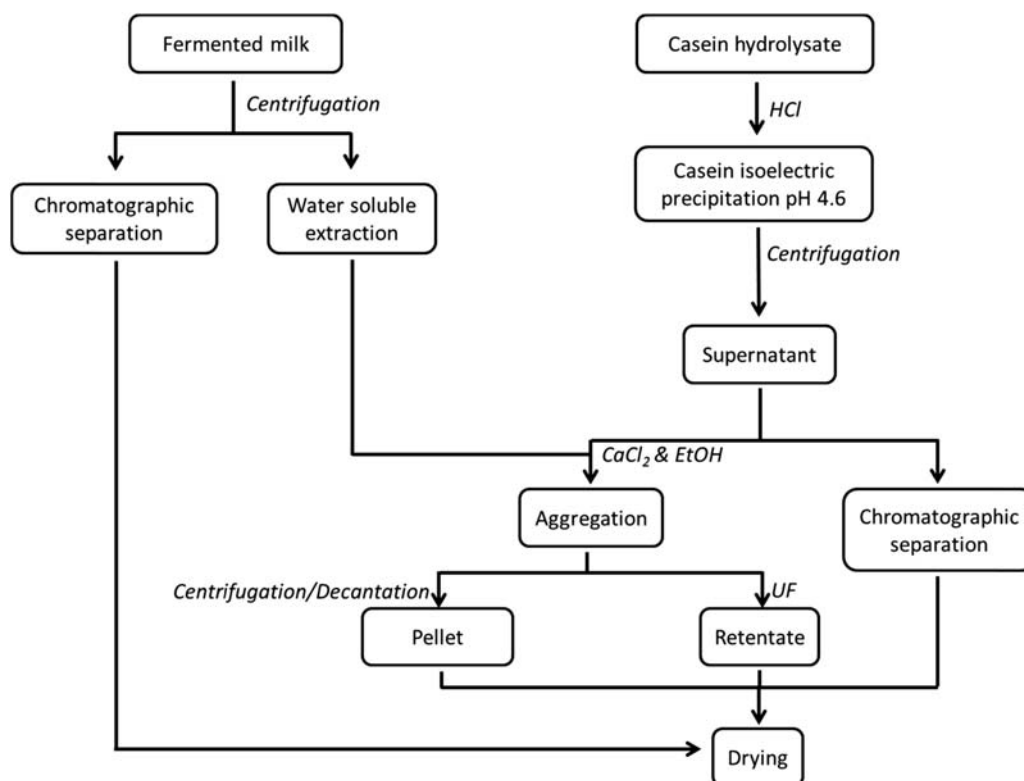
f(55-64)4P Gly-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser(P)-Ala-Glu-Val

### ■ $\beta$ -casein

f(1-25)4P Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser-Ile-Thr-Arg

f(12-23)4P Ile-Val-Glu-Ser(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser-Ile

**Figure 1** Example of bovine milk-derived caseinophosphopeptides (CPPs) containing the “acidic motif” (fragment underlined). Adapted from FitzGerald (1998), Meisel and FitzGerald (2003), and Reynolds (1998). Ser(P): phosphorylated Ser residue.



**Figure 2** Outline of the procedures used during the generation of caseinophosphopeptide (CPP) preparations. Adapted from [Adt et al. \(2011\)](#), [Ferrazzano et al. \(2011\)](#), and [Reynolds et al. \(1994\)](#). EtOH: ethanol; UF: ultrafiltration.

appear to be the most frequently used enzyme activities for the generation of CPPs ([FitzGerald, 1998](#); [Nongonierma and FitzGerald, 2012](#); [Vegarud et al., 2000](#)). However, [McDonagh and FitzGerald \(1998\)](#) demonstrated that a broad range of mammalian, bacterial and plant-derived enzyme preparations (27 different enzyme preparations were evaluated) could be employed to prepare CPPs.

Differences in the composition of CPP products prepared under various conditions have been highlighted. Trypsin and Alcalase™ were employed under the same conditions to generate CPP preparations. The CPP sequences obtained from the Alcalase™ hydrolyzate were generally shorter (6–15 amino acid residues) when compared to those obtained in the trypsin hydrolyzate (11–37 amino acid residues), which was attributed to the broader specificity of Alcalase™ ([Adamson and Reynolds, 1996](#); [Reynolds et al., 1994](#)). Enzymatic hydrolysis has conventionally been conducted with free enzymes, however, encapsulated enzyme preparations have also been described during the generation of CPPs ([Rocha-Martin et al., 2018](#)). The advantages of encapsulation lie in the possibility of recycling the enzyme and carrying out repeated hydrolysis reactions. This may help to reduce costs associated with the generation of CPP preparations and possibly lead to the application of less severe heat treatments during enzyme inactivation steps.

Following enzymatic hydrolysis of caseins, enrichment steps are generally applied ([Fig. 2](#)) to remove unhydrolyzed caseins from the hydrolyzate and subsequently to separate non-phosphorylated peptides from CPPs. The selective precipitation of caseins at their isoelectric point ( $pI = 4.6$ ) renders them insoluble and allows their subsequent removal from the hydrolyzate by centrifugation or decantation. The soluble fraction contains non-phosphorylated peptides and CPPs. In a second step, mineral-bound CPPs are prepared using mineral salts (e.g.,  $CaCl_2$ ,  $BaCl_2$  and  $FeCl_2$ ) and the complexes formed may be precipitated by pH adjustment (generally to pH 7.0) and the addition of ethanol. The CPP complexes are recovered by decantation, centrifugation or ultrafiltration (UF). The hydrodynamic radius of Ca-CPP ( $\alpha_{s1}$ -casein (f59–79)5P and  $\beta$ -casein (f1–25)4P) aggregates was determined to be of 60 nm ([Gravaghi et al., 2007](#)). The relatively large size of CPP aggregates aids in their subsequent recovery by UF separation. Chromatographic separation involving anion-exchange resins (e.g. hydroxyapatite) has also been used to isolate CPPs from milk protein hydrolyzates ([Fig. 2](#)). The advantage of chromatographic separation is the ability to isolate individual CPP sequences. CPPs may also be extracted from fermented dairy products (cheese and yoghurt), generally by preparing a water-soluble extract of cheese or from yoghurt supernatants. CPP preparations are generally dried using either spray- or freeze-drying processes ([Fig. 2](#) and [Table 2](#)).

The recovery obtained during CPP manufacture is typically reported for all phosphorylated peptides obtained during the extraction process regardless of whether they contain the “acidic motif” or not. The yield obtained during CPP generation is calculated using the following formula ([Zhao et al., 2007](#)):



**Table 2** Examples of the different conditions used at laboratory- and pilot-scale to generate caseinophosphopeptide (CPP)-enriched preparations

Hydrolysis conditions	CPP enrichment steps	CPP yield (%)	References
<ul style="list-style-type: none"> <li>• sodium caseinate (10% (w/v))</li> <li>• trypsin (E:S 2.0% (w/w); (Sigma))</li> <li>• pH 8.0, 20 °C, 18 h</li> </ul>	<ul style="list-style-type: none"> <li>• selective precipitation of caseins at pH 4.6</li> <li>• pH adjustment to 3.5, 4.6 or 8.0</li> <li>• CPP precipitation with <math>\text{CaCl}_2</math> or <math>\text{BaCl}_2</math> and ethanol (50% (v/v))</li> <li>• freeze-drying</li> </ul>	10.5 ± 0.4 (for precipitation at pH 3.5)	Reynolds et al. (1994)
<ul style="list-style-type: none"> <li>• sodium caseinate (10% (w/v))</li> <li>• Alcalase™ (E:S 0.5% (w/w); (Novozymes))</li> <li>• pH 8.0, 50 °C, 2 h</li> </ul>	<ul style="list-style-type: none"> <li>• selective precipitation of caseins at pH 4.6</li> <li>• CPP precipitation with <math>\text{CaCl}_2</math> (100 mM) and ethanol (50% (v/v)) at pH 7.0</li> <li>• drying</li> </ul>	12.27 ± 0.27	Adamson and Reynolds (1996)
<ul style="list-style-type: none"> <li>• sodium caseinate (8% (w/v))</li> <li>• 27 different enzyme preparations</li> <li>• pH 7.0–8.0, 50 °C, 2–3 h</li> </ul>	<ul style="list-style-type: none"> <li>• selective precipitation of caseins at pH 4.6</li> <li>• CPP precipitation with <math>\text{CaCl}_2</math> (1% w/v) and ethanol (50% (v/v)) at pH 7.0</li> <li>• freeze-drying</li> </ul>	3.4–16.0	McDonagh and FitzGerald (1998)
<ul style="list-style-type: none"> <li>• sodium caseinate (10% (w/v))</li> <li>• Trypsin PTN 3.0 (E:S 0.5% (w/w); (Novozymes))</li> <li>• pH 8.0, 50 °C</li> <li>• DH = 18.8 ± 0.6%</li> </ul>	<ul style="list-style-type: none"> <li>• selective precipitation of caseins (2 h at 40 °C, pH 4.60)</li> <li>• UF &amp; DF</li> <li>• agarose anion-exchange resin eluted with 0.2 M NaOH</li> <li>• concentration by RO 6 times (50 °C and 60 bars)</li> <li>• mixing of the RO retentate with <math>\text{CaCl}_2</math></li> <li>• DF &amp; RO</li> <li>• spray-drying</li> </ul>	20	Ellegård et al. (1999)
<ul style="list-style-type: none"> <li>• casein (15% (w/v))</li> <li>• Alcalase™</li> <li>• pH 8.0–9.0, 60 °C, 2 h</li> </ul>	<ul style="list-style-type: none"> <li>• selective precipitation of caseins (2 h at 40 °C, pH 4.6)</li> <li>• CPP precipitation with <math>\text{CaCl}_2</math> (1.15% w/v) and ethanol (50%–70% (v/v))</li> <li>• freeze-drying</li> </ul>	24	Zhao et al. (2007)
<ul style="list-style-type: none"> <li>• casein (6% (w/v))</li> <li>• trypsin (E:S 2% (w/w); Biocatalysts)</li> <li>• pH 8.0, 50 °C, 30, 60 and 120 min</li> </ul>	<ul style="list-style-type: none"> <li>• selective precipitation of caseins at pH 4.6</li> <li>• CPP precipitation with <math>\text{CaCl}_2</math> and ethanol at pH 4.0, 6.0 and 8.0</li> <li>• freeze-drying</li> </ul>	nd	Cruz-Huerta et al. (2015)
<ul style="list-style-type: none"> <li>• casein (15 g)</li> <li>• Alcalase™</li> <li>• pH 8.0–9.0, 60 °C, 2 h</li> </ul>	<ul style="list-style-type: none"> <li>• CPP precipitation with <math>\text{CaCl}_2</math> and ethanol</li> <li>• freeze-drying</li> </ul>	28.7	Prakash and Lakshmi (2015)
<ul style="list-style-type: none"> <li>• casein (5% (w/v))</li> <li>• (1) immobilized trypsin (Biocatalysts) then (2) immobilized thermolysin (Biocatalysts)</li> <li>• pH 8.0, 50 °C, 3.5 h (1)/pH 8.0, 40 °C, 16 h (2)</li> </ul>	<ul style="list-style-type: none"> <li>• CPP precipitation with <math>\text{CaCl}_2</math> (100 mM) and ethanol (50% (v/v)) at pH 7.0</li> <li>• freeze-drying</li> </ul>	22–24	Rocha-Martin et al. (2018)

DF: diafiltration; DH: degree of hydrolysis; E:S: enzyme to substrate ratio; RO: reverse osmosis; UF: ultrafiltration.

$$\text{Yield} = \frac{\text{Amount of N in the CPPs}}{\text{Amount of N in the protein substrate}} \times 100 \quad (1)$$

Different extraction yields have been reported following CPP generation. Yields vary and depend on the hydrolysis conditions employed as well as the parameters (i.e., pH, mineral concentration, ethanol content, etc.) used during the aggregation steps (Table 2). They also depend on the peptide sequences released as these dictate overall mass of the phosphorylated peptides generated. The susceptibility to precipitation under certain conditions also depends on the CPP sequence. Therefore, conditions such as mineral concentration and pH value should be appropriately chosen to selectively precipitate the CPPs present within specific casein hydrolyzates (Adamson and Reynolds, 1996). In addition, the duration of the hydrolysis reaction and the pH value employed during CPP precipitation have been shown to affect the recovery of individual CPPs (Cruz-Huerta et al., 2015). These parameters, therefore, need to be optimized to maximize the yield of specific CPPs. When comparing 27 commercial enzyme preparations, the highest yield of CPPs (i.e., 16.0%) was obtained from a Bioprotease N100L (a *Bacillus subtilis*-derived preparation, Quest International, Carrigaline, co. Cork, Ireland) sodium caseinate hydrolyzate (McDonagh and FitzGerald, 1998). CPP yields as high as 28.7% (w/w) have been reported with an Alcalase™ hydrolyzate of sodium caseinate (Prakash and Lakshmi, 2015).

Interestingly, the calcium binding ability of the CPP preparations did not appear to be related to the yield as the highest binding value (i.e., 24 mg  $\text{Ca}^{2+}$  mg<sup>-1</sup> CPP) was obtained with Pancreatin™ (McDonagh and FitzGerald, 1998). The binding affinity of  $\text{Ca}^{2+}$  was shown to depend on the CPP sequence. For example, a higher  $\text{Ca}^{2+}$ :peptide ratio was reported for  $\beta$ -casein (f1-25)4P than for  $\alpha_{s1}$ -casein (f59–79)5P, i.e., 4 and 1  $\text{Ca}^{2+}$ :peptide, respectively. Similarly, the  $\text{Ca}^{2+}$  binding constant was higher for  $\beta$ -casein (f1-25)

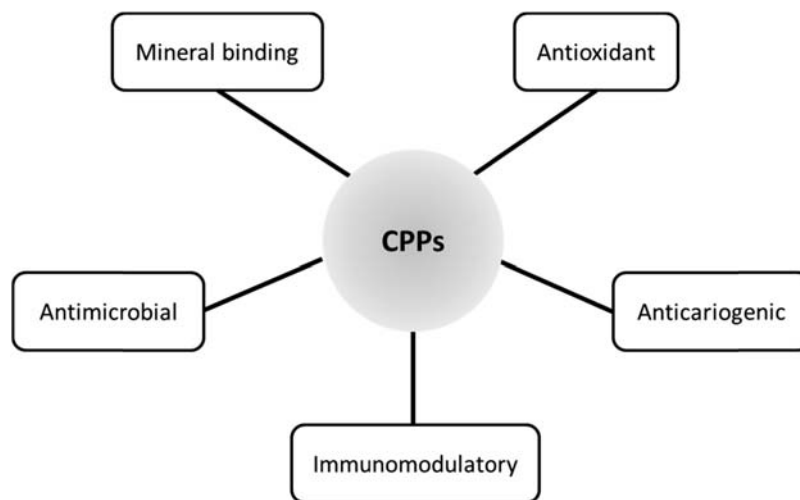
4P than for  $\alpha_{s1}$ -casein (f59–79)5P, i.e., 0.63 and 0.85 mM<sup>-1</sup>, respectively (Meisel and Olieman, 1998). Optimization experiments have been performed to maximize the amount of mineral bound by CPPs. In this context, optimization of binding of Fe<sup>2+</sup> to a commercial CPP preparation (VIVID Casein Phospho Peptide-H, Zhengzhou Friend Biological Engineering Co., Zhengzhou, Henan, China) has recently been investigated using a central composite design combining pH, Fe:CPP ratio and holding time (Delshadian et al., 2018). Optimization of these three parameters was achieved using response surface methodology (RSM). The most important parameter for Fe<sup>2+</sup> binding was the pH value. The optimum conditions (pH 6.5, 0.14 Fe:CPP ratio and a holding time of 72 min) yielded a product containing  $67.62 \pm 0.95$  mg Fe<sup>2+</sup> g<sup>-1</sup> CPP preparation.

The CPP format generally used in dentistry and oral hygiene products consists of CPP-amorphous calcium phosphate (ACP). CPP-ACP has been used since in the presence of water, it converts into hydroxyapatite, the precipitated form of calcium and phosphate at the surface of dental enamel (Boskey and Posner, 1973; Skrtic et al., 2003). In several instances, fluoride has been included with the ACP to obtain amorphous calcium fluorophosphate (ACFP). CPP-ACFP complexes are claimed to have a greater remineralizing effect than CPP-ACP. This is linked to the fact that tooth enamel, where fluoroapatite is deposited, is claimed to be particularly resistant to demineralization. This has been explained by the poor aqueous solubility of fluoroapatite (Ostrom et al., 1984). The manufacture of CPP-ACP and CPP-ACFP complexes has also been described in the patent literature (Reynolds, 2008b, 2011). The starting point for the release of CPPs still involves enzymatic hydrolysis of caseins followed by isoelectric precipitation of unhydrolyzed caseins at pH 4.6 as outlined in Fig. 2. However, differences are found during the aggregation step of the CPPs. The addition of CaCl<sub>2</sub> to CPPs is performed slowly while NaOH is added to set the pH value within 5.0 and 9.0 (Walsh, 2009). An aqueous solution of Na<sub>2</sub>HPO<sub>4</sub> is then mixed with the CPP-Ca sample, leading to the formation of CPP-ACP complexes. CPP-ACFP complexes are prepared by adding NaF to CPP-ACP. Membrane separation, such as microfiltration or dialysis through a 1 kDa UF membrane, allows separation of the CPP-ACP or CPP-ACFP complexes from the other peptides (Cross et al., 2004). The CPP-ACP complexes formed consist of [ $\alpha_{s1}$ -casein (f59–79) (ACP)<sub>7</sub>]<sub>6</sub> and [ $\beta$ -CN(f1–25) (ACP)<sub>8</sub>]<sub>6</sub>. The [ $\alpha_{s1}$ -casein (f59–79) (ACP)<sub>7</sub>]<sub>6</sub> complexes have an average hydrodynamic radius of 2.12 nm (Cross et al., 2004). The average hydrodynamic radius of [ $\beta$ -CN(f1–25) (ACP)<sub>8</sub>]<sub>6</sub> is between  $1.526 \pm 0.44$  and  $1.923 \pm 0.082$  nm (Cross et al., 2007; Cross et al., 2016). CPP-ACFP complexes are reported to have an average diameter of 41 nm (Sun and Chow, 2008). Recently, Cross et al. (2016) demonstrated that ACP interacts with the entire CPP sequence and not only with the “acidic motif” to form CPP-ACP complexes.

## Bioactive Properties of CPPs

### Mechanisms of Action of CPPs

Using *in vitro* protocols and small animal studies, a number of bioactive properties have been identified with CPPs (Fig. 3). These comprise mineral binding, anticariogenic, antimicrobial, antioxidant and immunomodulatory properties (Bouhallab and Bouglé, 2004, 2011; FitzGerald, 1998; Kitts, 2005, 2006; Nongonierma and FitzGerald, 2012; Vegarud et al., 2000). Most of the reported bioactive properties of CPPs have systemic targets (i.e., associated with bone, the circulatory system and various internal organs). Therefore, CPP bioavailability is necessary in order for their bioactive properties to be observed. Numerous *in vitro* studies have evaluated the effect of CPPs on intracellular mineral uptake (for reviews, see: Bouhallab and Bouglé, 2011; Sun et al., 2016). A range of cell models including intestinal (Caco-2 and HT29 cells) (Cao et al., 2017; Cosentino et al., 2010a; Cosentino et al., 2010b; Ferrarretto et al., 2003; Gravaghi et al., 2007; Kibangou et al., 2005; Perego et al., 2012; Perego et al., 2015), human hepatoma cells



**Figure 3** Main bioactive properties of caseinophosphopeptides (CPPs). Adapted from Bouhallab and Bouglé (2004), FitzGerald (1998), Kitts (2005), (2006), Nongonierma and FitzGerald (2012), and Vegarud et al. (2000).

(HuH7) (García-Nebot et al., 2015) and osteoblasts (SaOS-2M and C3T3-E1) (Donida et al., 2009; Tulipano et al., 2010) have been used to demonstrate the role of CPPs as enhancers of mineral uptake at cellular levels.

Bovine  $\alpha_{s1}$ -casein (f59–79)5P and  $\beta$ -casein (f1–25)4P are the most studied CPPs in the scientific literature. Both peptides contain the “acidic motif”. This “acidic motif” is rich in negatively charged groups at alkaline pH (Rose, 2000). The negative charges arise from the phosphate groups and Glu residues (Fig. 1) (Kitts, 2006; Mekmene and Gaucheron, 2011). These negative charges confer the ability of CPPs to bind bivalent minerals ( $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Mn}^{2+}$ .) and a range of trace elements (barium, selenium, nickel, cobalt and chromium) (Bouhallab and Bouglé, 2004; FitzGerald, 1998; Kitts, 2006). The central role of the “acidic motif” in mineral binding has been demonstrated by Ferrarretto et al. (2003) during a study on truncated and/or dephosphorylated peptides originating from bovine  $\beta$ -casein (f1–25)4P. Inorganic mineral salts such as calcium phosphate and ferrous sulfate have low solubility in water (Skibsted, 2016). Binding of minerals to CPPs causes an increase in their water solubility. Their mineral solubilizing properties have, therefore made CPPs attractive for the development of mineral supplements with enhanced bioavailability. The presence of negative charges within CPPs and their complexation with minerals have also been linked to their purported resistance to gastrointestinal digestion (Boutrou et al., 2010; Kitts, 2005; Meisel and Frister, 1988; Vegarud et al., 2000).

The anticariogenic properties of CPP-ACP and CPP-ACFP have been reviewed in several publications (Bijle et al., 2018; Li et al., 2014; Lopatiene et al., 2016; Nongonierma and FitzGerald, 2012; Peters, 2010; Wang et al., 2017). Due to its low solubility, calcium phosphate is inefficient at remineralizing tooth surfaces (Reynolds, 2008a). CPPs have been shown to increase calcium and phosphate solubility and stabilize these compounds at the tooth surface, thereby promoting enamel remineralization (Peters, 2010; Reynolds, 1997). In the oral cavity, CPP-bound calcium allows the maintenance of a supersaturation of  $\text{Ca}^{2+}$  at the tooth surface. This in turn leads to remineralization of tooth enamel and repair of enamel lesions (Adamson and Reynolds, 1996). The anticariogenic properties of CPP-ACP and CPP-ACFP involve various modes of action including (a) reduction in the rate of tooth demineralization, (b) remineralization of dental enamel and (c) anti-plaque activity through their buffering capacity and antimicrobial effects (El Mehdi et al., 2016; Nongonierma and FitzGerald, 2012; Pinto et al., 2012a; Reynolds, 1998). Some contradictory reports in the literature have, however, raised several questions in terms of the efficiency of CPPs as tooth remineralization agents in humans (Azarpazhooh and Limeback, 2008; Bijle et al., 2018; Sonesson et al., 2016).

The antimicrobial activity of CPPs has been mostly shown against strains found in the dental plaque (i.e., aciduric bacteria and *Streptococcus mutans*) (Beerens et al., 2010; Rose, 2000). The mechanism of action involves binding of CPPs to the dental plaque. The negative charges of the bound CPPs induce repulsive forces, preventing the binding of microorganisms to the enamel surface (Rahiotis et al., 2008). Recent studies have shown that CPPs display an antiviral activity *in vitro* against human norovirus, which is involved in gastroenteritis (Lebetwa et al., 2017). In this study, the presence of phosphate groups in CPPs was shown to be a requirement to observe the antiviral activity.

CPPs also display immunomodulatory properties such as in the stimulation of immunoglobulin production and lymphocyte proliferation (for review, see: Meisel and FitzGerald, 2003). CPPs generated during tryptic hydrolysis of bovine milk proteins (i.e.,  $\alpha_{s1}$ -casein (f59–79)5P and  $\beta$ -casein (f1–25)4P) demonstrated a mitogenic activity and a stimulatory effect on immunoglobulin production in mouse spleen and rabbit Peyer's patch cells (Hata et al., 1998). Immunoglobulin release may be influenced by the increase in  $\text{Ca}^{2+}$  uptake, which has a mitogen-induced proliferative action for lymphocytes (Hata et al., 1998; Hosono et al., 2002).

The antioxidant properties of CPPs have also been shown *in vitro* (Kitts, 2005). CPPs can act both as primary (scavenging of antioxidants) and secondary (sequestering of potential metal pro-oxidants) “site-specific” antioxidants. The antioxidant properties of CPPs have been linked with phosphoserine residues, and their proximity, within CPP sequences. The arrangement of phosphoserine residues within CPP sequences results in anionic and polar domains, which have the ability to sequester cationic metal ions (Kitts, 2005). CPPs also protect cells (Caco-2) against  $\text{H}_2\text{O}_2$  oxidation (García-Nebot et al., 2011; Laparra et al., 2008). The CPPs are reported to act at different levels, i.e., having a cytoprotective effect (increase in cell viability) and a regulatory effect on cellular redox systems (e.g., glutathione production, stimulation of catalase activity and reduction of lipid peroxidation).

### Bioavailability of CPPs

In the context of dietary mineral deficiencies (e.g., osteoporosis and anemia), it appears that an increased bioavailability for  $\text{Ca}^{2+}$  and  $\text{Fe}^{2+}$  is a requirement to enhance their uptake in the body. The increased bioavailability of minerals bound to CPPs has been linked with the enhanced solubility of minerals in the ileum (Kitts and Yuan, 1992; Zidane et al., 2012). Consequently, CPPs must be able to arrive intact at, or be released in the small intestine. The theoretical maximum luminal yield of  $\alpha_{s1}$ -casein (f43–58), (f59–79) and  $\beta$ -casein (f1–25) has been determined to be 3.24, 4.58% and 4.81% (w/w protein), respectively (Meisel, 1998). CPP sequences have been found in *in vitro* simulated gastrointestinal digests of milk and cheese (Cattaneo et al., 2017; Picariello et al., 2010, 2013; Sánchez-Rivera et al., 2014; Sanchón et al., 2018) and CPPs (Ait-Oukhatar et al., 2000; Bouhallab et al., 1999; García-Nebot et al., 2010; Miquel et al., 2006). In addition, CPPs have been identified in the gastrointestinal tract of mammals following casein or CPP ingestion (for reviews, see: Boutrou et al., 2015; FitzGerald, 1998; Nongonierma and FitzGerald, 2017).

The phosphorylated region of CPPs is reported to confer resistance to gastrointestinal enzyme-mediated degradation (Cattaneo et al., 2017; Picariello et al., 2010; Sánchez-Rivera et al., 2014; Vegarud et al., 2000). The bioavailability of CPPs has been reported in

several *in vivo* studies (Ait-Oukhatar et al., 2002; Bennett et al., 2000). CPPs were detected in the feces of rats fed with diets containing 50–500 mg kg<sup>-1</sup> casein or commercial CPP preparations (CPP I and CPP III, Meiji Seika, Tokyo, Japan) (Kasai et al., 1995). Detection of CPPs has also been reported in the gastrointestinal contents of pigs fed with a diet containing 145 g skimmed milk powder (Barbé et al., 2014) and rats fed with 17.2 µg of CPP III (Hirayama et al., 1992). Detection of CPPs in feces of animals following the consumption of milk proteins or CPPs indicates their relative stability in the gastrointestinal tract (Barbé et al., 2014; Hirayama et al., 1992; Meisel and Frister, 1988). However, contradictory studies have been shown in other animal studies, reporting no improvement in the bioavailability of minerals. This was the case when a diet containing 120 µg CPP-Zn was administered to rats (Matsui et al., 2002).

A limited number of human intervention studies have been conducted with CPPs *per se* or dairy products as a source of CPPs. The format and outcomes of the human intervention studies carried out to date are summarized in Table 3. Early studies (before 2010) have mainly focused on the role of CPPs on mineral (Ca<sup>2+</sup>, Fe<sup>2+</sup> and Zn<sup>2+</sup>) absorption and uptake (principally in bone) following dietary intervention. The results obtained are contradictory as some studies have reported beneficial effects of CPPs on mineral absorption following intake of 175 mg CE90CPP (DMV International, Veghel, the Netherlands) (Adolphi et al., 2009). However, other studies reported no differences compared to a control diet when amounts between 250 and 2000 mg CPPs were administered (Ait-Oukhatar et al., 2002; Hansen, Sandström, Jensen and Sørensen, 1997a; Hansen, Sandström, Jensen and Sørensen, 1997b; López-Huertas et al., 2006; Narva et al., 2003). One study reported a reduction in fractional Ca<sup>2+</sup> absorption following ingestion of ~100 mg CPP-Ca (Teucher et al., 2006). Other human studies have reported enhancement effects by CPPs (amounts ingested between 87.5 and 2000 mg) on Ca<sup>2+</sup> absorption, the effects being population- (Heaney et al., 1994) or diet-dependent (Hansen et al., 1997a).

The reported lack of an observed beneficial effect of CPP intake in certain studies may arise from different factors, including the CPP format (free CPPs vs. intact or partially hydrolyzed milk proteins), the population tested (healthy vs. mineral deficient subjects), the diet (low vs. phytate-rich), food format (liquid vs. solid), timing of ingestion, CPP dose and CPP:mineral ratio (Nongonierma et al., 2016). Antinutritional components such as phytates can bind bivalent minerals and, therefore reduce their overall bioavailability. This may be the reason in the case of a rice-based meal, which is low in phytates, why CPPs (1000 and 2000 mg) were observed to increase Ca<sup>2+</sup> and Zn<sup>2+</sup> absorption by ~30% in human subjects in contrast with the whole grain meal, which contained phytates (Hansen et al., 1997a). Furthermore, no effect on Ca<sup>2+</sup> and Zn<sup>2+</sup> absorption were found on the ingestion of CPPs (250 and 1000 mg) with white bread, which is also a phytate-rich food (Hansen et al., 1997b). Another factor which may modify the effect of CPPs is the stability of CPPs in the gastrointestinal tract. It has been shown that milk proteins appear to have a protective effect on CPP integrity, which translates to the prevention of CPP degradation during gastrointestinal transit. This was demonstrated following the consumption of CPP preparations or milk. Higher levels of CPPs were detected in the ileostomy fluid of humans fed with cow's milk (250 g) than with CPP preparations (1520 mg), i.e., 7.10 and 0.2 nmol mL<sup>-1</sup>, respectively (Meisel et al., 2003). The timing of CPP intake may also play a role on their effects on mineral absorption. Nocturnal intake of inulin-type fructans (ITF) and CPPs (175 mg) had a positive effect on Ca<sup>2+</sup> absorption in post-menopausal women, compared to fermented milk with or without a Ca<sup>2+</sup> supplement (Adolphi et al., 2009). The dietary status of the subjects is also another important factor, which needs to be taken into account. Heaney et al. (1994) identified differences in Ca<sup>2+</sup> absorption in post-menopausal women depending on their Ca<sup>2+</sup> status. Women having low basal Ca<sup>2+</sup> absorption could absorb more Ca<sup>2+</sup> in the presence of CPPs (87.5 mg) as opposed to women with high basal Ca<sup>2+</sup> absorption (Heaney et al., 1994).

The other human studies outlined in Table 3 are linked to the detection of CPP sequences in humans. Most of these studies have reported on the detection of CPPs in the small intestine (duodenum, jejunum and ileum) following the ingestion of bovine milk proteins (Boutrou et al., 2013; Chabance et al., 1998; Meisel et al., 2003). Recently, Caira et al. (2016) reported the identification of CPPs in the plasma of human subjects who ingested 100 g Parmigiano Reggiano cheese daily for 7 days. Significant interindividual differences were found regarding the detection of CPPs in the plasma. This was the case for α<sub>s1</sub>-casein (f43–50)2P, α<sub>s2</sub>-casein (f7–12)2P and (f8–12)3P, which were only detected in the plasma of one out of four subjects (Caira et al., 2016). It is also interesting to note that the detection of CPPs in humans has been carried out post ingestion of intact or partially hydrolyzed milk proteins (i.e., cheese and yoghurt). As previously stated, the stability of CPPs in the gastrointestinal tract may be low (Meisel et al., 2003). Therefore, CPPs appear to survive digestion when they are ingested in their encrypted format, i.e., within intact or partially digested milk proteins. This may be part of the reason why most human intervention studies which have evaluated the effect of CPP intake in the precursor substrates for CPP release (intact or partially hydrolyzed milk proteins), as opposed to ingesting CPPs *per se*, have reported a positive effect on mineral absorption (Table 3).

In summary, major differences are reported in the relative stability of CPPs during *in vivo* and *in vitro* digestion (García-Nebot et al., 2010; Meisel et al., 2003; Perego et al., 2015). These findings highlight the need to assess/validate both the stability and the bioactivity of biofunctional peptides within specific food delivery matrices. Therefore, there may be a need for the application of specific reformulation and/or protective methodologies (e.g., encapsulation) if the biofunctional peptide is not stable during gastrointestinal transit in a given food matrix.

## Functional Properties in Foods

CPPs are manufactured commercially and the process for their production has been extensively patented over the years (for review, see: Nongonierma and FitzGerald, 2012). A range of CPP preparations are sold as food-grade ingredients both for food and oral

**Table 3** Overview of human intervention studies which have evaluated the bioavailability or bioactive properties of caseinophosphopeptides (CPPs)

Starting substrate	Study design	Locus	Main outcomes	References
Gelatin capsules of CPPs (87.5 mg; Meiji Seika)	<ul style="list-style-type: none"> <li>• 35 post-menopausal ♀ (59 ± 3.8 y)</li> <li>• randomized cross-over study after o/n fast:               <ul style="list-style-type: none"> <li>- meal (1) = 250 mg CaCO<sub>3</sub> with or without CPPs;</li> <li>- meal (2) = 250 mg CaHPO<sub>4</sub> with or without CPPs</li> </ul> </li> </ul>	blood 5 h post absorption	<ul style="list-style-type: none"> <li>• fractional Ca absorption: no significant effect of CPPs.</li> <li>• better Ca<sup>2+</sup> absorption with CPPs in subjects with low basal Ca<sup>2+</sup> absorption</li> </ul>	
CPPs (MD Foods)	<ul style="list-style-type: none"> <li>• 22 subjects (23 y median)</li> <li>• controlled parallel study at breakfast:               <ul style="list-style-type: none"> <li>- (1): rice-based infant gruel with 0, 1 or 2 g CPP</li> <li>- (2): whole-grain infant gruel with 0, 1 or 2 g CPP</li> </ul> </li> </ul>	whole body radioisotopic <sup>65</sup> Zn and <sup>47</sup> Ca retention & urine (24 h)	<ul style="list-style-type: none"> <li>• rice-based cereal:               <ul style="list-style-type: none"> <li>- Ca<sup>2+</sup>: no effect of CPPs on fractional absorption &amp; increase in total absorption</li> <li>- Zn<sup>2+</sup>: increase of fractional &amp; total absorption</li> </ul> </li> <li>• whole-grain cereal: no effect of CPPs on Zn<sup>2+</sup> &amp; Ca<sup>2+</sup> absorption</li> </ul>	Hansen et al. (1997a)
CPPs (MD Foods)	<ul style="list-style-type: none"> <li>• 31 subjects (19–30 y)</li> <li>• RCT:               <ul style="list-style-type: none"> <li>- bread (1): low phytate/high Ca<sup>2+</sup> with 0, 250 or 1000 mg CPP</li> <li>- bread (2): high phytate/high Ca<sup>2+</sup> bread with 0, 250 or 1000 mg CPP</li> <li>- bread (3): high phytate/low Ca<sup>2+</sup> bread with 0, 250 or 1000 mg CPP</li> </ul> </li> </ul>	whole body radioisotopic <sup>65</sup> Zn and <sup>47</sup> Ca retention & urine (24 h)	no effect of CPPs on Ca <sup>2+</sup> and Zn <sup>2+</sup> absorption	Hansen et al. (1997b)
Skimmed milk	<ul style="list-style-type: none"> <li>• 6 subjects (24–49 y)</li> <li>• 500 mL reconstituted skimmed milk (6.2 g protein L<sup>-1</sup>) at breakfast</li> </ul>	duodenum	detection of β-casein (f7-16) & β-casein (f7-18)	Chabance et al. (1998)
β-casein (f1-25)-Fe enrich milk	<ul style="list-style-type: none"> <li>• 10 ♀ (20–30 y)</li> <li>• 250 mL milk containing 3 mg iron (FeSO<sub>4</sub> or β-casein (f1-25)-Fe) at breakfast after o/n fast</li> </ul>	iron in blood & organs (liver, spleen, bone marrow)	<ul style="list-style-type: none"> <li>• Fe<sup>2+</sup> absorption: β-casein (f1-25)-Fe = FeSO<sub>4</sub></li> <li>• Fe<sup>2+</sup> tissue uptake: β-casein (f1-25)-Fe &gt; FeSO<sub>4</sub></li> </ul>	Ait-Oukhatar et al. (2002)
Milk CPPs (1 g; DMV International)	<ul style="list-style-type: none"> <li>• 9 post-menopausal ♀ (49–66 y)</li> <li>• double blind randomized cross over study 1 at breakfast:               <ul style="list-style-type: none"> <li>- milk</li> <li>- milk + CPPs</li> </ul> </li> <li>• double blind randomized cross over study 2 at breakfast:               <ul style="list-style-type: none"> <li>- fermented milk + CPPs</li> <li>- milk + CPPs</li> </ul> </li> </ul>	serum (0–6 h) & urine (0–24 h)	<ul style="list-style-type: none"> <li>• no effect of CPPs on Ca<sup>2+</sup> metabolism (i.e., seric and urinary Ca<sup>2+</sup>)</li> <li>• no effect of fermentation on Ca<sup>2+</sup> metabolism</li> </ul>	Narva et al. (2003)

(Continued)

**Table 3** Overview of human intervention studies which have evaluated the bioavailability or bioactive properties of caseinophosphopeptides (CPPs)—cont'd

Starting substrate	Study design	Locus	Main outcomes	References
Full fat cow's milk (250 g) & CPP preparations (1.52 g)	<ul style="list-style-type: none"> <li>• 4 subjects with an ileostomy (43–60 y)</li> <li>• RCT sample consumption at breakfast after o/n fast:               <ul style="list-style-type: none"> <li>- full fat cow's milk</li> <li>- CPP preparation 1</li> <li>- CPP preparation 2</li> </ul> </li> </ul>	ileostomy fluid (0–10 h post sample ingestion)	<ul style="list-style-type: none"> <li>• detection of <math>\beta</math>-casein (f13–23)</li> <li>• CPP bioavailability = 1.8% for milk</li> </ul>	<a href="#">Meisel et al. (2003)</a>
CPP-Ca preparations (100 mg)	<ul style="list-style-type: none"> <li>• 15 subjects (18–45 y)</li> <li>• RCT with intake of:               <ul style="list-style-type: none"> <li>- drink (1) = 400 mg calcium lactate;</li> <li>- drink (2) = (1)+CPP preparation 1;</li> <li>- drink (3) = (1)+ CPP preparation 2</li> </ul> </li> </ul>	blood & urine	fractional $\text{Ca}^{2+}$ absorption from drink (2) = drink (3) < drink (1)	<a href="#">Teucher et al. (2006)</a>
CE 90 CPP/L (2g; DMV International)	<ul style="list-style-type: none"> <li>• 15 subjects (25–36 y)</li> <li>• RCT double blind cross-over with intake at breakfast of:               <ul style="list-style-type: none"> <li>- drink (1) = semi-skimmed milk</li> <li>- drink (2) = drink (1)+12.6% <math>\text{Ca}^{2+}</math> from milk solids</li> <li>- drink (3) = drink (1)+15.5% <math>\text{Ca}^{2+}</math> from <math>\text{Ca}_3(\text{PO}_4)_2</math></li> <li>- drink (4) = drink (1)+ FOS (5 g)</li> <li>- drink (5) = drink (1)+CPPs</li> </ul> </li> </ul>	blood & urine (24 h)	no effect of CPPs on $\text{Ca}^{2+}$ absorption	<a href="#">López-Huertas et al. (2006)</a>
CE90CPP (175 mg; DMV International)	<ul style="list-style-type: none"> <li>• 85 post-menopausal ♀ (48–67 y)</li> <li>• controlled parallel double-blind study (14 days), bed-time consumption of:               <ul style="list-style-type: none"> <li>- drink (1): fermented milk (175 mL)</li> <li>- drink (2): drink (1)+ Ca</li> <li>- drink (3): drink (2)+ ITF + CPPs</li> </ul> </li> </ul>	fasting plasma and serum & urine (48 h)	<ul style="list-style-type: none"> <li>• nocturnal <math>\text{Ca}^{2+}</math> absorption from (3) &gt; (1) &amp; (2)</li> <li>• no difference in bone resorption in the 3 groups</li> </ul>	<a href="#">Adolphi et al. (2009)</a>
Casein or whey (30 g)	<ul style="list-style-type: none"> <li>• 16 subjects (21–38 y)</li> <li>• daily intake of milk proteins</li> </ul>	jejunal contents (on day 8)	identification of a CPP ( $\alpha_{s1}$ -casein (f106–119))	<a href="#">Boutrou et al. (2013)</a>
Parmigiano Reggiano (containing 1% water soluble CPPs)	<ul style="list-style-type: none"> <li>• 4 subjects (2 ♂ &amp; 2 ♀; 24–49 y)</li> <li>• daily intake of 100 g cheese for 7 days</li> </ul>	fasting plasma	identification of CPPs: <ul style="list-style-type: none"> <li>- <math>\alpha_{s1}</math>-casein (f43–50)2P, (f43–52)2P</li> <li>- <math>\alpha_{s2}</math>-casein (f6–12) 3P, (f7–12)2P, (f7–12)3P, (f8–12)2P &amp; (f8–12)3P</li> </ul>	<a href="#">Caira et al. (2016)</a>

FOS: fructo-oligosaccharides; ITF: inulin-type fructans; nd: not determined; o/n: overnight; RCT: randomized control trial.



**Table 4** Examples of commercially available caseinophosphopeptide (CPP)-containing ingredients

<i>Commercial name</i>	<i>Manufacturer</i>
CE90CPP	DMV, International (Veghel, The Netherlands)
Capolac	Arla Foods (Stockholm, Sweden)
Lacprodan DI-2021	
Peptigen 110	MD Foods (Viby, Denmark)
CPPB	Armor Protéines (Saint-Brice-en-Coglès, France)
CPPC	
CPP I	Meiji Seika (Tokyo, Japan)
CPP II	
CPP III	
VIVID Casein Phospho Peptide-H	Zhengzhou Friend Biological Engineering Co. (Zhengzhou, Henan, China)
Hyvital	Friesland Campina (Amersfoort, The Netherlands)
Recaldent	Cadbury Adams (Parsippany, New Jersey, USA)

hygiene applications (Table 4). To date, CPPs appear to have been mainly used in oral hygiene products such as toothpaste, mouthwash and chewing-gums. However, foods formulated with CPPs have also been developed, especially in beverage format (Nongonierma and FitzGerald, 2012; Pinto et al., 2012b).

Different studies have shown that components within the food matrix may play a significant role in the bioavailability of dietary minerals. As already discussed, foods which are rich in antinutritional compounds such as phytates can reduce the bioavailability of minerals. Similarly, the bioavailability of  $\text{Ca}^{2+}$  from CPP-Ca is lowered in the presence of inorganic phosphate (Erba et al., 2001). The food matrix may have a protective effect on CPP stability to digestive enzymes. This has been demonstrated when CPPs were ingested in their precursor in the format of milk proteins or fermented products. Other matrices such as a fruit beverage have also been shown to protect CPPs against degradation during simulated gastrointestinal digestion (García-Nebot et al., 2010).

The antioxidant properties of CPPs have been exploited to bind  $\text{Fe}^{2+}$  and therefore inhibit lipid peroxidation in oil-in-water emulsion systems and in meat (Díaz and Decker, 2004; Díaz et al., 2003). The antioxidant activity of CPPs was, however, not linked to their phosphorylated fragments, it was suggested that Glu and Asp residues were responsible for the  $\text{Fe}^{2+}$  chelation (Díaz et al., 2003).

Studies have linked casein phosphorylation with an enhancement in certain technofunctional properties such as foaming, emulsifying and solubility (for review, see: Broyard and Gaucheron, 2015). Alterations in these properties have been explained by the increased charges associated with the phosphate groups. This in turn is linked to a reduction in the isoelectric point of the caseins and in their surface hydrophobicity. Due to their high charge contents, CPPs *per se* may present similar tensioactive properties. However, studies on the foaming and emulsifying properties of CPPs do not currently appear to exist in the published literature.

## Conclusions

Several *in vitro* and small animal studies have demonstrated that CPPs are effective as mineral carriers. Therefore, CPPs may have the ability to be exploited as food-grade ingredients with mineral-binding and possibly delivery properties. To date, most human intervention studies, which have been conducted with CPPs have failed to demonstrate a real effect as bone remineralizing agents. However, the format in which the CPP is ingested appears to play a major role in their bioavailability. Human intervention studies reporting beneficial mineralization effects of CPPs have employed CPPs, which were encrypted within intact caseins or in partially hydrolyzed caseins. In these formats, it seems that CPPs are less likely to be degraded in the gastrointestinal tract. The detection of CPPs in the small intestine and in the circulation of humans following ingestion of intact or partially hydrolyzed milk proteins is well documented. However, different research approaches may need to be taken to demonstrate the biological activity of CPPs. These include the development of processes for the manufacture of CPP precursors (partially hydrolyzed milk-protein derived peptides containing the “acidic motif”) yielding more stable and thus ultimately bioavailable CPPs. The encapsulation of CPPs is another approach, which may be explored in the delivery of CPP ingredients that remain stable until they reach the small intestine where maximal absorption of dietary minerals takes place in humans.

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# Food for Eye Health: Carotenoids and Omega-3 Fatty Acids

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## Introduction

### Eye Health and Its Related Diseases

The eye is a unique sensory organ which needs more attention to ensure a productive and healthy lifestyle (Abdel-Aal et al., 2013). However, less attention was given to eye health as several eye diseases during the early stage did not show any signs or symptoms (pain or change in vision) until it transformed to an advanced stage. Hence, the ophthalmologist always recommends regular eye examinations (at least once in a year) to cross check eye health status (Ono et al., 2010; Sloan et al., 2005). Therefore, the importance and the awareness of eye health help everyone to preserve vision and thus can lead to better quality of life (Scott et al., 2016). A global action plan was adopted by the World Health Organization (WHO) to decrease global vision impairment (blindness) as well as to increase the rehabilitation for already visually impaired people (Bourne et al., 2017). Copious number of studies have concluded that the prevalence of age-related visual impairments and blindness are considerably increased in developed or high-income countries as the life expectancy has increased significantly (Chan et al., 2017; Finger et al., 2011). WHO, in their 2010 survey concluded that about 253 million people are experiencing various degrees of visual impairment. Nonetheless, only 39 million people are completely blind based on best-correction visual acuity (BCVA) score. It has been estimated that by 2050 the number of visually impaired would be tripled (WHO, 2012; Bourne et al., 2017).

Moreover, the economic burden of visually impaired or blind people was substantially increased in recent times in developing and developed countries. It is predicted that in the year 2050 the economic burden for visual impairment would cross \$70 billion in developed countries especially in US, UK, Germany and Canada (Rein et al., 2009; Koberlein et al., 2013). Most of the age-related eye diseases including Age-related macular degeneration (AMD), cataract, and glaucoma have a negative impact on the quality of life as they need utmost care (basic needs) as well as expensive treatments (Eckert et al., 2015; Kundtson et al., 2005). Currently, many researchers are more focused on eye diseases that include age-related macular degeneration (AMD) and cataract as they contribute to almost 50%–60% of total blindness or visual impairment globally in the elderly population (Flaxman et al., 2017; Resnikoff et al., 2004). Therefore, for the current study, we included only age-related eye diseases like AMD and cataract and its correlation with carotenoids and  $\omega$ -3 fatty acid supplementation as the oxidative stress (light induced) is the common contributor for both types of vision impairment.

### Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is characterized by the progressive loss of macular pigment (in macula) or photoreceptors, which subsequently results in blindness (Vujosevic et al., 2017; Van Lookeren Campagne et al., 2014). AMD is one of the major causes of vision impairment or blindness globally in the elderly population (over 65 years) next to cataract and glaucoma (WHO, 2012; Abdel-Aal et al., 2013). Two main types of AMD are dry (atrophic) and wet (neovascular or exudative). Dry type AMD is quite common, which affects about 80%–90% individuals. The exact reason is still unknown, but drusen deposition (white or yellowish deposits) beneath the macula could be responsible for the macular deterioration/degeneration with large choroidal vessels (Van Lookeren Campagne et al., 2014). Whereas, wet type (neovascular) AMD only affects 10%–15% of individuals, but very dangerous as it contributes to 90% of blindness in AMD patients (Abdel-Aal et al., 2013). Wet type AMD is mainly caused by an abnormality in a retinal blood vessel (bleed, leak fluid) growth, which leads to damaging the macula and eventually lowers the macular density and thus results in blindness.

There are 3 stages of AMD including early, intermediate and late or advance AMD. In the early stage of AMD, the patient might not experience any vision loss, without any symptoms. Early-stage AMD is diagnosed by the presence of smaller or medium-sized (<125  $\mu$ m in diameter) drusen (white or yellowish deposits) beneath the macula of the retina (Coleman et al., 2008). In contrast, at the intermediate stage of AMD, patients may experience slight vision loss or blurred vision (central vision loss) without any noticeable symptoms, but the density of macular pigment might have decreased as compared with the normal eye. Intermediate stage AMD is diagnosed by the presence of medium or large sized (>125  $\mu$ m in diameter) drusen (Vujosevic et al., 2017). During advanced or late stage of AMD, the patient could experience vision loss due to large-sized drusen with thin macular pigment density/bruch membrane and the chances of reverting to normal level is very low (Vujosevic et al., 2017; Ach et al., 2015).

AMD affects approximately 30–35 million worldwide and the numbers are increasing lately in developed and developing countries owing to modified lifestyle (Kawasaki et al., 2010; Friedman et al., 2004). AMD patients encounter various difficulties in their day to day life as they depend on others for all basic needs and thus the quality of life is significantly hampered (Choudhury et al., 2016). AMD is strongly correlated with environmental, behavioral and genetical factors. Some causative factors include smoking, obesity, gender (females are highly prone to AMD), colour of eye (especially lighter eye colour), race (Caucasians are more likely to develop AMD) and genetical factors (complement H and B) (Lim et al., 2012; Kawasaki et al., 2010; Friedman et al., 1999). The



prevalence of AMD in black and Hispanic are lesser than in the white population (Klein et al., 2006). Nevertheless, the exact pathophysiology of AMD is still unexplored, but few researchers have demonstrated that oxidative stress and inflammation might contribute to the onset of AMD (Klein et al., 2014; Van Lookeren Campagne et al., 2014). Especially, photoreceptors and retinal pigment epithelium (RPE) of the retina are highly exposed to UV and blue light, which trigger excessive production of A2E (lipofuscin fluorophore) during the aging process and subsequently results in drusen formation. Hence, supplementation of antioxidants and anti-inflammatory agents may considerably lower the onset of early AMD (Chang et al., 2017; Peng et al., 2016; Buschini et al., 2015).

Currently, no known cure or medication are available for AMD, but the risk of AMD and its progression rate can be reduced by altering lifestyle modification (regular exercise and smoking cessation), less exposure to blue or UV light, intravitreal injection of anti-VEGF (ranibizumab and bevacizumab) or laser treatment as well as increased intake of carotenoids (antioxidant or micronutrients) to maintain macular density (Jager et al., 2008). *In vitro* studies have shown that presence of lutein and zeaxanthin might lower the A2E (lipofuscin) production in RPE cell model (Bhosale et al., 2009; Sundelin and Nilsson, 2001). Ample amount of preclinical and clinical studies (meta-analysis) have concluded that consumption of carotenoids (lutein, zeaxanthin or meso-zeaxanthin) alone or combined with antioxidants (Vit C, E or  $\omega$ -3-Fatty acids) can significantly lower the incidence of AMD and revert the late onset AMD to early stage AMD (Ma et al., 2012; Chong et al., 2008).

## Cataract

Cataract (clouding of the lens) is another common age-related (degenerative) eye disease in which eye lens gradually become opaque (impaired protein fibers leads to the crystalline lens), and thus contributes to blindness than any other eye diseases (WHO, 2012; Gupta et al., 2003). The three main types of age-related cataracts are nuclear, cortical and subcapsular. Nonetheless, nuclear cataract is a common one, which profoundly affects women than men (Congdon et al., 2004). Approximately, 80 million people with cataract are known with impaired vision (reduced vision) and is a causative factor for blindness in about 18 million people. The number of cataract patients is increasing day by day, which is attributable to modified lifestyle (lack of carotenoids) and increased life expectancy (Weikel, 2014; Manayi et al., 2016). Surgery is an effective procedure to treat cataract, but it's too expensive (for an ordinary man) and thus contributes to economic burden in most of the countries (Hodge et al., 2005).

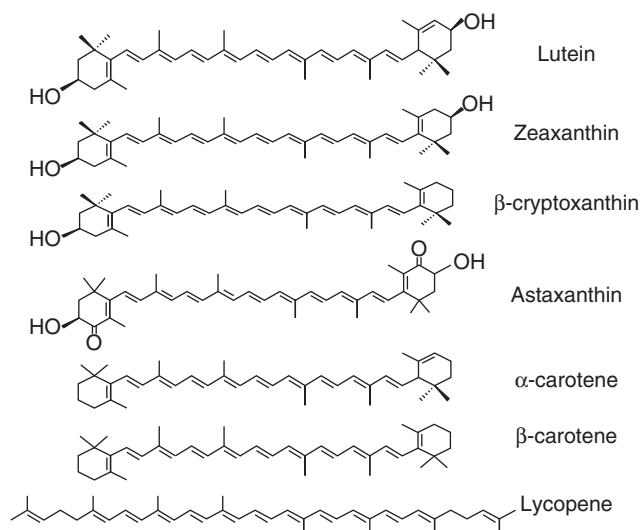
It has been identified that aging, smoking, hypertension, diabetes mellitus, malnutrition and UV/sunlight are the major risk factors for the development of cataract (Roberts et al., 2011). Nuclear cataract is a multifactorial disease, but oxidative stress is the main culprit that alters the protein fibres and subsequently triggers the onset of cataract (Bai et al., 2016; Karppi et al., 2012). Therefore, natural antioxidants like carotenoids (lutein, zeaxanthin,  $\beta$ -carotene, lycopene) are extensively used for attenuating the nuclear cataract development (Ma et al., 2014; Karppi et al., 2012; Christen et al., 2004; Olmedilla et al., 2003). Moeller et al. (2008) concluded that intake of diets rich in lutein and zeaxanthin are associated with decreased incidence of nuclear cataract. In addition, Vu et al. (2006) enrolled 3271 nuclear cataract patients in his trial and treated them with an increased concentration of lutein and zeaxanthin, which led to 36% reduction in nuclear cataract formation. A study conducted by Chang et al. (2011) reported that AREDS (age-related eye disease study) patients after consumption with AREDS formula showed a slight decrease in the risk of nuclear cataract. The correlation between lens clarity with macular pigment implies the importance of lutein and zeaxanthin to treat cataract, which has been confirmed by the clinical trial conducted by Chew et al. (2013a), using AREDS2 supplements (rich in lutein and zeaxanthin) and demonstrated that supplementation of lutein/zeaxanthin could lower the risk of cataract, but no significant changes were noted.

## Carotenoids

Carotenoids are the plant pigments (polyphenol) responsible for different colours (orange, yellow or red) in many vegetables and fruits with various beneficial properties like antioxidant (quenching of free radicals) as well as light harvesting (quenching of excessive light) and photosynthesis (Gong and Bassi, 2016; Demmig-Adams and Adams, 2002). Carotenoids are highly hydrophobic with C40 backbone structure of isoprene units (tetraterpenoid) and usually binds to the membrane with specific membrane proteins. Carotenoids are not synthesized by our body and hence should be supplied with food or supplement (Roberts et al., 2009). Six major carotenoids found in human plasma or tissue are lutein (meso-zeaxanthin)/zeaxanthin,  $\beta$ -cryptoxanthin, astaxanthin, lycopene,  $\alpha$ -carotene,  $\beta$ -carotene (Landrier et al., 2012). The structures of all major carotenoids are shown in Fig. 1. The conventional sources of carotenoids are fruits and vegetables including carrots, papaya, pumpkin, watermelon, sweet potato, yam, tomato, bell peppers, capsicum, paprika, kale, mango, kiwi, spinach, broccoli, cantaloupe, avocado, and orange (Saini et al., 2015; Fernandez-Garcia et al., 2012; Thurnham, 2007). Other sources include algae like *Dunaliella*, *Chlorella*, *Haematococcus*, *Muriellopsis*, *Chlamydomonas*, *Spirulina* spp., (Gong and Bassi, 2016; Guedes et al., 2011), fungi/yeast including *Phytophthora blakesleanus*, *Blakeslea trispora*, *Neurospora crassa* (Mata-Gomez et al., 2014; Pegklidou et al., 2008) and bacteria such as *Halophilic*, *Myco/myxobacterium*, *Paracoccus*, and *Streptomyces* spp. (Schweiggert and Carle, 2016).

Carotenoids are broadly used in food industry as a colorant, additives, feeds (aquaculture) and in the cosmetic industry for treating sunburns or skin related disorders (Schweiggert and Carle, 2016). Moreover, recent studies have indicated that carotenoids display





**Figure 1** Chemical structure of all major carotenoids.

a broad spectrum of biological activities including antioxidant, anti-inflammation, anti-cancer, anti-aging, immunomodulatory as well as vision improvement, cardioprotective, neuroprotective, hepatoprotective functions (Chuyen and Eun, 2017; Zhang et al., 2014; Fiedor and Burda, 2014). Moreover, few carotenoids ( $\beta$ -carotene and  $\beta$ -cryptoxanthin) act as precursors for vitamin A (provitamin A activity). Therefore, many pharmaceutical industries have commercialized carotenoids as health or nutritive supplements. Currently,  $\beta$ -carotene, astaxanthin, and lutein (with zeaxanthin) are the well-recognized carotenoids in the nutraceutical market (Gong and Bassi, 2016). Carotenoids exist in free or esterified form (with fatty acids), and both forms are well absorbed in the intestine and transported (free form) by lipoprotein (high-density lipoprotein-cholesterol, HDL-c or low-density lipoprotein-cholesterol, LDL-c) in blood. Hence, commercialized carotenoids (supplements) are available in free or esterified form (Nolan et al., 2016; Norkus et al., 2010). Carotenoids are classified into two types as xanthophyll's and carotenes (Yaroshevich et al., 2015).

### Xanthophylls (With $O_2$ Has Hydrocarbon- Yellow)

Xanthophylls are an oxygenated derivative of carotenes, which are relatively hydrophilic in nature owing to many hydroxyl group (OH) and keto group ( $=O$ ) as compared with carotenes. The major xanthophylls are lutein, zeaxanthin,  $\beta$ -cryptoxanthin, astaxanthin (Rao and Rao, 2007). Of which lutein, zeaxanthin,  $\beta$ -cryptoxanthin are classified as primary xanthophylls as they produced directly, whereas astaxanthin and canthaxanthin are classified as secondary xanthophylls as they are synthesized from primary xanthophylls.

### Lutein (Meso-zeaxanthin) and Zeaxanthin (Macular Xanthophylls)

Lutein and its stereoisomer zeaxanthin (different OH position) cannot be synthesized by our body and must be supplied through foods (fruits and vegetables) or commercial supplements (Calvo, 2005). Lutein alone or with zeaxanthin have been reported to show potent antioxidant, anti-cancer, anti-inflammatory activities as well as neuroprotective, and cardioprotective properties (Vijayapadma et al., 2014; Abdel-Aal et al., 2013). Nevertheless, lutein and zeaxanthin have a central role in maintaining eye health (Liu et al., 2015; Huang et al., 2008). Lutein or meso-zeaxanthin (produced from macular lutein) and zeaxanthin are the macular carotenoids found in retina (macular lutea-yellow spot) at a ratio of 1:1:1 and contribute to macular pigment (MP) composition, thereby preserving central vision through protecting retina (RPE) from blue light by efficiently scavenging excessive free radicals (Manayi et al., 2016; Ahmed et al., 2005).

However, lutein (also called eye vitamin) is mainly focused as it is prevalent in the diet (especially in spinach, carrot, kale, marigold) with higher bioavailability (with a fatty meal) and better blue light filtering property than zeaxanthin (Sindhu et al., 2010). Lutein is reported to protect the retina from blue light via inhibiting lipid peroxidation, neuronal nitric oxide synthase (inflammation) and c-fos expression (Sasaki et al., 2012). Meanwhile, for macular deposition (centre) the levels of meso-zeaxanthin are considerably higher, but lutein dominates the peripheral macula (Bone et al., 2007). Both lutein and zeaxanthin could quench most of the free radicals including superoxide, peroxyxynitrite, singlet oxygen, hydroxyl radical (Li et al., 2010) as well as upregulate various antioxidant enzyme systems via Nrf2 signaling pathway in the retina (Wu et al., 2015; Zou et al., 2014). Furthermore, zeaxanthin and meso-zeaxanthin are attached to glutathione-S- transferase isoform (GSTp1) and thus maintain the glutathione levels in the macula (Bhosale and Bernstein, 2005).

Meso-zeaxanthin is supplemented to improve macular health (increase the macular pigment density) especially in AMD patients because the machinery for conversion of lutein to Meso-zeaxanthin is impaired (Bernstein et al., 2016). Epidemiological studies have indicated that supplementation of lutein and zeaxanthin would considerably elevate plasma lutein and zeaxanthin level, which in turn significantly abolish the risk of AMD and cataract (Delcourt et al., 2006; Mares, 2004). Several experimental and conservative studies have demonstrated that intervention with lutein and zeaxanthin could considerably increase the macular pigment density (levels) in the retina and thus enhance eye function (Bone et al., 2007; Ma et al., 2012; Liu et al., 2015). Furthermore, lutein and zeaxanthin with omega-3 fatty acids were added to AREDS2 (age-related eye disease study 2) formula with the original AREDS formula including vitamin C and E with zinc, copper, and  $\beta$ -carotene (Chew et al., 2013a).

### $\beta$ -Cryptoxanthin

$\beta$ -Cryptoxanthin is a type of natural xanthophyll carotenoid pigment and hence used as a coloring agent. The primary sources for  $\beta$ -cryptoxanthin are oranges, tangerines, persimmons, papaya, red peppers, apples, and peaches. It structurally resembles  $\beta$ -carotene, lutein and zeaxanthin with high bioavailability (Burri et al., 2016; Burri, 2015). It possesses antioxidant, anti-inflammation and anti-cancer activities (Lorenzo et al., 2008; Lian et al., 2006). Moreover, it improves ocular health owing to its pro-vitamin A, antioxidant properties as well as structural similarity with lutein and zeaxanthin. Recent studies showed that  $\beta$ -cryptoxanthin could efficiently form retinol using the enzyme system involved in  $\beta$ -carotene to retinol conversion (Burri et al., 2016; Burri, 2015).

A clinical trial conducted by Turner et al. (2013) concluded that  $\beta$ -cryptoxanthin-rich tangerines were better absorbed than  $\beta$ -carotene-rich sweet potatoes. A cross-section study conducted in India revealed that low antioxidant ( $\beta$ -cryptoxanthin) concentration was associated with increased risk of cataract formation (Dherani et al., 2008). Lyle et al. (1999) showed a marginal inverse association of lutein and  $\beta$ -cryptoxanthin intake with nuclear cataract, however few other studies indicated no correlation exists between intake of  $\beta$ -cryptoxanthin and the risk of cataract or AMD (Zhou et al., 2011; Delcourt et al., 2006). Hence, more research (preclinical and clinical studies) is required to confirm the direct involvement of  $\beta$ -cryptoxanthin in ocular health.

### Astaxanthin

Astaxanthin is a yellow or orange xanthophyll (terpenes) mostly present in the aquatic environment. It is commonly present in salmon, microalgae, shrimp, and trout (Olaizola et al., 2007). It is also used as a coloring agent as well as feed additives (aquaculture) and thus extensively used in the food industry (Avalos and Limon, 2015). Astaxanthin is one of the best antioxidant carotenoids and hence preferred by the pharmaceutical industry for marketing (Dhankhar et al., 2012; Hussein et al., 2006). It also displays anti-inflammatory, anti-cancer, anti-diabetic, anti-aging activities as well as cardio, neuro and derma protective (Park et al., 2010; Fassett and Coombes, 2009; Pashkow et al., 2008). Astaxanthin is recommended for treating AMD, cataract and other ocular diseases along with lutein and zeaxanthin attributing to its antioxidant, anti-inflammatory and UV filtering activities (Lennikov et al., 2012; Parisi et al., 2008; Olaizola et al., 2007).

Pre-treatment of human lens epithelial cells with xanthophylls (Astaxanthin) led to a significant reduction in the UVB-induced lipid peroxidation and stress signaling and thus showcase that astaxanthin possess strong UV-filtering activity (Chitchumroonchokchai et al., 2004). Astaxanthin is reported to reduce ocular inflammation (uveitis) by suppressing the expression of various pro-inflammatory markers via inhibiting NF- $\kappa$ B signaling pathway in rat endotoxin-induced inflammatory model (Suzuki et al., 2006). Astaxanthin also helped in the prevention of ocular hypertension, cataract and eye strain or fatigue (asthenopia) in various models (Cort et al., 2010; Liao et al., 2009; Nagaki et al., 2006; Shiratori et al., 2005). Moreover, a double-blind clinical trial conducted by Nagaki et al. (2002), demonstrated that astaxanthin considerably improved the accommodation amplitude (adjustment of the lens) in visual display terminal workers.

### Carotenes (With O<sub>2</sub>- Orange)

Carotenes ( $\alpha$  and  $\beta$ -carotene) are terpenoids without oxygen and mainly responsible for orange/red color, with 40 carbon molecules. Both  $\alpha$  and  $\beta$ -carotene are the critical carotenes, which act as a provitamin A (retinal/retinol/retinoic acid) and thus directly involved in improving ocular health as deficiency of vitamin A could lead to night blindness (Strobel et al., 2007; Christen et al., 2000). However,  $\beta$ -carotene (trans form) has higher potential to produce vitamin A than  $\alpha$  carotene or  $\beta$ -cryptoxanthin and thus has been extensively studied (Fernandez-Garcia et al., 2012; Grune et al., 2010).  $\beta$ -carotene is the most abundant carotenoids in nature, which is used as a food colouring agent/additives; it also exhibits numerous pharmaceutical properties including antioxidant, anti-obesity, anti-cancer, anti-aging, anti-atherosclerotic and anti-sunburn properties as well as hepatoprotective, neuroprotective and improved vision and night blindness prevention (Virtamo et al., 2014; Dufosse et al., 2005).

$\beta$ -carotenes are highly absorbed in duodenal region (small intestine) and transported via VLDL, CM and LDL and subsequently stored in the liver and other peripheral tissues; finally, the stored form is converted to active vitamin A using various retinoid metabolizing enzymes (retinal/retinol/retinoic acid/retinyl esters) as required (Li et al., 2014; Fernandez-Garcia et al., 2012). Studies have

shown that vitamin A (retinol) can effectively protect the surface of the eye (cornea) from various pathogenic infections like bacteria, virus, and fungi and is thus recommended for treating dry eyes. In addition, vitamin A is also prescribed for lowering ocular inflammatory conditions like superior limbic keratoconjunctivitis/retinitis (Toshida et al., 2017; Strobel et al., 2007; Smith and Steinemann, 2000). Hence,  $\beta$ -carotenes were included in the AREDS trial sponsored by the National Eye Institute, to improve ocular health. Chronic supplementation of  $\beta$ -carotene could increase the prevalence of lung cancer in smoking population owing to excessive oxidation of  $\beta$ -carotene (Tanvetyano and Bepler, 2008). Few randomized clinical trials (RCTs) have indicated that no significant association exists with the risk of cataract (Cui et al., 2013; Christen et al., 2003); however when combined with vitamins or antioxidants,  $\beta$ -carotenes showed a significant reduction in the risk of cataract (Wang et al., 2014a; Ravindran et al., 2011).

## Lycopene

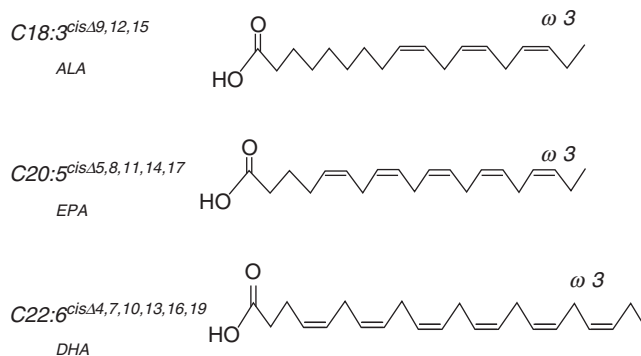
Lycopene is one of the pivotal carotenoids, which is responsible for bright red color in various fruits and vegetables including tomatoes, watermelon, apricots, and papaya (Chan and Hung, 2014). Tomatoes are the best sources of lycopene (*cis*-isoform), which are highly absorbed after cooking. Lycopene has antioxidant, anti-inflammatory, anti-cancer (prostrate), osteoprotective, and neuroprotective activities as well as vision performance improvement particularly against cataract and AMD (Yang et al., 2016; Wei and Giovannucci, 2012). Moreover, lycopene has higher bioavailability than  $\beta$ -carotene with better free radical scavenging property, which makes lycopene a potent candidate to preserve visual function by attenuating macular degeneration (Gupta et al., 2003).

Lycopene is present in human RPE-choroid and ciliary body (Chan and Hung, 2014). The effect of lycopene on ocular health (owing to its antioxidant, anti-inflammatory and anti-angiogenic activities) is ambiguous because few reports have showed positive results (Yang et al., 2016; Abdulazeez et al., 2012; Cardinault et al., 2005) against various eye diseases, whereas others showed negative results (Cui et al., 2013; Brown et al., 1999). An *in vitro* study conducted by Yang et al. (2016) concluded that lycopene treatment could considerably abolish oxidative stress and inflammation and thereby protect retinal pigment epithelium in ARPE-19 cell model and might be recommended for treating AMD in combination with other standard drugs. A study conducted among the Indian population indicated an inverse correlation between the blood antioxidant (lutein, lycopene,  $\beta$ -cryptoxanthin) concentration and the risk of cataract formation (Dherani et al., 2008).

## Omega-3-Fatty Acids

Omega( $\omega$ )-3-fatty acids belong to polyunsaturated fatty acids (PUFA) and are also classified as essential fatty acids. Since these  $\omega$ -3 fatty acids cannot be synthesized by our body, humans rely on dietary sources (Chong et al., 2008). The major omega-3-fatty acids are  $\alpha$ -linolenic acid (ALA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Fig. 2). ALA is a precursor of DHA and EPA, but its synthesis in our body is very limited (SanGiovanni and Chew, 2005). Omega fatty acids are reported to show several beneficial effects including antioxidant, anti-inflammatory, immunomodulatory, hypolipidemic, anti-cancer as well as cardioprotective, neuroprotective and hepatoprotective activities (Swanson et al., 2012; Hooper et al., 2006). Studies have shown that intake of diets rich in  $\omega$ -3 fatty acids and fish is inversely proportional to the risk for AMD development (Querques and Souied, 2014; Seddon et al., 2003). Especially, DHA (structural lipid) deficiency is reported to initiate the onset of AMD as it is present in outer segments of retina, especially photoreceptor membranes (Chong et al., 2008). Moreover, DHA plays a vital role in altering the G-protein coupled signaling pathway and thus contributes to rhodopsin regeneration and thereby healthy vision maintenance (Litman et al., 2001).

Few studies have confirmed that EPA might play a vital role in retinal function by acting as a precursor for many signaling molecules (Chow et al., 2013b; Augood et al., 2008). Furthermore,  $\omega$ -3 fatty acids play a key role in maintaining optical function by protecting macular region from oxidative stress (lipid peroxidation), inflammation, as well as regulate retinal blood vessel growth



**Figure 2** Chemical Structure of Omega Fatty Acids.

(new choroidal vessel formation) and thus suppress the risk of both early and advanced AMD (Christen et al., 2011; SanGiovanni et al., 2008). A meta-analysis conducted by Chong et al. (2008) with 3202 AMD subjects that consumed diets rich in  $\omega$ -3 fatty acids (at least twice a week) showed a reduced risk of late/advanced AMD. Likewise, a cross-section study also showed that eating oily fish (at least once a week) could protect against neovascular AMD (Augood et al., 2008). In addition, omega-3-fatty acids can lower the risk of cataract and computer vision dry eye syndrome via regulating tear production and evaporation rate (Bhargava et al., 2015; He and Bazan, 2010; Lu et al., 2005).

### Holistic Activity of Lutein/Zeaxanthin and $\omega$ -3 Fatty Acid

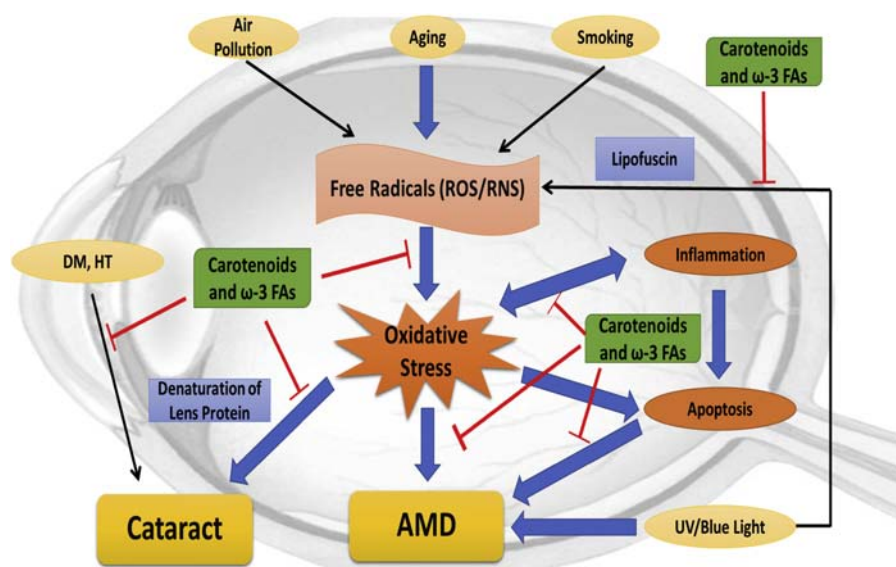
Epidemiological studies have shown that increased intake of carotenoids like lutein/zeaxanthin and  $\omega$ -3 fatty acids (FA) was found to be associated with decreased risk of cataract and AMD (Wang et al., 2014b; Huang et al., 2008; Delcourt et al., 2006; Moeller et al., 2000). Many clinical trials have proved that consumption of a diet rich in carotenoids (lutein/zeaxanthin) and  $\omega$ -3 fatty acid (DHA/EPA) can considerably reduce the risk of neovascular AMD and nuclear cataract (Ho et al., 2011; Olmedilla et al., 2003). Another study conducted by Johnson et al. (2008) demonstrated that DHA might facilitate the macular deposition of lutein (especially central part of fovea), which synergistically combats AMD or cataract. The AREDS research group (2001a) concluded that daily oral supplementation with AREDS formula (Vit C, E,  $\beta$ -carotene, copper, and Zinc) could considerably lower the risk of developing advanced AMD by 25%. Likewise, AREDS research group (2001b), demonstrated that daily oral supplementation with AREDS formula did not show any statistical significance on age-related lens opacities (cataract) or visual acuity. Based on experimental and observatory studies, it has been hypothesized that addition of macular carotenoids (lutein, zeaxanthin) and  $\omega$ -3-fatty acids with AREDS formula might further reduce the risk of developing advanced AMD.

Chew et al. (2013b) conducted an AREDS2 study between 2006–12 with 4203 participants (aged 50–85) at risk of progression to advanced AMD and found that supplementation with AREDS2 formula (Vit C, E, lutein, zeaxanthin,  $\omega$ -3-fatty acids and Zinc) did not show any further decrease in the risk of progression to advanced AMD or visual acuity in comparison with AREDS. Nevertheless, lutein and zeaxanthin replacement with  $\beta$ -carotene in AREDS2 formulation could potentially decrease the incidence of lung cancer in former smokers, which has been noted in the AREDS study. Similarly, AREDS2 formula was used to evaluate the beneficial effect of age-related cataract, but no marked changes were observed on age-related cataract (Chew et al., 2013a).

Overall, AREDS2 formula is highly preferred for abolishing the risk of developing advanced AMD, but not for cataract. Dawczynski et al. (2013) concluded that long-term supplementation with lutein, zeaxanthin,  $\omega$ -3 PUFA (LUTEGA) would significantly increase the MP, which in turn improves and stabilize the vision (BCVA) in AMD patients. Rotterdam study conducted by Ho et al. (2011), demonstrated that high dietary intake of carotenoids (antioxidants) and  $\omega$ -3-fatty acids suppressed the prevalence of early AMD. The schematic representation of current review is illustrated in Fig. 3.

### Conclusion

This contribution summarized that supplementation of micronutrients or antioxidants with modified lifestyle (regular exercise, quit smoking) could considerably improve ocular health. Carotenoids (lutein, zeaxanthin) can act as a protective shield (improving



**Figure 3** The schematic representation of current review: DM, Diabetes Mellitus; HT, Hypertension; ROS, Reactive oxygen species; RNS, Reactive nitrogen species; AMD, age-related macular degeneration;  $\omega$ -3 FAs, omega-3 fatty acids.

macular pigment density as well as scavenging excessive free radicals) and thus prevent the formation of AMD (neovascular AMD) and cataract (nuclear cataract). Whereas  $\omega$ -3 fatty acids did not effectively protect retina/macula (insufficient evidence), and therefore, it might be preferred with other antioxidants (carotenoids) or micronutrients for better protective action against AMD or cataract. This is because DHA might enhance the macular deposition of lutein in the macula (synergic effect). However, large-scale studies are needed in future to evaluate the effective dose of these carotenoids, alone or in combination with  $\omega$ -3 fatty acids against AMD or cataract as well as the exact mechanisms of action need to be explored.

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# Cholesterol-Reducing Foods: Proteins and Peptides

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## Glossary

**Bioabsorption** The process whereby substances are absorbed by the tissues and organs of organisms.

**Bioactive peptides** Bioactive peptides are a key category of molecules useful for functional food application. Most known bioactive peptides are fragments that are encrypted in the primary sequences of proteins and provide physiological functions beyond basic nutritional benefits.

**Mass spectrometry** An analytical technique that ionizes chemical species and sorts the ions based on their mass-to-charge ratio.

**Modulation of metabolic pathway** Modulation of the activity of one and/or more targets involved in the same intracellular metabolic pathway.

**Peptidomics** The branch of molecular biology that studies the set of peptides of an organism or a biological sample.

## Nomenclature

**HMGCoAR** 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase

**AMPK** Adenosine monophosphate-activated protein kinase

**AP** Apical

**BL** Basolateral

**CE** Capillary electrophoresis

**CID** Collision induced dissociation

**ECD** Electron capture dissociation

**ETD** Electron transfer dissociation

**ESI** Electrospray ionization

**ER** Endoplasmic reticulum

**(GSK)-3 $\beta$**  Glycogen synthase kinase 3 $\beta$

**HPLC** High Pressure Liquid Chromatography

**IEX** Ion-exchange

**LDLR** LDL receptor

**LDL** Low-density lipoprotein

**MALDI** Matrix-assisted laser desorption/ionization

**MRM** Multiple reaction monitoring

**PI3K** Phosphatidylinositol-3-kinase

**PCSK9** Proprotein convertase (PC) subtilisin/kexintype 9

**Akt** Protein kinase B

**RO** Reverse osmosis

**RPC** Reverse-phase

**SEC** Size exclusion

**SCAP** SREBP cleavage-activating protein

**SREBPs** Sterol-responsive element binding protein

**UF** Ultrafiltration

## Introduction

The edible seeds of numerous plants are major staple foods in most countries. Their importance is currently increasing owing to the growing awareness of their lower environmental impact when compared to animal foods. Moreover, the interest of consumers is attracted by the numerous health benefits they provide, particularly in the area of hypercholesterolemia and hypertension prevention. The first published papers refer to soybean, but other seeds are also promising in these areas, although available literature is still scarce. The seed components responsible for the observed activities are probably numerous: in particular, different papers have underlined the importance of protein, soluble and insoluble fiber, polyunsaturated lipids, phytosterols, tocopherols, and other

phytochemicals, such as polyphenols. The final observed effects derive probably from a combination of the specific activities of all these components probably also with a synergistic behavior.

The importance of plant proteins has been underlined by numerous clinical studies performed on soybean protein. A recent meta-analysis (Harland and Haffner, 2008) has shown that the consumption of 25 g per day of soybean protein leads to a reduction of mean total cholesterol by 0.22 mmol/L (95% CI -0.142 to -0.291,  $p < 0.0001$ ), mean LDL-cholesterol by 0.23 mmol/L (95% confidence interval, CI -0.160 to -0.306,  $p < 0.0001$ ), and mean blood triglycerides by 0.08 mmol/L (95% CI -0.004 to -0.158,  $p = 0.04$ ), concluding that a modest amount of soybean protein in the diet of mild hypercholesterolemic patients results in a small but significant 6% reduction of LDL-cholesterol.

Other plants have been investigated, particularly grain legumes. Hypocholesterolemic effects have been observed when suitable animal models have been treated with white lupin, narrow-leaf lupin, pea, and broad bean proteins versus casein. On the contrary, some clinical studies have been performed on the protein of narrow-leaf lupin, that produced only a moderate decrease of total and/or LDL cholesterol in mild hypercholesterolemic subjects who had consumed about 30 g per day of lupin protein included in different model foods for 4–12 weeks.

Since proteins undergo an extensive hydrolysis during gastrointestinal digestion, the actual bioactive species should be specific peptides encrypted in their sequences that are released by digestive enzymes. These peptides are sometimes referred to as cryptides. The experimental approach to sort out these peptides involves a series of steps: 1) release of the bioactive sequences; 2) initial screening for targeted bioactivities, mainly in suitable cell models; 3) purification and separation; 4) further determination of the biological activity of purified peptides; 5) peptide identification by mass spectrometry (MS); 6) final *in vitro* and *in vivo* validation of the biological activity (often performed on synthetic peptides).

As far as cholesterol metabolism is the topic of interest, HepG2 cells are the preferred reference model, which has permitted experimental demonstration that protein hydrolysates from soybean, lupin bean, hempseed, and amaranth are able to modulate cholesterol metabolism. These initial results have stimulated numerous investigations aimed at assessing: a) the actual bioactive species; and b) the mechanism of action.

## Technological Strategies in Peptidomics

### Production and Purification of Peptides From Plant Foods

The most common methods to produce bioactive peptides from plant proteins are by enzymatic hydrolysis or fermentation (Lee and Hur, 2017). Generally, the use of enzymatic hydrolysis is preferred over microbial fermentation due to the short reaction time, easy scalability, and predictability. More than a single proteolytic enzyme (purified or crude) can be used for protein hydrolysis to produce hydrolysates containing short peptide sequences. However, the optimized mode of enzyme addition (whether simultaneously or sequentially) depends on the optimal pH and temperature conditions for each enzyme. The selection of the best enzymes and hydrolytic conditions is a critical aspect for producing bioactive hydrolysates, since peptide sequences and their biological activities may differ depending on the type of enzyme used. Many known bioactive peptides deriving from plant proteins have been produced using a single enzyme, such as pepsin or trypsin or a combination of them in order to simulate the gastrointestinal digestion, as recently reported for the investigation of hemp seed protein hydrolysates (Girgih et al., 2014a).

However, the large number of peptides contained in the hydrolysates, produced either by enzymatic hydrolysis or by fermentation, suggests the need to perform some preliminary separation before identification by MS. In addition, peptides vary in size, hydrophobicity and net charge. In most cases, multi-step procedures are needed, since their natural diversity can make it very difficult to perform a comprehensive isolation of peptides from a complex mixture using a single purification method. Peptide enrichment is primarily conducted using membrane filtration and chromatographic methods. Ultrafiltration (UF), nanofiltration, and reverse osmosis (RO) are frequently used membrane-based technologies employed for bioactive peptide separation. However, membrane filtration can result in incomplete protein removal (Lemes et al., 2016) due to the possible aggregation of some peptides and peptide loss in the precipitate. HPLC has become therefore, the preferred technique for the separation of peptides, because of its versatility, efficiency, and automation capabilities. Reverse-phase (RPC), ion-exchange (IEX), size exclusion (SEC), and affinity chromatography are currently used in peptide fractionation. Specific innovative methods are applied in the area of “phosphopeptidomics” (Leitner et al., 2010) using metal oxide affinity chromatography.

### The Role of Mass Spectrometry in Peptidomics

MS-based peptidomic approaches set a new standard in the discovery and quantification of nutritionally relevant bioactive peptides, becoming the undisputed tool in bioactive peptide analysis, which reduces the use of other identification techniques, such as amino acid analyzers or Edman sequencing. High sensitivity and accurate mass information, likely structure elucidation, and short time analysis are the main benefits of the use of MS in peptide identification.

The electrospray ionization (ESI) source and matrix-assisted laser desorption/ionization (MALDI) are the most important ionization sources used in peptidomics. Despite MALDI ionization is an alternative source used in peptidomic and proteomic investigations, the majority of peptidomic studies are conducted using LC-ESI-MS/MS approach. In fact, about a half of the known bioactive peptides are shorter than 10 amino acids and therefore fall into the low mass range where MALDI matrix interference

is overwhelming (Panchaud et al., 2012). This severely limits the applicability of MALDI MS in peptidomic studies. In addition to LC, capillary electrophoresis (CE) has also been extensively used for peptide separation.

MS experiments can be addressed directly to measure the molecular mass of derived peptides (single-stage MS) or used to generate AA-sequence information from tandem mass spectra (MS/MS or MSn), obtained in the hybrid arrays by collision induced dissociation (CID). Very recent technological improvements further enlarge the capabilities of tandem mass spectrometry strategies. Electron transfer dissociation (ETD) and electron capture dissociation (ECD) have emerged as new tools to sequence peptides in very complex samples. For example, hybrids LC-ESI-Q-IT has been used to identify the major hypocholesterolemic peptides generated by the *in vitro* hydrolysis of *Amaranthus cruentus* protein (Soares et al., 2015). In particular, GGV, IVG, and VGVV have been identified by coupling ultrafiltration steps to MS analysis.

In peptidomics, there are two complementary kinds of strategies for the discovery and/or quantification of potentially bioactive peptides: the hypothesis-free approach and the hypothesis-driven targeted one. The former strategy, referred to as shotgun or data-dependent analysis (DDA), is widely used for obtaining a comprehensive peptide profile of biological systems in a complex peptide mixture. For example, this strategy, including database searching, was adopted to characterize some hemp seed protein hydrolysates produced by using single enzymes or a combination of proteases in order to mimic the gastrointestinal digestion. Specifically, the number of peptides identified ranged from 90 belonging to 33 species-specific parent proteins in the peptic hydrolysate to 9 belonging to 6 proteins in the pancreatin digest. The peptic and tryptic hydrolysates provided the best hypocholesterolemic candidates (Zanoni et al., 2017a).

The latter strategy, referred to as targeted multiple reaction monitoring (MRM), is used to probe a predefined subset of peptides. LC-MRM was used to obtain quantitative information of the degree of transport of some hypocholesterolemic soy peptides, i.e. IAVPGEVA, IAVPTGVA, LPYP, across Caco-2 cell model as well as to elucidate the metabolic degradation due to the action of brush border peptidases, to which they may be subjected when exposed to the microvilli surface. LC-MRM is used for the identification of small peptides, even in the presence of complex mixtures consisting of di-, tri- and tetra-peptides, containing amino acids such as Val, Leu, and Ile (where Leu and Ile are isobaric amino acids), which have similar chromatographic behaviors.

The application of integrated multiple-omics information, such as structural information provided by MS and bioinformatics driven-modeling, to a peptic hydrolysate from lupin protein allowed the identification of LILPKHSDAD and LTFPGSAED, two specific peptide sequences very powerful in the modulation of cholesterol metabolism (Zanoni et al., 2017b).

In conclusion, mass spectrometric measurement with high-energy collision-induced dissociation (CID) tandem MS data (yielding immonium ion and other MRM-relevant data) can provide sufficient information for peptide sequences, when combined with retention times and relevant protein sequence databases and bioinformatics-driven approaches.

## Absorption of Bioactive Peptides

Food-derived bioactive peptides have to cross the gastrointestinal barrier and enter the circulation in order to reach their target sites for exerting their biological activities. Therefore, the transport through intestinal cells is a major factor influencing their bioavailability and is a very critical issue that needs to be addressed. The hypothesis that small peptides may escape complete digestion and be transported from intestinal lumen into blood circulation is gaining acceptance, mainly due to numerous studies describing the *in vitro* trans-epithelial transport of bioactive peptides. In fact, the primary *in vivo* function of the small intestine is to absorb nutrients from the lumen and to transport them to blood circulation. This absorption is mainly performed by enterocytes, which are continuously exposed to food components able to modulate some of their physiological functions, although the mechanism of passage across the intestinal mucosa is still largely unknown.

In light of these observations, the Caco-2 cell model is very often employed in order to study the ability of small peptides (2–20 amino-acidic residues) to permeate the gastro-intestinal barrier. Despite its tumoral origin, this human intestinal cell line (originally isolated by J. Fogh from a human colon adenocarcinoma) has been extensively used over the past 30 years and represents the best available cellular model to investigate intestinal barrier functions, absorption mechanisms, and metabolic activities (Rubas et al., 1996).

When seeded in culture inserts fitted with polycarbonate filters, Caco-2 cells undergo a process of spontaneous differentiation leading, after 21 days, to the formation of a monolayer of polarized cell, coupled by tight junctions and expressing several morphological and functional features of small intestinal enterocytes. Differentiated Caco-2 cells on filters create a two-compartment system, where the apical (AP) side of the cell monolayer (*in vivo* corresponding to the intestinal lumen) is separated from the basolateral (BL) side (*in vivo* facing the intestinal vascular and lymphatic circulation).

Recently, this model has been used for evaluating the bioavailability of antioxidant and hypocholesterolemic peptides from soybean  $\beta$ -conglycinin and lupin protein (Amigo-Benavent et al., 2014). Both studies provided innovative information on the absorption of these peptides, through the analysis of the efflux in the BL compartments from the AP side by using suitable analytical techniques based on mass spectrometry approach.

Briefly, the former study (Amigo-Benavent et al., 2014) evaluated the bioavailability of antioxidant peptides released by gastrointestinal digestion from soybean 7S globulins. In total, 25 different peptides were identified in AP sample, out of which 22 were found also in the BL chamber, indicating that they are absorbed by the Caco-2 cells. Among the 22 identified peptides,



YVNPNDNEN and YVNPNNEN were demonstrated to have the capacity to modulate cholesterol metabolism in hepatic cells (Amigo-Benavent et al., 2014).

A similar study performed on tryptic and peptic hydrolysates from lupin protein has shown that 11 tryptic peptides and 8 peptic ones are bioavailable in the same Caco-2 cell model (Lammi et al., 2016a). Considering the overall features of absorbed peptides, they are very heterogeneous being constituted by 9–23 amino acid residues and mostly belong to  $\beta$ -conglutin, the acidic 7S globulins of lupin. The mechanisms of absorption across intestinal epithelium may vary from passive diffusion of hydrophobic molecules across membranes to active transport, either by membrane transporters or vesicular-mediated transcytosis, and finally to permeation across tight junction complexes. Moreover, the transport of these peptides to the BL compartment takes place with a certain degree of selectivity. Interestingly, the same paper showed that the absorbed peptides maintained the hypocholesterolemic activity previously observed in the parent tryptic and peptic lupin hydrolysates.

## Bioactivity

### Cholesterol Metabolism Pathway

From a physiological point of view, the main cholesterol-lowering organ in our body is the liver. In fact, the majority of plasma cholesterol is transported by the low-density lipoprotein (LDL) fraction, and the cellular uptake of LDL is mediated by the LDL receptor (LDLR) mainly expressed at hepatic levels. The circulating level of LDL cholesterol is determined in large part by its rate of uptake through the LDLR pathway. In general, the LDLR expression is finely tuned by changes in intracellular cholesterol. In fact, since the transcription of the LDLR and the genes required for cholesterol and fatty acid synthesis are controlled by membrane-bound transcription factors called sterol-responsive element binding protein (SREBPs), the intracellular cholesterol acts with a negative feedback inhibition mechanism. In particular, the SREBP-2 isoform is responsible for the LDLR and 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase (HMGCoAR) transcription, and the SREBP-2 maturation is regulated by the intracellular cholesterol homeostasis. Moreover after synthesis, SREBP-2 forms a complex with the SREBP cleavage-activating protein (SCAP) and is localized in the endoplasmic reticulum (ER), as an inactive precursor (pro-SREBP-2). Sterol deficiency results in the release of SREBP-2/SCAP complex from ER and transport to the Golgi, where pro-SREBP-2 is processed further, allowing the N-terminal fragment (mature SREBP-2) to enter the nucleus and up-regulate transcription of LDLR and HMGCoAR (Goldstein et al., 2006; Sato, 2010).

HMGCoAR is the enzyme, which plays a key role in intracellular cholesterol biosynthesis, because it is the rate-controlling enzyme in the mevalonate pathway, and therefore it constitutes the target of numerous investigations aimed at lowering the rate of cholesterol biosynthesis. Emerging evidences suggest that the phosphatidylinositol-3-kinase (PI3K)/Akt/Glycogen synthase kinase (GSK)-3 $\beta$  pathway is implicated in the regulation of lipid metabolism through the activation of SREBP-2 with a positive effect on the LDLR levels at cellular membranes. Moreover, the adenosine monophosphate-activated protein kinase (AMPK) pathway is also involved in the regulation of the cholesterol metabolism through its effect on the phosphorylation of HMGCoAR, which is its main target. In particular, when AMPK is activated through an increase of its phosphorylation on the threonine 172 residue, it is able to inhibit the HMGCoAR activity through increased phosphorylation of serine 872 residue.

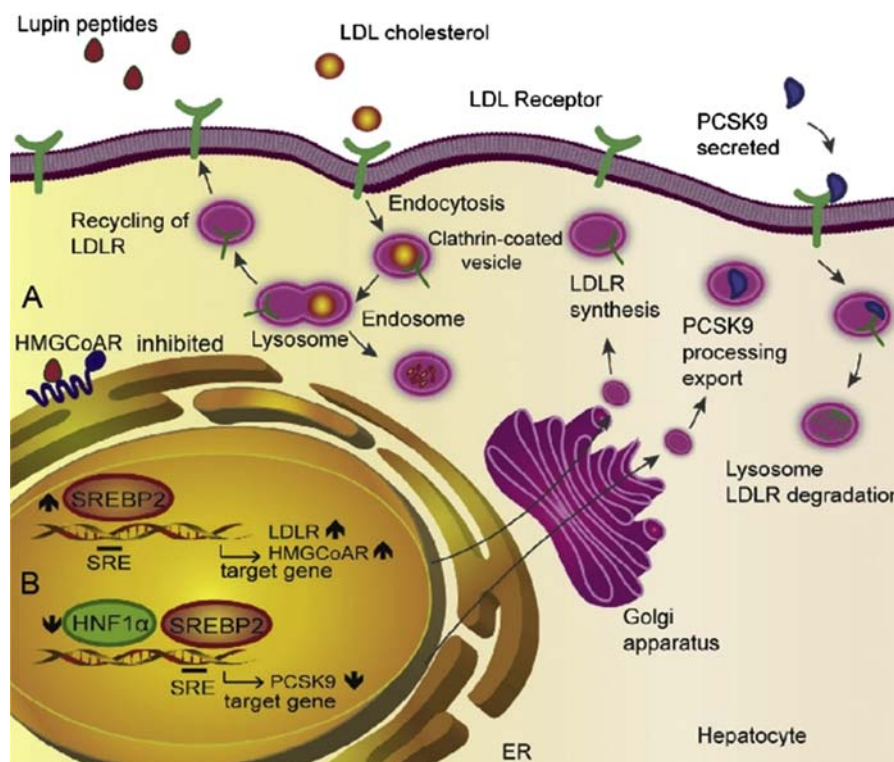
Recently, proprotein convertase (PC) subtilisin/kexintype 9 (PCSK9) has been identified as a key player in the regulation of the cholesterol metabolism through a direct effect on the LDLR activity (Horton et al., 2007). Briefly, in the absence of PCSK9, hepatic LDLR is shuttled back to the plasma membrane after cholesterol delivery to the lysosome for degradation. PCSK9 binding, instead, prevents this LDLR shuttling and targets it for degradation. PCSK9 primarily acts on the LDLR as circulating plasma protein and several studies have underlined the positive relationship between circulating PCSK9 and plasma LDL cholesterol levels. On the contrary, PCSK9 inhibition significantly reduce the LDL cholesterol levels reducing the risk of cardiovascular event. For this reason, PCSK9 is considered an attractive target for the management of hypercholesterolemia.

### Lupin Peptides

A study on human hepatic HepG2 cells has provided a detailed elucidation of the molecular mechanism by which lupin peptides exert the hypocholesterolemic activity observed in animal models and in clinical trials. Both the tryptic and peptic hydrolysates from lupin protein are able to interfere with the HMGCoAR activity, up-regulating the LDLR due to the activation of SREBP-2, through the modulation of the Akt/GSK3 $\beta$  pathway (Lammi et al., 2014). In addition, from a functional point of view, both peptic and tryptic peptides increase the LDLR protein levels inducing an increased LDL-uptake by HepG2 cells. The research was subsequently concentrated on P5 (LILPKHSDAD) and P7 (LTFPGSAED), two peptides containing 10 and 9 amino acid residues, respectively, deriving from the hydrolysis of lupin protein with pepsin. Both peptides belong to  $\beta$ -conglycinin and are potentially absorbable at intestinal level since they can be transported through the Caco-2 cell system. Interestingly, an *in silico* approach had suggested that both P5 and P7 might be able to bind the HMGCoAR catalytic site inhibiting the enzyme activity. Indeed, biochemical and cellular studies have confirmed that these peptides are able to modulate in a positive manner cholesterol metabolism leading to an increase of the LDLR protein levels (Zanoni et al., 2017b).

A recent clinical study has shown that the consumption of 30 g/day lupin protein for 3 months led to a 12.7% reduction in plasma PCSK9 level (Lammi et al., 2016b). The molecular mechanism through which lupin peptides modulate PCSK9 was





**Figure 1** Hypocholesterolemic mechanism of action mediated by lupin peptides in human hepatic HepG2 cells. Upon cell penetration, peptic and tryptic lupin peptides act as competitive inhibitors of HMGCoAR, leading to a reduction of intracellular cholesterol synthesis. When intracellular cholesterol level decreases, the transcription factor SREBP2 is activated and LDLR and HMGCoAR genes are transcribed with subsequent increase of LDLR and HMGCoAR protein levels and localization of LDLR in plasma membrane (A). In parallel, in a synergic way, lupin peptides reduce the PCSK9 protein level production and secretion. In particular, through the reduction of HNF1- $\alpha$  protein level, they lead to a decrease of intracellular precursor and mature PCSK9 protein levels. In agreement, the PCSK9 down-regulation is translated in a reduction of HepG2 cell ability to secrete mature PCSK9 in the extracellular medium, with the consequent stabilization of active LDLR on hepatic cellular membrane (B). For this reason, the distinct modulation of the two pathways leads to hypocholesterolemic effects through an improved and synergic activity of LDLR, which can bind and carry the extracellular LDL in HepG2 cells. Reprinted with the permission from Lupin protein exerts cholesterol-lowering effects targeting PCSK9: From clinical evidences to elucidation of the *in vitro* molecular mechanism using HepG2 cells. Lammi, C., Zannoni, C., Calabresi, L., Arnoldi, A., 2016. Journal of Functional Foods 23, 230–240. Copyright 2017, Elsevier.

investigated using HepG2 cells, demonstrating that both peptic and tryptic lupin hydrolysates decrease the mature PCSK9 protein levels and secretion at the extracellular environment (Fig. 1). An investigation on absorbable peptides indicate that P5 is mainly responsible of this mechanism of action. In addition, P5 is also able to inhibit the protein–protein interaction (PPI) between PCSK9 and LDLR with an  $IC_{50}$  equal to 1.6  $\mu$ M. Using bioinformatic tools, it was possible to build an *in silico* docking model of the P5 interaction with the PCSK9 LDLR binding site, which permitted elucidation of how this peptide impair the PPI between these two crucially important proteins (Lammi et al., 2016c).

### Hemp Seed Peptides

Hemp seed contains about 25%–30% proteins and is currently gaining a big interest in human nutrition (Girgih et al., 2014b). A recent paper has shown that a peptic hydrolysate of total hemp seed protein is able to mediate a hypocholesterolemic effects on human hepatic HepG2 cells by inhibiting the HMGCoAR activity and regulation with a statin-like mechanism. Moreover, the same hydrolysate activated the AMPK pathway which led to intracellular inhibition of HMGCoAR activity and induced LDLR protein level augmentation with consequence of an increased ability of HepG2 cells to absorb the LDL from the extracellular environment (Zannoni et al., 2017a). Similarly to statin, the hemp seed protein hydrolysate increased the mature PCSK9 protein levels versus the untreated samples. This is the main divergence of action between hemp seed and lupin peptides.

### Soybean Peptides

Different authors have pointed out that both glycinin and  $\beta$ -conglycinin contain some hypocholesterolemic peptides encrypted in their sequences. Two peptides, LPYP and IAVPGEVA, were isolated and characterized after digesting glycinin with trypsin and

pepsin, respectively. Both peptides were able to inhibit HMGCoAR activity. Moreover, the alignment of IAVPGEVA with the glycinin sequence permitted the identification of another inhibitory peptide, IAVPTGVA. *In vitro* experiments using the catalytic domain of HMGCoAR showed that these peptides acted as competitive inhibitors. Further experiments in HepG2 cells showed that the inhibition led to an increased LDLR protein levels by the activation of the SREBP2 pathway and to an enhanced LDL-uptake in the same cells. Moreover, these peptides were able to increase the phosphorylation level of HMGCoAR on Ser 872 (the inactive form of HMGCoAR) via the activation of the AMPK-pathway (Lammi et al., 2015). Another study has instead reported evidence on the hypocholesterolemic activity of YVNPNDNEN and YVNPNNEN, two peptides that are derived from  $\beta$ -conglycinin. Research in HepG2 cells aimed at investigating their effects on cholesterol metabolism showed that they are able to up-regulate the LDLR protein levels and behave as competitive inhibitors of HMGCoAR activity with a statin-like mechanism. Interestingly, the former is a fragment of LRVPA GTTFYVNPNDNENLRMIA, previously shown to increase the LDL-uptake and degradation in hepatocytes.

### Amaranth Peptides

A recent investigation (Soares et al., 2015) provided evidence underling the ability of peptides derived from amaranth protein hydrolysis to exert hypocholesterolemic effect. In particular, *in vitro* experiments using the catalytic domain of HMGCoAR demonstrated that the peptides GGV, IVG, and VGV are able to inhibit the reductase activity. These findings suggest that these peptides act as HMGCoAR competitive inhibitors, similarly to the soybean (IAVPGEVA, IAVPTGVA, LPYP, YVNPNDNEN and YVNPNNEN) and lupin (P5 and P7) peptides. However, more detailed studies should be performed in order to better characterize the hypocholesterolemic mechanism of action at cellular level and to find out the potential bioavailability of these peptides.

### Conclusion

The search of hypocholesterolemic peptides from food proteins is certainly an intriguing topic, although it has not reached any practical application yet. To achieve this, the open issues to be solved are: a) the selection of the best enzymes, b) the analysis of very short peptides; c) the stability verification and implementation by tools such as microencapsulation; c) the assessment of the bioavailability; d) the validation of the mechanism of action; and e) the confirmation of the activity by clinical trials. Several research groups are actively working on different protein sources to address all these issues all over the world.

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## Food for Male Reproductive Tract Health: Omega-3 Fatty Acids

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### Glossary

**Oligospermia** a condition of low sperm concentrations defined as less than  $15 \times 10^6$  sperm per mL.

**Asthenozoospermia** a condition defined as low sperm motility with less than 32% progressive motility in a semen sample.

**Teratozoospermia** a condition of abnormal sperm morphology defined as less than 4% spermatozoa with normal morphology.

**Normozoospermic** a condition defined having equal to or above lower reference limit set by World Health Organization in total number of spermatozoa, and percentage of progressively motile and morphologically normal spermatozoa

**Oligoasthenozoospermia** a condition defined having low sperm concentrations, combined with low sperm motility moving progressively in a semen sample

### Nomenclature

AA Arachidonic acid (20:4n-6)

AI Adequate Intake

ALA  $\alpha$ -linolenic acid (18:3n-3)

AMDR Acceptable Macronutrient Distributions Range

DHA Docosahexaenoic acid (22:6n-3)

DRI Dietary Reference Intake

EPA Eicosapentaenoic acid (20:5n-3)

LA Linoleic acid (18:2n-6)

PUFA Polyunsaturated fatty acids

WHO World Health Organization

## Introduction

The testis is a central organ responsible for male fertility. Abnormal testis function results in decreased human semen quality, low sperm count, or both, which are associated with more than 90% of male infertility cases. Among the several potential risks of male infertility, such as genetic, chemical, and physical environmental toxins, the quality of food we consume is getting recognized as a major contributing factor. The human testis and sperm are structurally and biochemically enriched with omega-3 (n-3) polyunsaturated fatty acids (PUFA), thus maintenance of optimal concentration of n-3 PUFA may be critical for normal physiological functions of testis and sperm.

## Overview of Testis and Sperm

### Testis

The testis has two major functions: steroidogenesis and spermatogenesis. In adult men, the testis weighs about 20 g with a testicular volume of 15–20 cm<sup>3</sup>. The testicular volume is known to be associated with testicular function. The testicular volume is decided by the thread-like seminiferous tubules occupying about 80% of testis. Cells responsible for steroidogenesis (Leydig cells) and spermatogenesis (germ and Sertoli cells) are located in the interstitial connective tissue, and seminiferous tubules, respectively. Spermatogenesis requires 65–75 days in humans to proceed from the germ cell to spermatozoa. Since the rate of cellular proliferation and differentiation are high during these periods, any external and internal insults could result in negative impacts on testis function. This is why testis is considered to be one of the most vulnerable tissues in our body.

## Sperm

Sperm quality is commonly accessed in the semen analysis, which is a key analysis for the evaluation of testicular function. According to the World Health Organization (WHO), four common terms are used to describe sperm quality in semen analysis (**Table 1**): i) oligozoospermia, a total number of sperm to be less than the lower reference limits of 15 million sperm/mL ejaculate; ii) azoospermia, defined as the absence of spermatozoa in the ejaculate; iii) asthenozoospermia, a semen sample testing motility, where less than 32% of sperm exhibit forward progression; iv) teratozoospermia, a semen condition of abnormal sperm morphology to have less than 4% morphologically normal sperm ([Cooper et al., 2010](#)). In morphology, many different abnormalities can occur, including abnormalities in the head neck or mid-piece, or tail. All of the above sperm characteristics including concentration, motility, and morphology, are reproductive challenges that need to be overcome for fertilization to occur. Sperm is the only cell designed to leave the male body and join with the female ovum for fertility, thus any challenges leading to poor semen quality and abnormal morphology could impact fertility success.

## Lipid Composition in Testis and Sperm

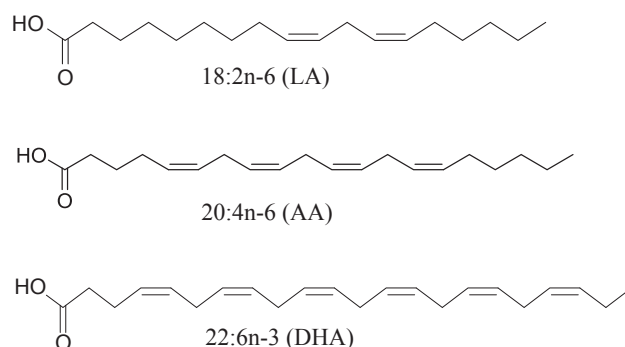
### Testis

The importance of lipids in testis structure and function has been recognized in the early 1930s in a rodent model ([Burr and Burr, 1930](#)). However, the lipids data from the human testis are still scarce. It has been reported that the total lipid in adult male testis are in the range of 25.6–39.0 (mg g<sup>-1</sup> wet tissue) and total phospholipids are 15.6–15.9 (mg g<sup>-1</sup> wet tissues) representing approximately 40%–60% of the total lipids ([Coniglio et al., 1975](#)), which indicates the testis to be a phospholipid enriched tissue. In terms of the fatty acid compositions, the total lipids of adult human testis is composed of 38%–45% saturated fatty acids, 19%–24%, monounsaturated fatty acids, 22%–32% of n-6 fatty acids, and 7%–10% n-3 fatty acids ([Coniglio et al., 1974, 1975](#); [Hoffmann et al., 2005](#); [Bieri and Prival, 1965](#)). Among PUFAs, arachidonic acid (AA, 20:4n-6, 11%–13%, w/w total lipids) and DHA (8%, w/w total lipids) are the predominate fatty acids of n-6 and n-3 PUFA, respectively (**Figure 1**; **Table 2**) ([Coniglio et al., 1974, 1975](#); [Hoffmann et al., 2005](#); [Bieri and Prival, 1965](#)). Interestingly, only DHA, not AA, has been found to decrease in testis during degeneration ([Coniglio et al., 1974](#)), indicating DHA is a significant factor for the development of germinal cells in the human testis.

**Table 1** WHO semen analysis reference with lower limits

Sperm characteristics	Reference value (lower ref limits)	Infertility conditions	
Semen volume (mL)	1.5–5.0 (1.4–1.7)		
Sperm conc (mL)	>15 × 10 <sup>6</sup> (12–16)	<12 × 10 <sup>6</sup>	oligozoospermia
Sperm conc (ejaculate)	>39 × 10 <sup>6</sup> (33–46)	absence	azoospermia
Total motile sperm (%)	>40 (38–42)		
Progressive sperm (%)	>32 (31–34)	<31	asthenozoospermia
Vitality (live spermatozoa, %)	58(55–63)		
Normal morphology (%)	>4 (3.0–4.0)	<3	teratospermia

Numbers in the bracket represents fifth centile and their 95% confidence intervals. Conc., concentration. [Cooper et al. \(2010\)](#)



**Figure 1** The major n-3 and n-6 polyunsaturated fatty acids found in human testis and sperm. LA, linoleic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; AA.

**Table 2** The overall fatty acid composition in testis and spermatozoa from normal and infertile males

Fatty acid	Testis <sup>a</sup>	Spermatozoa <sup>b</sup>	
		Normozoospermic	Infertile men <sup>c</sup>
Palmitic acid (16:0)	28.4 ± 2.6	30.2 ± 1.1	33.4 ± 5.1
Stearic acid (18:0)	11.2 ± 0.6	9.9 ± 5.0	15.3 ± 1.2
Oleic acid (18:1n-9)	18.0 ± 2.1	9.9 ± 0.5	11.8 ± 0.6
Linoleic acid (18:2n-6)	6.0 ± 1.1	5.8 ± 3.1	4.0 ± 0.5
Arachidonic acid (20:4n-6)	13.3 ± 2.8	4.4 ± 3.8	3.3 ± 1.7
Docosahexaenoic acid (22:6n-3)	7.4 ± 0.9	21.3 ± 7.3	13.4 ± 3.0
Total SAT	40.5 ± 2.8	48.9 ± 4.4	51.0 ± 4.2
Total Mono	21.3 ± 1.6	17.2 ± 2.6	19.1 ± 2.7
Total n-6 FA	25.3 ± 3.8	14.4 ± 6.7	13.9 ± 3.7
Total n-3 FA	8.5 ± 1.3	17.4 ± 2.4	15.8 ± 2.9
Ratio of n-6/n-3 FA	3.1 ± 0.9	0.7 ± 0.4	1.0 ± 0.3

Values are mean ± SD.

<sup>a</sup>An average of 4 studies (% w/w testis total lipids) (Coniglio et al., 1975; Hoffmann et al., 2005; Coniglio et al., 1974; Bieri and Prival, 1965).

<sup>b</sup>An average of 3 studies (mole% of spermatozoa) (Zalata et al., 1998; Martínez-Soto et al., 2013; and Tavilani et al., 2006). Zalata and Tavilani used phospholipids.

<sup>c</sup>Infertile men includes Fatty acids were combined for men with oligospermia, asthenozoospermia and oligoasthenozoospermia.

## Sperm

Like testis, sperm lipids are composed of a heterogeneous mixture of glycolipids, sterols, phospholipids, saturated and PUFA, which are believed to play an important role in the viability, maturation, motility and fertilizing ability of the sperm. Human sperm lipid profiles are generally determined on ejaculated sperm which have undergone epididymal maturation, thus the lipid profile may differ from testicular spermatozoa which have yet to mature. Among the lipids, the fatty acid composition of sperm has received significant attention due to its direct relationship with fertility. Several studies have carried out detailed fatty acid profiling of human spermatozoa (Table 2). The major saturated and monounsaturated fatty acids are palmitic acid (16:0, 30.2%) and stearic acid (18:0, 15.3%), and oleic acid (18:1, 9.9%). The PUFAs are mainly linoleic acid (18:2n-6, 5.8%), arachidonic acid (20:4n-6, 4.4%) and then remarkably high amounts of DHA (21.3%), ranging from 14%–32% of total fatty acid in phospholipids (Zalata et al., 1998; Martínez-Soto et al., 2013; Tavilani et al., 2006). Most studies agree with the distinctively high content of DHA in spermatozoa, which is thus considered as a marker for infertility. Interestingly, DHA is not evenly distributed in sperm. Learning from monkeys, DHA is significantly enriched in the tail of sperm (19.6%, w/w total fatty acids) in comparison to the head (1.1%, w/w total fatty acids) (Connor et al., 1998), suggesting that DHA is involved in sperm movement (Figure 2).

Unlike normozoospermic males who have above the lower reference limits in sperm characteristics described in Table 1, men with oligospermia, asthenozoospermia, and oligoasthenozoospermia had significantly lower concentration of DHA in their spermatozoa (Tavilani et al., 2006; Connor et al., 1998; Safarinejad et al., 2010) (Table 2). Men with these conditions were found to have increased levels of saturated and monounsaturated fatty acids, and lower PUFA levels (Coniglio et al., 1975; Zalata et al., 1998; Aksoy et al., 2006), compared with the normozoospermic men. This indicates that decreased sperm fertility potential is closely associated with alteration of sperm lipid content and composition.

## n-3 Fatty Acid Containing Foods or Supplementation in Healthy and Infertile Population

Emerging evidence to date has shown a possible relationship between diet and semen parameters. It is well known that membrane fatty acids are affected by dietary fat types, which are directly related to the maintenance of cell membrane for its optimal functions. Dietary fat intake thus, can affect the male reproductive function by modulating the fatty acid composition of testis and spermatozoa.

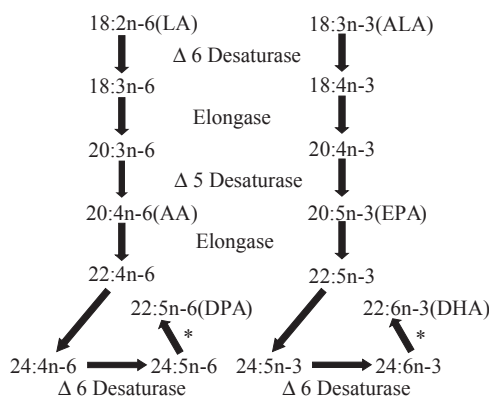
## Observational Studies

### n-3 Fatty Acid Food

Based on a few observational studies, higher intake of saturated fats is associated with lower sperm counts and concentration, whereas higher intake of n-3 PUFA is related to higher normal sperm morphology with lowering the sperm head defect (Attaman et al., 2012) (Table 3). Even in healthy young men, higher intake of fish as a part of a heart healthy diet was positively associated with sperm motility (Gaskins et al., 2012). In sub-fertile and asthenozoospermic men, higher intake of n-3 PUFA, fish and sea foods







**Figure 3** The n-3 and n-6 polyunsaturated fatty acid synthesis from the dietary essential fatty acids. Linoleic acid (LA) and  $\alpha$ -linolenic acid (ALA) from the diet are enzymatically desaturated and elongated to longer-chain, more unsaturated fatty acids via the same desaturases and elongases. Due to the same enzymes involved in desaturation of the ALA and LA, the high n-6 to n-3 fatty acid ratio in the Western diet inhibits the desaturation of ALA, leading to the reduction of DHA synthesis. EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. \* Presents one-step  $\beta$ -oxidation.

### Intervention Studies

A limited number of studies assessing diets high in n-3 fatty acids for semen quality in both healthy men and men with infertility conditions exist (**Table 4**). For healthy men, 75 g of walnut a day (a source of ALA) for 12 weeks improved sperm morphology, motility and vitality (**Robbins et al., 2012**). However, healthy men receiving 50 mL menhaden fish oil for 4 weeks did not find a significant difference in sperm quality (**Knapp, 1990**).

Limited evidence is available to determine if dietary supplementation with n-3 PUFA, as fish oil or DHA, improves sperm parameters in males with fertility issues (**Table 4**). Evidence from a relatively large study of infertile males ( $n = 238$ ) with idiopathic oligoasthenoteratospermia, a condition including oligospermia, asthenozoospermia and teratozoospermia, showed that 1.84 g supplementation of fish oil (1.84 g: 0.72 g of eicosapentaenoic acid (EPA, 20:5n-3), 0.48 g of DHA) per day for 32 weeks improved sperm concentration, motility and morphology, which suggests fish oil as a cheap and non-aggressive treatment strategy for these patients (**Safarinejad, 2011**). Another study performed with 27 infertile men receiving 1 g DHA in addition to 0.35 g n-6 fatty acids and vitamins for 24 weeks, showed that the sperm concentrations were increased with a concomitant increase in pregnancy in their female partner (**Comhaire et al., 2000**). However, not all studied agreed with these positive effects of n-3 PUFA consumption. Asthenozoospermic men supplemented with 0.4 or 0.8 g DHA per day for 12 weeks did not show any changes in their sperm parameters

**Table 4** Intervention studies showing the effect of n-3 PUFA supplementation on healthy and infertile population

Population	Participant #	n-3 PUFA treatment	Study length	Semen quality
Healthy men <sup>a</sup> Walnut vs. control	$n = 59$ vs $n = 58$	75 g d <sup>-1</sup> Walnut vs usual diet without tree nuts (control)	12 wks	Increased sperm motility, normal morphology and vitality in those receiving walnut than the control group consuming their normal diet
Healthy men <sup>b</sup>	$n = 10$	menhaden oil (50 mL/d)	4 wks	There was no significant effect with or without menhaden fish oil consumption on sperm motility and count
Asthenozoospermic men <sup>c</sup> in three groups	$n = 28$	DHA (0.4 g d <sup>-1</sup> and 0.8 g d <sup>-1</sup> ) vs no DHA (control)	12 wks	No significant effect on sperm parameters between 2 DHA interventions and control
Infertile men <sup>d</sup>	$n = 27$	1 g DHA, 0.25 g LNA and 0.10 g AA	24 wks	Increased sperm concentrations, with improved sperm membrane fluidity and acrosome reaction
Oligoasthenoteratospermia <sup>e</sup> Fish oil vs. controls	$n = 119$ vs $n = 119$	Fish oil (1.12 g d <sup>-1</sup> EPA + 0.72 g d <sup>-1</sup> DHA) vs. corn oil (control)	32 wks	Increased sperm concentration, motility and morphology in fish oil treatment group than controls

DHA, docosahexaenoic acid; LNA, linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid.

<sup>a</sup>Robbins et al. (2012).

<sup>b</sup>Knapp (1990).

<sup>c</sup>Conquer et al. (2000).

<sup>d</sup>Comhaire et al. (2000).

<sup>e</sup>Safarinejad (2011).

(Conquer et al., 2000). Although the level of DHA in serum and seminal plasma increased dose-dependently, dietary DHA was unable to incorporate into spermatozoa phospholipid in asthenozoospermic men. It raises a question of whether the quality of sperm can be improved if the intervention period is longer as other studies in the above.

### n-3 Fatty Acids Dietary Sources and Recommendations

In order to have adequate dietary n-3 fatty acids, especially DHA, knowledge regarding their dietary sources is required. Dietary n-3 fatty acids can be found in three main forms in the diet: ALA, EPA and DHA. In addition to the plant-source of DHA precursor, food-based approach for achieving EPA and DHA is recommended. Seafoods, particularly marine fish and shellfish, are the main sources of DHA. The American Dietetic Association/Dietitians of Canada recommended two servings of 8 oz of fatty fish (herring, salmon, sardine; accounting > 0.5 g DHA/100 mg) per week for health promotion and reduced risk of various chronic diseases (Kris-Etherton and Innis, 2007). DHA also can be found in the liver of some special lean fish (cod). Halibut and Pollock are known as moderate sources of DHA corresponding to 0.3–0.5 g of DHA/100 mg.

Nutritional supplements or fortified food with fish oil or DHA, such as eggs containing 0.15 g and algae supplements, are also needed to meet current recommendations for some individuals with limited or lack of fish intake in their diet. Although vegetable oils are not a good source of EPA and DHA, some are rich sources of ALA, such as flax oil.

The general male population has been encouraged to consume n-3 fatty acids-rich foods to benefit their reproductive tract health. Institute of Medicine of the National Academies issued a Dietary References Intake (DRI) for ALA to be 1.6 g d<sup>-1</sup> providing 0.6%–1.2% of energy, for men 19 to >70 years (Otten et al., 2006). DHA is then synthesized endogenously from ALA in the body, but its dietary intake is also recommended due to the low efficiency in DHA production. There are no DRI for DHA or EPA, but some recommendations have been issued for different population groups based on evidence demonstrating the various health benefits of these n-3 PUFA. The American Dietetic Association/Dietitians of Canada recommended 500 mg day<sup>-1</sup> of EPA + DHA (Kris-Etherton and Innis, 2007). Although this amount is required for normal growth and neural development, as well as preventing nutrient deficiencies, it is questionable if it is enough for males with reproductive health issues. Regardless, to achieve the recommendation for DHA intake, it is required to increase the dietary intake of DHA in a preformed state in addition to its plant-derived precursors.

### Conclusions

DHA is a significant structural and functional component of testis and sperm. Only few studies have measured the effects of n-3 PUFAs and DHA supplementation on male reproductive functions in both healthy and sub-fertile or infertile populations. Although more interventional studies are required to fully elucidate the relationship between n-3 PUFA and male reproductive health, consistent findings of reduced DHA in the sub-fertile or infertile populations provide potential to DHA for being used as an intervention strategy for male reproductive function. To have robust data, future intervention plans should consider the effects of dose, duration, and types of the n-3 PUFA supplementation, as well as the n-6/n-3 fatty acid ratio on these males. This will lead to developing an effective daily dietary recommendation of DHA-rich food or supplementation for optimal male fertility. Based on the epidemiological studies providing evidence of high dietary n-3 PUFA foods improving semen quality, habitual consumption of n-3 PUFA may overall improve the male reproductive health.

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## Hydrolysable Tannins

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### Glossary

**Hydrolyzable tannins** Secondary plant metabolites, which are also phenolic compounds.

**Gallic acid** Trihydroxybenzoic acid, a type of phenolic acid. A compound of gallotannins (see Fig. 1).

**Ellagic acid** A compound of ellagitannins. It is an ilactone of hexahydroxydiphenic acid (see Fig. 1).

**Gallotannins** Natural polymers formed by the subsequent esterification of hydroxyl groups of D-glucose and gallic acid.

**Ellagitannins** Esters of hexahydroxydiphenic acid and polyols, like glucose or quinic acid.

**Food antioxidant** Chemical compound that inhibits the oxidation of food constituents (*e.g.*, lipids, proteins).

**Astringency** Dry, puckering mouthfeel caused by tannins.

### Classification of Hydrolysable Tannins

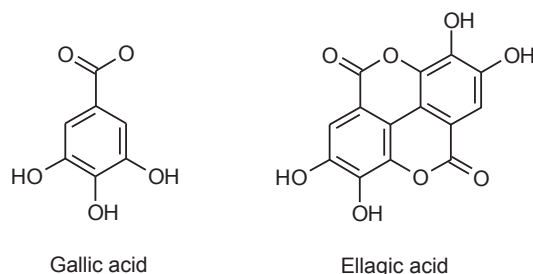
Hydrolyzable tannins are secondary plant metabolites, which are also phenolic compounds. Interest in hydrolyzable tannins has grown over the past decade due to their biological activity. According to their structure, hydrolyzable tannins are classified into three subclasses: simple gallic acid derivatives, gallotannins (GTs), and ellagitannins (ETs). The hydrolysis of GTs yields gallic acid whereas ETs yields ellagic acid. The chemical structures of gallic and ellagic acids are depicted in Fig. 1.

#### Simple Gallic Acid Derivatives

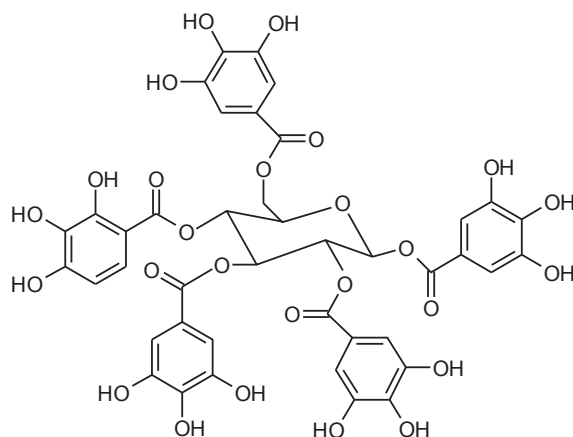
Simple gallic acid derivatives are composed of five or less galloyl groups that are esterified to either glucose or quinic acid. Examples include 1,2,3,4,6-penta-*O*-galloyl- $\beta$ -D-glucose (PGG) (Fig. 2). Fruits of *Galla chinensis*, the kernel and peel of *Mangifera indica* L. (mango), and the seeds of *Sideritis raseri* (a mountain tea) are rich sources of PGG (Tian et al., 2009; Pljevljaković et al., 2011; Luo et al., 2014).

#### Gallotannins

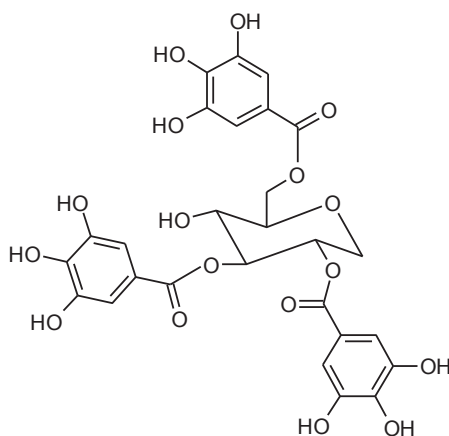
Gallotannins are natural polymers formed by the subsequent esterification of hydroxyl groups of D-glucose and gallic acid in polymeric chains, in which the galloyl moieties are linked by so called “depside” bonds. The term “depside” refers to polyphenols composed of two or more monoaromatic units linked via an ester bond, and include a wide array of compounds. Other types of gallotannins are formed by the esterification of shikimic acid and quinic acid with gallic acid. Gallotannins of these types contain one free carboxylic group in the quinic moiety and, as in glucose-derived gallotannins, a varying number of depside bonds between their galloyl residues, leading to high structural diversity (Karas et al., 2017). In glucitol-core containing gallotannins (GCGs), galloyl groups are bonded to a 1,5-anhydro-D-glucitol center (Fig. 3). GCGs have only been isolated from maple (*Acer*) species, including the red maple (*Acer rubrum*).



**Figure 1** Chemical structure of gallic acid and ellagic acid.



**Figure 2** Chemical structure of PGG.



**Figure 3** Chemical structure of GCG isolated from red maple.

## Ellagitannins

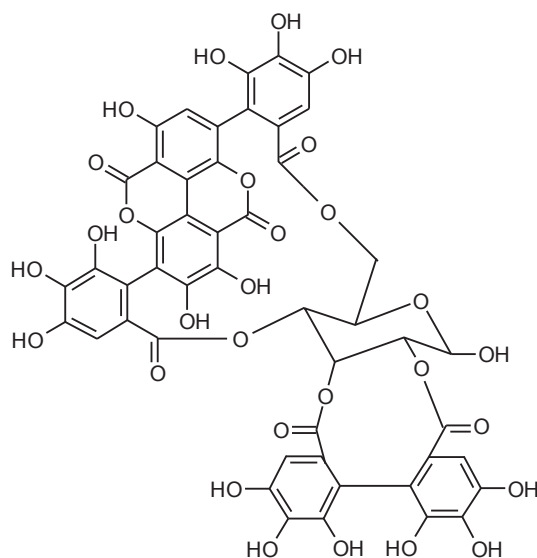
Ellagitannins (ETs) are esters of hexahydroxydiphenic acid and polyols, like glucose or quinic acid. After hydrolysis, the liberated ET molecule from hexahydroxydiphenic acid is spontaneously rearranged into ellagic acid, which is a water-insoluble compound. Methylation, glycosylation, and methoxylation lead to numerous derivatives of ETs in plants.

Chemical structure of ETs of pomegranate and walnut is depicted in [Figs. 4 and 5](#).

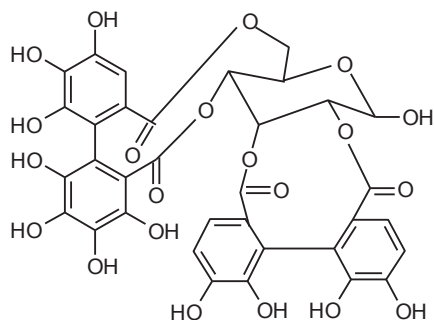
## Sources of Hydrolysable Tannins

The content of ETs in several plant products is high. Ellagitannin-containing plant species of economic importance as foodstuffs include cashew nuts, pistachios, mangos, hazelnuts, persimmons, chestnuts, walnuts, guavas, cloves, pimento, pomegranates, plums, apricots, peaches, bird cherries, strawberries, raspberries, blackberries, blackcurrants, gooseberries, teas, grapes, and muscadine grapes ([Clifford and Scalbert, 2000](#)). To illustrate, a glass of pomegranate juice can provide 1 g of ETs; 100 g of raspberries provide ~300 mg ETs; 100 g of strawberries ~70 mg; and four walnuts about ~400 mg of ETs ([Larrosa et al., 2010](#)). Daily consumption of two servings (250 mL each) of a functional beverage, based on a dealcoholized red wine matrix fortified with a pomegranate extract, provides 82 mg of total ellagitannins (ETs), corresponding to the sum of punicalagin A and B as well as ellagic acid ([Tárrega et al., 2014](#)). Juices produced only from the edible portion of pomegranates possess lower quantities of ETs compared to those extracted from the entire fruit ([Fischer et al., 2011](#)). The major contributors of ETs intake in Western diets are red fruits such as strawberries, raspberries, and blackberries. The main dietary source in France is undoubtedly strawberries. On average, the French ingest 1.7 kg of fresh strawberries yearly, and equally as much in the form of processed products ([Landete, 2011](#)).





**Figure 4** Chemical structure of punicalagin (ET of pomegranate).



**Figure 5** Chemical structure of pendunculagin (HT of walnut).

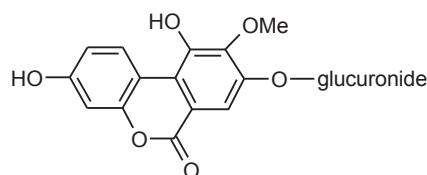
### Biological Activity of Hydrolysable Activity

Gallotannins have been reported to exhibit anti-cancer, anti-angiogenic, antioxidant, anti-inflammatory, and anti-ulcerative activities, as well as to inhibit *P*-glycoproteins (Karas et al., 2017). The tannin fraction isolated from the leaves of bearberry (*Arctostaphylos uva-ursi* L. Sprengel), which contain gallotannins comprising glucose and three, four, and five residues of gallic acid, showed anti-proliferative activities in a concentration-dependent manner against five carcinoma cell lines, namely MCF-7 (estrogen receptor-positive breast carcinoma), DU-145 (androgen receptor-negative prostate carcinoma), HT-29 (colon carcinoma), SK-MEL-5 and MDA-MB-435 (melanoma; skin carcinoma) (Amarowicz and Pegg, 2013).

PGG exhibited anti-cancer, anti-viral, anti-microbial, anti-inflammatory, and anti-diabetic activities (Torres-León et al., 2017). The anti-radical activity of PGG against the DPPH radical was reported by Shaikh et al. (2016): it exhibited an effective concentration that scavenged 50% of the radical ( $EC_{50}$ ) value of 20.5  $\mu$ M. The activity against reactive nitrogen species was also noted and reported: the inhibitory concentration that reduced 50% of the species ( $IC_{50}$ ) values for NO and ONOO<sup>-</sup> were 0.20 and 0.06  $\mu$ M, respectively (Kiss and Naruszewicz, 2012).

The GCGs showed superior activities when compared to the synthetic antiglycating agent, aminoguanidine, at the early, middle, and later stages of glycation (Ma et al., 2016). Furthermore, the GCGs containing 1 to 4 galloyl groups were noncompetitive inhibitors of  $\alpha$ -glucosidase (Ma et al., 2015), and should be further investigated for their antidiabetic potential.

Ellagitannins are not absorbed *in vivo*, but reach the colon and release ellagic acid that is metabolized by the human microflora to urolithins (García-Villalba et al., 2013). These compounds exhibit both estrogenic and antiestrogenic activities (Larrosa et al., 2006a). The results of Larrosa et al. (2006b) suggest that the anticarcinogenic effect of dietary ETs could be mainly due to ellagic acid liberated from ETs, which induces apoptosis via the mitochondrial pathway in colon cancer Caco-2 cells, but not in normal colon cells. In an experiment using Caco-2 cells, punicalagin (the chief ET of pomegranates) was hydrolyzed to yield ellagic acid, which then entered Caco-2 cells. After methylation and conjugation with glucuronic acid, urolithin derivatives (Fig. 6) were detected (Larrosa et al., 2006b).



**Figure 6** Chemical structure of urolithin C-methylether-glucuronide – ellagic acid metabolite found in plasma and urine after ETs intake.

### Hydrolysable Tannins as Natural Antioxidants

Mullen et al. (2002) listed sanguine H-6 (*i.e.*, an ET) as an important contributor to the antioxidant capacity of raspberries compared to that of vitamin C and anthocyanins. The findings of Kähkönen et al. (2012) indicated that cloudberry and red raspberry ETs were highly effective as free-radical scavengers. Berry ETs also showed significant antioxidant activity toward oxidation of both human LDL and methyl linoleate emulsions. Yet, only weak or moderate antioxidant activity was observed by ETs toward oxidation of bulk oils. In the raspberry, the ETs sanguine H-6 and lambertianin C were responsible for 44.7% and 13.6% of the total antioxidant capacity of the berries, respectively (Borges et al., 2010).

Thermal hydrolysis of gallotannins enhanced their antioxidant properties. Furthermore, such prepared gallotannins exhibited a synergistic antioxidant effect with  $\alpha$ -tocopherol, ascorbyl palmitate, and citric acid in an edible oil-based system (Terán-Hilares et al., 2018). In terms of their antioxidant activity, analyses of the gallotannins of *G. chinensis* showed that with higher degrees of galloylation these tannins exhibited stronger antioxidant activities than those with low degrees of galloylation. Moreover, for the first group, the antioxidant activities increased with a greater number of galloyl moieties (Tian et al., 2009). The antioxidant potential of raspberry jam is generated by the presence of ellagic acid, 4-arabinosylellagic acid, 4-acetylxylellagic acid, 4-acetyl arabinosylellagic acid, and two other derivatives of ellagic acid. The antioxidant activities of the aforementioned compounds range from 0.5 to 4.3 mM Trolox equivalent/mM compound (Zafilla et al., 2001).

### Astringency

Hydrolysable tannins just like condensed tannins (*i.e.*, proanthocyanidins) are astringent. The astringency derives from their interaction with salivary proteins that leads to precipitation of the formed complexes in the oral cavity. The more galloylated tannins are in grape seeds, which are then extracted during the process of maceration and fermentation, the stronger their contribution to wine astringency (Aron and Kennedy, 2007). Additionally, the GTs and ETs released from oak barrels can markedly influence wine astringency (Glabasnia and Hofmann, 2006). It is interesting to note that the complexes formed between a representative salivary protein and hydrolysable tannins are more soluble than those of condensed tannins (Lu and Bennick, 1998). The organoleptic properties were significantly impacted by the content of ETs, as the wine with the lowest ET level was described as less astringent, bitter, woody, and smoky/toasty (Michel et al., 2016). The results of Engström et al. (2016) showed that the oxidizability of ETs was directly correlated with the tendency of the tannins to form highly stabilized complexes with bovine serum albumin at an increased pH (7.6). However at a slightly lower pH (6.7), other tannin features, such as the size and flexibility of the tannin, appeared to dictate the formation of highly stabilized tannin–protein complexes.

### Influence of the Technological Process and Storage on Hydrolysable Tannins

The content of ETs in food and beverages can be changed by technological processing and during storage. In experiments with raspberries, on average 68.1% of the ETs were retained in the press cake, especially in its seedless fraction. Moreover, a significant negative correlation was found between the molecular mass of ETs and their transfer to juice. An increase in molecular mass from 1568 to 2805 Da resulted in a more than a 10-fold decrease in ET transfer (Sójka et al., 2016).

The impact of thermal pasteurization on hydrolysable tannins of pomegranate juice has been scarcely studied. With regard to this, Mena et al. (2013) demonstrated that the content of punicalagins increase after thermal treatment, while other compounds such as punicalagin-like and ellagic acid (both free and in glucoside forms), decrease. Overall, other researchers have reported that thermal pasteurization does not modify the content and form of ETs appreciably, apart from punicalagin isomers, pointing to depolymerization of higher-molecular-weight ETs (Hager et al., 2010). During processing of berries into jams, the total ellagic content decreased by 20% in all jam samples (Hakkinen et al., 2000). Thermal processes triggered a significant breakdown of hydrolysable tannins from witch hazel, *Hamamelis virginiana*, with 6 to 10 galloyl units to give pentagalloyl glucose. The release of high concentrations of free gallic acid, especially in long-term thermally processed samples, leads to an increase in the antioxidant capacity of heated *H. virginiana* extracts. Such an increase was evidenced by an increment in the reducing and free-radical scavenging capacities as well as by an improvement in the antioxidant effectiveness for inhibiting lipid oxidation of processed fatty fish muscle (González et al., 2010).

The results of an investigation by Qu et al. (2014) showed that liquid pomegranate peel extracts, containing, punicalagin A, punicalagin B, and ellagic acid, had acceptable thermal stability after sterilization (121 °C for 10 s) and storage up to 180 days.

During typical industrial processing of a blackberry-based beverage using glass bottles, ETs underwent severe losses during blanching and hot-filling of the final blackberry beverage. Indeed, blanching reduced the contents of hydrolyzable tannins (e.g., lambertianin C and sanguin H-6) by almost 20%, as they were hydrolyzed, during heat treatment, partially into insoluble ellagic acid. After hot-filling and holding, additional losses were as high as 59% for lambertianin C and 17% for sanguin H-6. Over the entire process, lambertianin C and sanguin H-6 ETs were reduced by 80% and 50%, respectively (Gancel et al., 2011).

In the research of Truchado et al. (2012), processing increased the quantity of free ellagic acid 2.5-fold in strawberries, but this had no effect on the transformation in urolithins by the gut microbiota or in the excretion of urolithin metabolites in urine (i.e., urolithin glucuronides). These results show that processing does not modify the potential health effects of strawberry polyphenols.

The effect of storage on the ET and ellagic acid contents in strawberries is not unambiguous. The content of free ellagic acid in strawberries during 10 days of post-harvesting at 5 °C increased significantly, although this was delayed at an elevated CO<sub>2</sub> concentration (Gil et al., 1997). Their storage at 6, 16, and 25 °C did not result in any significant change to free ellagic acid under the conditions employed (Cordenussi et al., 2005). The freezing of raspberries and their long term storage at −20 °C for 12 months decreased the total content of ellagic acid from 21% to 14%. The change, however, depends on the raspberry cultivar investigated (De Ancos et al., 2000) and could be related to the release of polyphenoloxidase linked to the cellular wall.

## Hydrolyzable Tannins and the Problem of Sediment and Haze

The use of fruits containing high ET as a fruit juice and for wine is limited by the problem of sediment and haze. Hydrolysis of ETs during processing and storage liberates less water-soluble ellagic acid that precipitates (Bakkalbasi et al., 2009). This problem is especially important when processing of muscadine grapes (*Vitis rotundifolia*) (Le and Talcott, 2002). According to Bakkalbasi et al. (2009), methods to avoid sediment in juice include the application of thermal processing or hydrolysis and filtration of precipitated ellagic acid before bottling; removal of skins and other fruit parts rich in ETs; and the selection of cultivars characterized by lower contents of ETs. Application of centrifugation and filtration can also be an effective method protected against the appearance of sediment in the final product (Siriwoham et al., 2005). Nanofiltration membranes with a molecular weight cut-off around 200 Da were evaluated to concentrate anthocyanins and ellagitannins in a blackberry juice (Acosta et al., 2017).

## Ellagitannins in Wine and Spirits

Ellagic acid and ETs are the most important phenolic compounds of wine and spirits aged in barrels. Their influence is not only on the color, astringency, and bitterness of wines and spirits, but also on the antioxidant capacity of these beverages (Bakkalbasi et al., 2009). During the aging of spirits in barrels, ETs are solubilized by the spirit. A part of the ETs can then be hydrolyzed to ellagic acid or degraded without formation of ellagic acid. Insoluble ETs present in barrel wood can also be hydrolyzed to yield ellagic acid. Then, it can diffuse from the barrel into the spirit (Viriot et al., 1993).

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# Food for Skin Health: Collagen Peptides

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## Background

The skin provides a protective barrier against the external environment. The skin consists of the epidermis, dermis, and subcutaneous tissue. Keratin is the main protein compound found in the epidermis. On the other hand, collagen and elastin are the main protein compounds present in the dermis. When the skin is mechanically damaged, blood clotting occurs and inflammation is subsequently induced, which attracts neutrophils and fibroblasts into the damaged area (Reinke and Sorg, 2012). The fibroblasts synthesize collagen and other extracellular matrix compounds, which replace the necrotic tissue and form granulation tissue. Keratinocytes cover the granulation tissue to regenerate the epidermis. The skin is also damaged by ultraviolet (UV) irradiation, exposure to dryness, aging etc. These factors induce epidermal thickening and barrier dysfunction of the epidermis and cause a decrease in the levels of extracellular matrix compounds, thus resulting in the decline of skin elasticity, roughness and dryness, wrinkle formation, and sagging (Ichihashi et al., 2009).

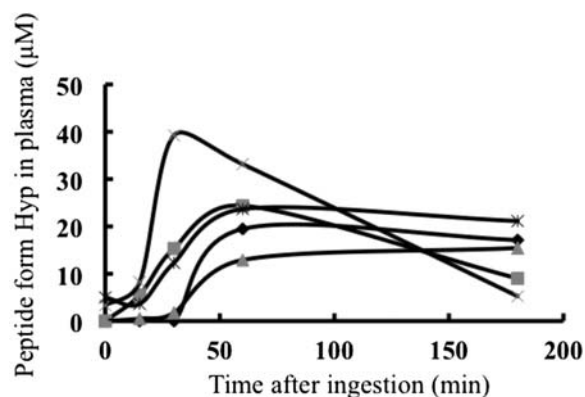
Collagen, one of the main constituents of the dermis, consists of three subunit chains called the  $\alpha$  chains, which form a triple helical structure. This collagen molecule has a rod-like shape and is derived from a molecular family consisting of more than 46 gene products (UniProtKB/Swiss-Prot - ExPASy). Collagen molecules are designated by 'type', followed by a Roman numeral, as type I to type XXVIII. Subunit chains are designated by Arabic and Roman numerals. For example,  $\alpha 1(I)$  represents the subunit 1 of type I collagen. The type I collagen consists of two  $\alpha 1(I)$  and one  $\alpha 2(I)$  subunits and is designated as  $[\alpha 1(I)]_2\alpha 2(I)$ . Some types of collagen (I, II, III, V, and XI) molecules self-assemble and form fibrils; these are stabilized by covalent cross-links. Collagen fibrils are associated with different types of collagen molecules and other extracellular matrix compounds, resulting in the formation of larger fibers. The collagen fibers provide mechanical strength to the dermis, while the elastin fibers provide elasticity. Type I collagen is the main protein constituent of the skin, bone, tendons and other non-cartilage tissues. In cartilage, type II collagen is the main protein constituent. Other types of collagen also play important roles in regulating the diameter and properties of the collagen fibrils. In addition, some of them form the basal lamina, although their content is relatively lower (van der Rest and Garrone, 1991). Collagen has unique amino acids; hydroxyproline (Hyp) and hydroxylysine (Hyl), which are generated after translation by prolylhydroxylase and lysylhydroxylase. Hyp accounts for approximately 10% of the total amino acids in collagen.

The triple helical structure of collagen collapses after heating with water and is converted to a globular structure, which is referred to as gelatin. Gelatin is soluble in water, while the collagen in tissues is not. Cooling of the gelatin solution induces partial formation of the triple helical structure between adjacent gelatin molecules, resulting in gel formation. Gelatin has been used in food, pharmaceuticals, photographic films, cosmetics, etc., owing to this gel-forming property. Gelatin can be degraded by protease. However, while the triple helical structure of collagen resists most of the protease. The gelatin hydrolysate or collagen peptides are prepared on an industrial scale and are widely used as food ingredients. Collagen peptides are water-soluble but lose their gel-forming ability in water. They do not have a bitter taste, unlike other protein hydrolysates such as soy protein and casein hydrolysates. Thus, relatively large doses (2–10 g) of collagen peptides can be easily consumed by dissolving in water or a similar beverage. Numerous reports suggest that ingestion of collagen peptides might improve skin condition and enhance wound healing. However, it was assumed that orally administered peptides were degraded into amino acids during the digestion and absorption processes. Thus, strong doubts were cast to the suggested beneficial effects of ingestion of collagen peptides. However, the observation of the presence of food-derived collagen peptides in human blood at unexpectedly high levels has dramatically changed this view. Some food-derived collagen peptides in human blood have been identified and demonstrated to exert significant biological activities on fibroblasts and keratinocytes. The structure, contents, and biological activities of the food-derived collagen peptides present in human blood and possible mechanism for the beneficial effects of ingestion of collagen peptides are addressed in the following sections.

## Detection of Collagen Peptides in the Body

In the 1960s, early studies demonstrated the presence of Hyp-containing peptides in human urine after ingestion of gelatin (Prockop and Sjoerdsma, 1961). This indicates the presence of collagen peptides in the blood after the ingestion of gelatin. However, this important finding was almost forgotten. In 2005, the presence of food-derived collagen peptides in human blood was rediscovered (Iwai et al., 2005). Blood was collected from the cubital vein before and after ingestion of collagen peptides (10 g). The contents of peptide form of Hyp in human blood plasma, namely, the collagen peptide content, was estimated by subtracting free Hyp from total Hyp in the HCl hydrolysate of the deproteinized fraction of blood plasma. As shown in Fig. 1, the collagen peptide content increased 30–60 min after the ingestion compared to the levels before the ingestion. Using this method, an increase in the collagen peptide levels in human blood after ingestion of collagen peptides has been demonstrated (Ohara et al., 2007, Shigemura et al., 2014, 2017). Both the maximum level and the area under curve of the collagen peptide content increased in a dose-dependent manner (Shigemura et al., 2014). The maximal levels of collagen peptides in human blood after ingestion of 10–20 g of collagen





**Figure 1** Contents of peptide form of hydroxyproline (Hyp) in human plasma after ingestion of 10 g of collagen peptides. This figure was adapted with permission from Iwai, K., Hasegawa, T., Taguchi, Y., Morimatsu, F., Sato, K., Nakamura, Y., Higashi, A., Kido, Y., Nakabo, Y., Ohtsuki, K., 2005. Identification of food-derived collagen peptides in human blood after oral ingestion of gelatin hydrolysates.

peptides were observed to be 20–100  $\mu\text{M}$ . These values are far higher than those previously reported for the peptides with in vitro angiotensin converting enzyme inhibitory activity (approximately 1 nM) (Matsui et al., 2002; Foltz et al., 2007).

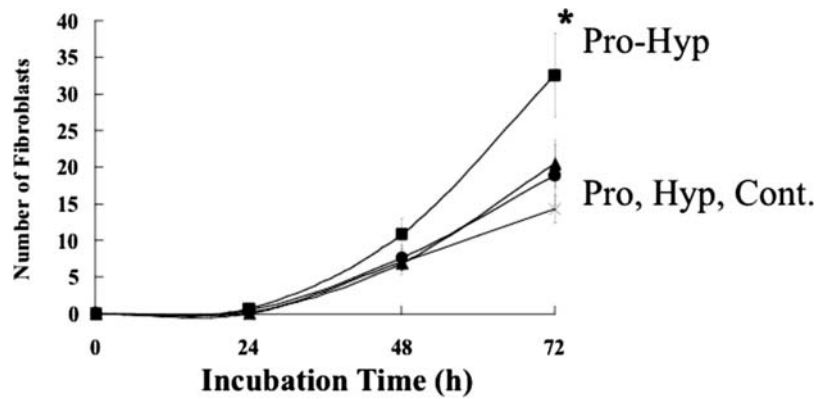
### Structure of the Food-Derived Collagen Peptides in Blood

Blood plasma contains numerous compounds. The direct injection of the deproteinized fraction of plasma into reversed phase-high performance liquid chromatography (RP-HPLC), the most powerful tool for the resolution of peptides, generated numerous unresolved peaks. Thus, it was difficult to isolate food-derived peptides in plasma by direct RP-HPLC analysis. To tackle this problem, pre-fractionation using size exclusion chromatography (SEC) was used. An oligo peptide fraction was prepared by SEC and used for the isolation of collagen peptides by RP-HPLC (Iwai et al., 2005; Ohara et al., 2007). Furthermore, a pre-column derivatization technique using phenyl isothiocyanate (PITC) was used to improve the resolution and detection of short chain peptides in human blood. PITC derivatives, namely phenyl thiocarbonyl (PTC)-peptides can be well resolved by RP-HPLC and the isolated PTC-peptide can be sequenced by the Edman degradation method (Aito-Inoue et al., 2006; Shigemura et al., 2011, 2017). This is possible, as the PTC-peptide is an intermediate of the Edman degradation. Using these techniques, some food-derived di- and tri-peptides, Pro-Hyp, Hyp-Gly, Pro-Gly, Glu-Hyp, Ala-Hyp, Ile-Hyp, Leu-Hyp, Phe-Hyp, Ser-Hyp-Gly, Ala-Hyp-Gly, Gly-Pro-Hyp, and Pro-Hyp-Gly have been identified. Among these, Pro-Hyp accounts for more than 50% of the total collagen peptides in the blood. Some research groups have confirmed the presence of Pro-Hyp and other peptides using liquid chromatography tandem mass spectrometric (LC-MS/MS) analysis (Ichikawa et al., 2010; Taga et al., 2014). An in vitro study using porcine brush-border membrane vesicles demonstrated that Pro-Hyp is transported via the peptide transporter (PepT1) on enterocytes (Aito-Inoue et al., 2007). In addition, peptides longer than tri-peptides have been demonstrated to pass through the Caco-2 cell monolayer by paracellular diffusion. This suggests that larger peptides are potentially absorbed into the blood circulating system (Shimizu et al., 2010). However, collagen peptides longer than tri-peptides have not been detected in the human blood circulating system after ingestion of collagen peptide. This observation is possibly due to their degradation by blood peptidase.

### In Vitro Activity of Pro-Hyp and Other Peptides

The effect of Pro-Hyp, the most abundant collagen peptide present in human blood, on skin fibroblasts has been examined. Generally, the fibroblasts of established cell lines are used for this purpose. However, in our preliminary experiments, the effect of Pro-Hyp on the growth of fibroblasts depended on the passage of the cell line. In order to obtain reproducible results, we opted to use a skin tissue culture system. The number of fibroblasts migrating from the skin into the serum free medium containing Pro-Hyp and constituting amino acids was evaluated (Shigemura et al., 2009). As shown in Fig. 2, Pro-Hyp significantly increased the number of fibroblasts migrating from the mouse skin, while the free Pro and Hyp did not show significant effects. This effect of Pro-Hyp on increase of number of outside fibroblasts was abolished in the presence of 10  $\mu\text{g}/\text{mL}$  of mitomycin C, which arrests the cell cycle and does not affect cell migration. Furthermore, Pro-Hyp enhances the growth of fibroblasts attached to the collagen gel in a dose-dependent manner (Shigemura et al., 2009). These results indicate that Pro-Hyp enhances the growth of fibroblasts in the skin. Hyp-Gly was also observed to enhance the growth of fibroblasts attached to the collagen gel (Shigemura et al., 2011). In addition, Pro-Hyp increases the production of hyaluronic acid from cultured human dermis fibroblasts (Ohara et al., 2010).

An animal study demonstrated that the ingestion of collagen peptides upregulated the keratin-associated proteins in mice, while the direct addition of Pro-Hyp to a keratinocyte culture system did not increase the expression of these proteins. However, upon



**Figure 2** Numbers of fibroblasts migrating from mouse skin in the presence of 200  $\mu$ M of Pro-Hyp, free Pro, or Hyp. This figure was adapted with permission from Shigemura, Y., Iwai, K., Morimatsu, F., Iwamoto, T., Mori, T., Oda, C. Taira, T., Park, E.Y., Nakamura, Y., Sato, K., 2009. Effect of prolyl-hydroxyproline (Pro-Hyp), a food-derived collagen peptide in human blood, on growth of fibroblasts from mouse skin.

addition of Pro-Hyp to a keratinocyte-fibroblast co-culture system, upregulation of the keratin-associated proteins was observed. These results indicate that Pro-Hyp can affect the production of proteins from keratinocytes by affecting the excretion of some biological substances from fibroblasts (Le Vu et al., 2015).

Recently, it has been demonstrated that Pro-Hyp is generated by the degradation of endogenous collagen in the inflammation and wound healing process without the ingestion of collagen peptide (Kusubata et al., 2015; Jimi et al., 2017). These observations indicate that Pro-Hyp and Hyp-Gly are not just inactive degradation products of collagen in food and in the body but are vital collagen derived-bioactive peptides.

### Effects of Collagen Peptide on the Skin

In 2003, a human clinical study demonstrated that ingestion of collagen peptides increases the moisture contents of the epidermis and promotes skin flexibility (Kikuchi and Matahira, 2003). However, these findings were not considered valid and not accepted by peer-reviewed journals, even though the conclusion was based on the results of a randomized double-blind placebo control study. After discovery of the presence of food-derived collagen peptides in human blood, a couple of peer-reviewed papers demonstrating the beneficial effects of collagen peptides on human skin have been published. These human trials have demonstrated that ingestion of collagen peptides increases skin moisture (Ohara et al., 2009; Asserin et al., 2015; Inoue et al., 2016), elasticity (Proksch et al., 2014a; Inoue et al., 2016), and decreases wrinkle volume (Proksh et al., 2014b; Inoue et al., 2016) compared to the placebo control. Furthermore, a study on human volunteers demonstrated that daily ingestion of 2.5 g collagen peptides significantly increased the gene expressions of type I collagen and elastin in the skin compared to that in the placebo control (Proksh et al., 2014b). These results suggest that an increase in the number of fibroblasts and/or enhancement of the production of type I collagen and elastin may be responsible for the reduction of wrinkle volume. Some human trials revealed that older subjects responded better to the ingestion of collagen peptides than younger subjects (Ohara et al., 2009; Proksch et al., 2014a). These findings suggest that the ingestion of collagen peptides could improve age-related deterioration of skin condition. Further, the skin conditions are also dependent on environmental conditions such as temperature and humidity in addition to the subject's age. Thus, the data from different studies should be carefully evaluated by understanding that the conditions of the subject and environment can affect the results. To my knowledge, there has been no animal study confirming the beneficial effects of collagen peptides on skin aging. On the other hand, some animal studies have demonstrated that ingestion of collagen peptides attenuates the UV irradiation-induced pathological changes of the skin, such as inflammation, thickening of the epidermis, dysfunction of the barrier function of the epidermis, water loss, and decrease of collagen (Pyun et al., 2012; Oba et al., 2013). Recently, a human trial using male volunteers demonstrated that ingestion of collagen peptides (5 g) suppressed UV irradiation-induced erythema in the subjects older 30 years (Koyama et al., 2014). These facts suggest that ingestion of collagen peptides has the potential to attenuate UV irradiation-induced skin damage.

Some human studies have also demonstrated that ingestion of collagen peptides enhanced the healing of pressure ulcers compared to the placebo control (Sugihara et al., 2015). Enhancement of the proliferation of fibroblasts by Pro-Hyp, a food-derived collagen peptide, might be associated with wound healing.

## Conclusions

Human and animal studies have demonstrated and suggested that ingestion of collagen peptides can improve subjective and objective skin conditions and attenuate UV irradiation-induced damage to skin. The efficacy of the collagen peptides depends upon background of the subjects and the environmental conditions. Elucidation of effects of the subject's background on the efficacy is necessary. Several *in vitro* studies have demonstrated that Pro-Hyp, a prominent peptide in human blood after ingestion of collagen peptides, exerts significant biological activities on fibroblasts and keratinocytes. These effects could be associated with the beneficial effects caused by the ingestion of collagen peptides. The effects of Pro-Hyp on the fibroblasts migrating from the skin are different from those on the fibroblasts in cell lines. Therefore, the effects of the degree of differentiation and cell division of fibroblasts on their response to Pro-Hyp could be potential subjects for further studies.

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## Nutrients for Bone Health

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### Introduction

Bone health is a lifelong concern because healthy bones form the foundation for a vital and energetic life. Skeleton provides mobility, flexibility, and mechanical support as well as protects various organs, stores minerals, and serves as a calcium reservoir to keep physiological calcium balance ([Office of the Surgeon General, 2004](#)). During childhood and adolescence, bone is continuously deposited until a maximum level is reached in the adult, which is also called peak bone mass, after which losses in bone mass begin ([Weaver, 2017](#)). Peak bone mass is the maximum amount of bony tissue present at the end of the skeletal maturation, which is the most important factor for bone health ([Bonjour et al., 1994](#)). Thus, two strategies to reduce the risk of bone health problem are to maximize peak bone mass within our genetic potential and to slow the rate of bone loss with age ([Weaver, 2017](#)). Meanwhile, other diseases can also damage bone health, such as arthritis, fractures, infections, osteoporosis and tumor; osteoporosis, also called “silent killer”, is one of the most serious health issues. Even though the annual incidence of osteoporotic fracture approaches 2 million in America and 1.5 million in Canada aged 40 years or older ([Weaver, 2017](#); [Osteoporosis Canada, 2017](#)), the public awareness of osteoporosis remains low. This might be because osteoporosis is difficult to realize before people get fracture, as well as its lower risk of mortality. However, hip fracture does increase the risk of dying, especially through pneumonia associated with immobility ([Weaver, 2017](#)). Moreover, the loss in quality of life with associated pain and reduced mobility and growing healthcare cost deserve more attention. The overall yearly cost to Canadian healthcare system of treating osteoporosis and associated fractures was over \$2.3 billion as of 2010 and this was estimated to rise to \$3.9 billion if a proportion of Canadians were assumed to be living in long-term care facilities ([Osteoporosis Canada, 2017](#)). Although genetics and preprogrammed body size is considered responsible for 60%-80% of bone mass ([Weaver, 2017](#)), diet and living style are vital to bone health management since these can be changed and modified. In this chapter, the relation between nutrient and bone health will be reviewed.

### Bone Physiology

The skeleton is initially formed by a series of programmed mesenchymal condensations followed by formation of a cartilaginous template for all major skeletal structures ([Dempster and Raisz, 2015](#)). In general, bone consists of two distinct layers: an outer compact layer called cortical bone and an inner lattice-like layer called trabecular bone ([Kini and Nandeesh, 2012](#)). Although bone is always viewed as settled, it is a dynamic and living tissue and consequently requires all essential nutrients for growth and maintenance. Bone is built mainly of two compounds: the collagen protein framework providing flexibility while the mineralized calcium phosphate (hydroxyapatite) offers strength and rigidity ([Heaney, 2012](#)). This combination of protein and hydroxyapatite allows bone to withstand considerable mechanical stress. Throughout life, bone constantly undergoes a cycle of tear down and repair, named remodeling, to remove old, microdamaged bone and replaces it with new, mechanically sound bone to preserve bone strength ([Kini and Nandeesh, 2012](#)). To mediate the remodeling process, three types of cells are composed in bone, including formation cells called osteoblasts and osteocytes, and resorption cells called osteoclasts ([Dempster and Raisz, 2015](#)). The remodeling may be in balance when the amount of newly-formed bone equals to the amount of resorbed, or maybe be imbalanced when the bone formation is either more or less than the bone resorption. The remodeling process is subject to systemic and local regulations, such as nutritional status, sex hormone status, parathyroid hormone level, and vitamin D status.

### Bone Diseases and Nutrition-Related Disorders

The body systems that control growth and maintenance of the skeleton can be disrupted in different ways that result in a variety of bone diseases and disorders. Since genetic effect plays a fundamental role in bone development, some bone diseases can occur at or before birth, such as genetic abnormalities and developmental defects (US Department of Health and Human Services, 2004). Genetic factors cause skeletal diseases either by affecting other tissues and involve the skeleton indirectly such as neurofibromatosis, Gaucher's disease, alkaptonuria, or directly affect skeleton, which is known as skeletal dysplasia or constitutional disorders of bone ([McCarthy, 2011](#)). The most common skeletal dysplasias are osteogenesis imperfect (OI), achondroplasia, and osteopetrosis ([McCarthy, 2011](#)). Compared to genetic-caused bone diseases, most bone problems occur during later life, which may be due to natural aging, nutrition disorder or improper lifestyle. Osteoporosis is one of the most common bone disorders that can have devastating consequences. Although osteoporosis is always considered as an age-related disease, it could also happen in children, adolescents and adults since osteoporosis could be a by-product of medication use like glucocorticoid-induced osteoporosis (US Department of Health and Human Services, 2004). Environmental abnormalities can also cause bone diseases such as rickets and osteomalacia because of vitamin D or phosphate deficiency (US Department of Health and Human Services, 2004). Moreover, some other diseases may also lead to bone health issues. Patients with chronic renal disease are not only at risk of developing rickets

and osteomalacia, but they are also at risk of a complex bone disease known as renal osteodystrophy (US Department of Health and Human Services, 2004). Genetic factors primarily contribute to the variance of the determinants of bone strength. Environmental factors also play an important role in bone health management, especially nutritional intakes. The prevalence of eating disorders has increased in last few decades which may cause the impairment of bone mass gain (Rizzoli, 2014). Bone mineral density is reduced at several skeletal sites in most of women with anorexia nervosa (AN), which is frequently observed in young women (Kasner et al., 2013).

## Nutrients and Bone Health

### Protein Intake and Bone Health

Bone is a composite tissue, made up of 60% minerals, 30% organic matrix, and 10% water (by weight), respectively. Protein makes up one-third of bone mass, and undergoes continuous formation and resorption (Heaney and Layman, 2008). Type I collagen, the major part of the organic matrix, represents 98% of total bone proteins that offers the basic structure of bone, binds to mineral crystals, and provides resilience. Other main non-collagenous proteins including osteocalcin, osteopontin, sialoprotein, and osteonectin can help to strengthen the collagen matrix, and further regulate its ability to bind minerals (US Department of Health and Human Services, 2004). Thus, dietary protein represents a key nutrient for bone health as the “brick supplier”. Although it is still repeatedly claimed that high dietary proteins may be a risk factor for bone health, a large amount of studies confirmed the positive influence of protein on bone health management, and the potential in preventing osteoporosis.

During growth, protein undernutrition from infancy to childhood and adolescence results in reduced bone mass and strength (Bonjour et al., 2015). Moore et al. (2008) studied the effects of average childhood protein intake on adolescent bone health and reported that high intakes of dairy, meats and other proteins were positively associated with high bone mineral content and bone area (Moore et al., 2008). High protein intake, particularly when associated with physical activity, favors healthy development and peak bone mass acquisition enabling individuals to reach their genetic potential (Bonjour et al., 2015). Meanwhile, resistance exercise and high-protein supplementation have been considered as a preventive therapy for osteoporosis associated with aging (Matsuo et al., 2003). A positive correlation between protein intake and bone mass has also been found in premenopausal women (Feskanich et al., 1996). Since bone health is a multifactorial musculoskeletal issue, muscle mass and strength is also important to the development and maintenance of bone health. Loss of bone mass (osteopenia) and loss of muscle mass (sarcopenia) that occur with age are closely related (Heaney and Layman, 2008). Factors that affect muscle anabolism, especially protein intake, also affect bone mass. Dietary protein is a widely accepted strategy for muscle synthesis for elder population (Paddon-jones et al., 2008). Thus, dietary protein could also protect bone health by benefitting muscle health.

One hypothesis indicated that dietary proteins could induce increase in urinary calcium excretion and thereby enhance bone resorption. However, there is a positive interaction between dietary protein, calcium-phosphate economy, and bone metabolism mediated by the anabolic bone trophic factor insulin-like growth factor 1 (IGF-1) (Bonjour et al., 2015). The changes in circulating IGF-1 have been observed in response to various protein intakes in both animal (Ammann et al., 2010) and human studies (Schurch et al., 1998). IGF-1 could increase the circulating level of 1,25-dihydroxyvitamin D, the active form of vitamin D, and boost the intestinal absorption of both calcium and inorganic phosphate (Bonjour et al., 2015). Ammann et al. (2010) found protein undernutrition-induced bone loss is associated with decreased IGF-1 level and/or estrogen deficiency, and leads to a consequent imbalance in bone remodeling. The effects of protein supplements on patients with recent hip fracture also indicated that the increase of serum IGF-1 induced by protein intake could attenuate bone loss (Schurch et al., 1998). Despite the effects of IGF-1 on mineral absorption, the possible mediating role of IGF-1 on the anabolic effect of subcutaneous administration of parathyroid hormone (PTH) was investigated and confirmed (Tahimic et al., 2013). As one of the most important regulating hormones for bone formation, PTH has already been used as an efficient drug for osteoporosis treatment. Eli et al. (2010) indicated that the higher serum IGF-1 levels could synergize PTH action on the skeleton (Eli et al., 2010). Thus, an adequate intake of protein should be recommended in the prevention and treatment of postmenopausal and age-dependent osteoporosis.

### Minerals and Bone Health

Bone consists of a mineral phase of an imperfect hydroxyapatite  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$  and other ions. There is no doubt that calcium and phosphate are two of the most important minerals affecting bone health. Dietary requirements for calcium are determined by the needs for bone development and maintenance, which vary throughout the life stage. It has always showed great needs during the periods of rapid growth in childhood and adolescence, pregnancy and lactation, as well as in elderly life (Flynn, 2003). Dietary calcium intake has an important impact on bone metabolism and bone health, especially when bone also serves as a reservoir for calcium. Chronic calcium deficiency resulting from inadequate intake or poor intestinal absorption is one of the several important causes of reduced bone mass and osteoporosis (Cashman, 2002). Currently, it has become a common sense that calcium supplements could benefit bone health and help to prevent osteoporosis. A number of calcium supplements or calcium-rich foods are also recommended in the dietary guidelines (Cashman, 2002). Meanwhile, consumption of functional foods, which contain ingredients such as lactulose (Seki et al., 2007), insulin (Weaver, 2005), or oligofructose (Weaver, 2005), might positively influence calcium absorption and lead to optimized calcium bioavailability from foods (Cashman, 2002). Like the calcium, maintaining physiological phosphate balance is important for bone health. Phosphorus is widely distributed in all natural foods, because of



its incorporation into the structure and machinery of most cellular tissues (Heaney, 2015). Major sources of phosphate are protein-rich foods, including milk, meat, fish, poultry eggs, and cereal grains (Penido and Alon, 2012). Although phosphorus is essential for maintaining bone structure and bone cell activity, serum phosphorus concentrations substantially above the normal range increase the risk of extraskeletal calcification, especially for people who have renal dysfunction or kidney disease (Moe and Chen, 2008). Also, it is sometimes argued that high phosphorus intakes lead to increased PTH secretion, an outcome presumed to be bad for bone health (Katsumata et al., 2014). High phosphorus intakes have even been proposed as contributor to pathogenesis of osteoporosis (Nieves, 2005). The recommended dietary allowance (RDA) in the US for phosphorus is 700 mg/day for adults (Heaney, 2015). Although there is no evidence for prevailing phosphorus deficiency, some studies reveal low intakes of phosphorus in adults (Heaney, 2015). Calcium supplements are widely recommended for osteoporosis prevention and recognized to bind food phosphorus. But, for individuals with already low phosphorus intake, the effect of calcium supplementation will be a reduction in available phosphorus, which could potentially induce hypophosphatemia (Heaney, 2015). Thus, individuals who are taking calcium supplements, and who are receiving anti-osteoporosis therapies should pay attention to their home diets and nutritional statutes to keep the balance of Ca:P ratio.

While predominant attention has been given to calcium (with vitamin D) and phosphorus, other nutrients also affect bone status, although they are less commonly factored or mentioned into dietary recommendations for the prevention of osteoporosis or the support of anti-osteoporosis therapy. Sodium, potassium, and magnesium influence bone status by affecting calcium economy as well as bone cells. Sodium is the best-studied nutrient among these four minerals. High salt intake is a well-recognized risk factor for osteoporosis because it induces calciuria (Teucher et al., 2008). Additionally, high salt intake also appears to increase bone resorption in postmenopausal women (Harrington and Cashman, 2003). However, in most cases when the papers speak of “sodium”, what is meant is “sodium chloride, which is the form about 90% of contemporary sodium intakes are ingested. This is probably a mistake to ignore the accompanying chloride anion (Heaney, 2015). Nevertheless, it remains true that contemporary intake of excess sodium chloride usually leads to hypercalciuria and harm for bone health.

Compared to calcium, only small amounts of potassium will be trapped when calcium phosphate is precipitated (Heaney, 2015). Zhu et al. (2009) found potassium intake shows positive association with bone density in elderly women, suggesting that increasing consumption of potassium-rich foods may play a role in osteoporosis prevention. In addition, potassium also shows effects on maintaining calcium homeostasis, particularly urinary calcium conservation and excretion (Heaney, 2015). Rafferty et al. (2005) asserted that increases in dietary potassium result in decreased urinary calcium, which helped to reduce the loss of calcium.

Magnesium is considered to condition bone mineral crystal solubility. Without enough magnesium, bone will be brittle and fragile (Castiglioni et al., 2013). The RDA for magnesium is 320 mg/day in women and 420 mg/day in men, but more than 70% of the adult population in the US fall below these recommended intakes (Heaney, 2015). It is unclear whether the magnesium deficiency has skeletal consequences, although several direct or indirect mechanisms contribute to the effects of low magnesium on bone density (Castiglioni et al., 2013). Magnesium deficiency leads to hypomagnesemia, which is in part buffered through the mobilization of surface magnesium from the bone (Creedon et al., 1999). Meanwhile, animal study revealed magnesium deficiency decreased bone formation by blocking osteoblastic activity (Rude and Gruber, 2004). Apart from direct effects on the structure and cells of skeleton, magnesium deficiency indirectly impact the homeostasis of parathyroid hormone (PTH) (Pironi et al., 2009) and 1,25(OH)<sub>2</sub> vitamin D (Gray et al., 1972). Hypomagnesemia also promotes inflammation, which could elevate bone resorption (Mazur et al., 2007). Although the evidence of magnesium and bone health is still fragmentary, most studies available now point out the contributor factor of magnesium to bone health.

## Fat and Bone Health

Body weight is one of the major determinants of bone mineral density. Population studies show a positive influence between body fat and bone density, leading to a positive association between adipocytes and bone strength (Kawai, de Paula and Rosen, 2012). Ellis et al. (2003) studied the bone mineral mass in overweight and obese children and concluded obese children do not show lower bone mineral mass compared with leaner children, even after adjustments for height, age, gender, and ethnicity. Goulding et al. (2008) also investigated the relationship of total body mass and bone area in New Zealand five-year-old children, and suggested that increased fat mass is associated with outward expansion of the total-body-less-head skeletal envelope independently of height and lean mass in very young children. However, controversy remains whether fat has a positive or detrimental effect on the bone. Goulding et al. (2000) reported that overweight and obese children have low bone mass and area for their weight, suggesting a negative effect of body fat on bone health. Another aspect to be taken into account in the evaluation of fat's influence on bone strength is adipocyte distribution (de Paula and Rosen, 2015). Lv et al. (2016) studied the fat distribution and bone quality in healthy Chinese men and found body fat mass, especially android fat and visceral fat, have negative effects on bone microstructure. Similar results have also been found in health postmenopausal Chinese women (Shao et al., 2015). Recently, the relation between regional fat mass and bone mineral was studied. Visceral adipose tissue is an independent inverse determinant of bone density in obesity and it was suggested that this association may be mediated by adipokines and a chronic inflammatory state (Russell et al., 2010). Surplus energy leads to fat accumulation not only on subcutaneous and visceral pads, but ectopic deposition of fat in muscle and liver contributes to the degenerative disorders associated with obesity (de Paula and Rosen, 2015). Bone marrow adipose tissues develop around 50%–70% of bone marrow volume in healthy adults (Cawthron and Scheller, 2017). Common conditions associated with osteoporosis including aging, menopause, and glucocorticoid therapy show increased marrow adiposity indicating

the relation between bone marrow fat and osteoporosis (Gallagher et al., 1979; Kohles et al., 1996). However, the nutritional relationship between bone mass, bone marrow fat, and white adipose tissue is much more complex and incompletely understood.

Dietary fats can influence bone health by affecting intestinal calcium absorption, and possibly renal calcium excretion (Corwin, 2003). Several studies have shown the importance of individual fatty acids on enterocyte membrane dynamics, vitamin D activity, and prostaglandin formation, which can have important effects on intestinal calcium absorption as well as urinary calcium excretion. The proper quantities of dietary fat show benefits on calcium absorption, while large amounts can increase intestinal and urinary calcium excretion. Hormones are critical to bone turnover, especially growth hormone, which is involved in the regulation of IGF-1 and bone formation (Corwin, 2003). Studies have found that secretion of growth hormones is inhibited by increases in serum free fatty acids (Lanzi et al., 1999; van Dam et al., 2000). In addition to serum lipids, dietary lipids can also influence growth hormones. Previous reports also showed that calcitonin level increased when high-fat diet was consumed (Defetos et al., 1989), while PTH did not change (Sanderson et al., 1997). Moreover, dietary fat could also affect bone remodeling by exacerbating the uncoupling of bone resorption and formation. Osteoblasts are derived from mesenchymal stem cells, which are also the progenitor of several other different cell types including adipocytes (Dempster and Raisz, 2015). Several studies indicated that when the differentiation process is directed to adipocyte formation, osteoblast formation might be compromised (Nuttall and Gimble, 2000; Nuttall et al., 1998). Fatty acids and their oxidation products serve as natural ligands for peroxisome proliferator-activated receptors (PPARs), the important nuclear transcription factor involved in adipocyte differentiation (Kliwer et al., 1997; Nagy et al., 1998), and an inhibitor of osteoblast differentiation.

Although the influence of dietary fat on bone has been rarely studied, evidence is accumulating that dietary lipids play an important role on bone health. Epidemiological data indicate that high-fat diets, especially those rich in saturated fatty acids, may contribute to reduced bone density and increased fracture risk, in both elder and younger people (Corwin, 2003). Although large amounts of fat may, especially saturated fatty acids (Corwin et al., 2006), have negative effects on bone health, fats composed of other fatty acids may have positive effects. Recent research highlights the role of polyunsaturated fatty acids (PUFAs) in inflammatory regulation of bone remodeling via cellular pathways (Mangano et al., 2013). Treatment with docosahexaenoic acid (DHA, n-3 FA) promotes proliferation of osteoblasts due to its binding affinity for peroxisome proliferator active receptor gamma (PPAR $\gamma$ ), which is a transcription factor with negative effects on bone homeostasis (Maurin et al., 2005; Wan, 2010). PUFA may also affect bone through attenuation of pro-inflammatory cytokines (LeBlanc et al., 2008), increase of nitric oxide production (Rahman et al., 2009), and promotion of osteoblastic differentiation by stimulating production of IGF-1 and PTH (Shen et al., 2006; Watkins et al., 2003). But the role of dietary PUFAs on bone turnover is controversial. Several studies showed protective associations with arachidonic acid and alpha-linolenic acid (Farina et al., 2011, 2012), while others showed no association (Orchard et al., 2010; Virtanen et al., 2010). Thus, more research works need to be conducted to confirm the effects of PUFA on bone health.

### Vitamins and Bone Health

Vitamin D, the sunshine vitamin, is well recognized for its significance in the development and maintenance of bone health throughout life (Holick, 2015). Vitamin D deficiency not only causes rickets in children and osteomalacia in adults, but also exacerbates osteoporosis including risk of bone fracture (Holick, 2012). The major source of vitamin D for children and adults is from sun exposure. Without proper solar ultraviolet B radiation, 1,25-dihydroxyvitamin D [1,25(OH) $_2$ D], the active form of vitamin D, cannot be formed. 1,25(OH) $_2$ D interacts with its vitamin D receptor in the intestine, which helps to maintain both serum calcium and phosphate concentrations (Holick, 2012, 2015). Thus, when dietary calcium is inadequate to satisfy the body's requirement for calcium, vitamin D becomes a catabolic hormone that mobilizes calcium stores from the skeleton and leads to bone loss (Holick, 2012). Meanwhile, vitamin D interacts with the vitamin D receptor in mature osteoblasts and induces the expression of alkaline phosphatase, osteocalcin, and RANKL (Khosla, 2001). There are very few foods that naturally contain vitamin D, including oily fish like mackerel, eel, and salmon, cod liver oil, sun- and UV-exposed mushrooms, and egg yolks (Holick, 2015). Several products have been produced to contain vitamin D, such as fortified dairy, fortified juice, and fortified breakfast cereals (Calvo et al., 2004). However, sensible sun exposure in combination with vitamin D supplementation is encouraged to prevent vitamin D deficiency.

Although calcium and vitamin D have been most widely studied as the essential nutrients in bone physiology, other vitamins appear to play a role in bone health as well. In 1998, Melhus et al. (1998) reported that greater intake of vitamin A is associated with reduced bone density and increased risk of hip fractures. Other studies also showed that excessive level of retinol intake might be associated with compromised bone health, which can lead to different types of bone abnormalities (Ahmadiéh and Arabi, 2011). But experiments also pointed out that insufficient level of retinol intake or vitamin A deficiency decreases bone mineral content and results in bone health problem as well (Ahmadiéh and Arabi, 2011; Baybutt et al., 2011). Thus, until additional evidence is available, it is still important to ensure sufficient vitamin A supplementation, especially in children.

Another potentially modifiable risk factor for osteoporotic fracture is the elevated homocysteine (Hcy) level. Individuals with high Hcy levels exhibit reduced bone mineral density, alteration in microarchitecture, and therefore lead to the increased risk of fracture (Fratoni and Brandi, 2015). The B vitamins serve as cofactors or substrates for the enzymes involved in Hcy metabolism (Dai and Koh, 2015; Fratoni and Brandi, 2015). Animal studies have shown that a deficiency in folic acid and vitamin B $_6$  and B $_{12}$  can lead to increased levels of Hcy and eventually cause osteoporosis (Fratoni and Brandi, 2015).

Ascorbic acid, more commonly known as vitamin C, is an essential vitamin required for the formation of stable collagen, which is important for bone formation (Aghajanian et al., 2015). Moreover, as an important antioxidant, it is not only involved in the regulation of development and function of bone cells (Aghajanian et al., 2015), but can limit bone resorption induced by oxidative

stress (Hart et al., 2015). Human trials also confirmed that high dietary intake and supplementation with vitamin C reduced the risk of hip fractures in postmenopausal women (Pasco et al., 2006). Same as vitamin C, vitamin E has also gained increasing scientific interest recently in bone health because of its high antioxidative activity (Mohamed et al., 2012). Vitamin E supplementation is able to protect bones from oxidative damage and maintain bone matrix (Feresin et al., 2013). In the ovariectomized rat model, vitamin E showed improvement of the bone microarchitecture, and less bone calcium loss (Norazlina et al., 2007). Additionally, recent studies also claimed the importance of vitamin K in age-related bone loss (Shea and Booth, 2015). This is mainly through five vitamin K-dependent proteins: osteocalcin, matrix gla protein, growth-arrest-specific gene 6 protein (Gas6), protein S, and periostin (Vermeer and Knapen, 2012). Many observational studies have demonstrated that poor vitamin K status is associated with low bone mineral density and increased fracture risk in older adults (Koshihara et al., 2003; Shah et al., 2014). But Booth et al. (2008) indicated that vitamin K supplementation did not confer any additional benefit for bone health when taken with recommended amount of calcium and vitamin D, suggesting that vitamin K might need to be accompanied by other nutrients to show positive effects on bone health.

## Lifestyle and Supplements

Understanding the role of nutrients, food components, and diet on bone health is important as a means available to individuals to build and maintain peak bone mass within their genetic potential. Smoking and alcohol consumption are two lifestyle factors that have been recognized as significant contributors to reduced bone mass and increased fracture risk (Sahni and Kiel, 2015). Hermann et al. (2010) studied the bone density of premenopausal smoking women and reported the negative effect of smoke on bone health. Baheiraei et al. (2005) investigated the bone mineral density and cigarette smoking among Iranian women in Australia as well, and confirmed that smoking weakens bone strength. Although the mechanism is still unclear, there are several research works that have investigated the direct and indirect effects of smoking on skeletal health and fracture risk. Smoking directly affects osteogenesis including alteration in the RANK-RANKL-OPG system, collagen metabolism, and bone angiogenesis. Meanwhile, smoking has indirect effects on bone by decreasing intestinal calcium absorption, dysregulation in sex hormone production and metabolism, alterations in metabolism of adrenal cortical and gonadal hormones as well as calciotropic hormones such as 25-hydroxy-vitamin D and PTH. The role of alcohol on skeletal health was not as well studied as smoking, but the direct effects of alcohol on bone and mineral metabolism have been described in both rats and humans (Sahni and Kiel, 2015). Chronic ethanol administration to growing rats was attended by decreased bone density and microarchitecture (Baran et al., 1980). Prabhakaran et al. (2014) studied the bone mineral metabolism and bone mineral density in alcohol related chronic pancreatitis patients and found majority of patients had low bone mineral density.

A wealth of animal and human data provides evidence for the relationship between physical activity and bone health at all ages. Although the goal of physical exercise is not always the same at different life stages, an exercise prescription is important for the prevention and treatment of osteoporosis at all ages. The goal of physical activity in relation to bone health in youth is to maximize peak bone mass, which is attained at various sites by age 16–26 years in most studies (Vuori, 1996), in order to potentially reduce the burden and delay the onset of osteoporotic fracture in adults (Singh, 2015). An initial emphasis on weight-bearing aerobic and high-impact activities in youth, shifting toward resistive loading and balance-enhancing exercise in old age, appears to address optimally the needs and capacities of the musculoskeletal system throughout the lifespan (Singh, 2015). Thus, a lifestyle including nonsmoking, a high physical activity level, and a high body weight reduces bone loss and fracture risk in both sexes, with increasing effect from peak bone mass to old age (Wilsgaard et al., 2009).

The use of dietary supplements or nutraceuticals is common and exceeds 50% of US adults with higher rates in individuals that suffer from chronic diseases (Nieves, 2015). Many adults resort to the use of supplements and nutraceuticals to improve their bone health. Calcium and vitamin D are the most common supplements to maintain bone health while various other nutrients may also provide benefits to the skeleton as discussed in **Nutrients and Bone Health** section. Although there are no clear or consistent results for the relationship between skeletal health and nutritional intake of protein, magnesium, phosphorus, various antioxidants, flavonoids, carotenoids, omega-3 fatty acids, and various vitamins, it is important for adults to consume adequate amounts of these nutrients in order to maintain and promote musculoskeletal health.

## Summary

Bone health is critically important to the overall health, vitality and quality of life; however, public awareness of bone health management remains low. Despite the significance of genetic factor, daily diet and nutrients play more important role in maintain skeletal health because it is a modifiable condition. Calcium and vitamin D play a clear role in bone health; it is important to have a well-balanced diet that is rich in calcium and vitamins. Meanwhile, proper intakes of protein, fat, mineral and various micronutrients are also crucial for bone strength. Although a great amount of supplementation and nutraceuticals are applied, the first choice is still to consume the nutrients from foods.

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## Structured Lipids for Foods

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### Nomenclature

ARA arachidonic acid  
BSTR batch stirred tank reactor(s)  
C6:0 caproic acid  
C8:0 caprylic acid  
C10:0 capric acid  
C12:0 lauric acid  
C18:0 stearic acid  
CB cocoa butter  
CBE cocoa butter equivalent(s)  
CLA conjugated linoleic acid  
CPL *Carica papaya* lipase  
DAG(s) diacylglycerol(s)  
DHA docosahexaenoic acid  
EPA eicosapentaenoic acid  
FA(s) fatty acid(s)  
FBR fluidized-bed reactor(s)  
FFA(s) free fatty acid(s)  
GLA gamma-linolenic acid  
HMFS human milk fat substitute(s)  
L long-chain fatty acid (C14–C24)  
LCTAG(s) long-chain triacylglycerol(s)  
Lipozyme RM IM commercial immobilized *Rhizomucor miehei* sn-1,3 regioselective lipase from Novozymes<sup>®</sup> A/S, Bagsvaerd, Denmark.  
Lipozyme TL IM commercial immobilized *Thermomyces lanuginosa* sn-1,3 regioselective lipase from Novozymes<sup>®</sup> A/S, Bagsvaerd, Denmark.  
Lyso-PL(s) lysophospholipid(s)  
M medium-chain fatty acid (C6–C12)  
MAG(s) monoacylglycerol(s)  
MCTAG(s) medium-chain triacylglycerol(s)  
MLM triacylglycerol presenting a medium-chain fatty acid at positions sn-1 and sn-3 and a long-chain fatty acid at position sn-2  
Novozym 435 commercial immobilized *Candida antarctica* lipase from Novozymes<sup>®</sup> A/S, Bagsvaerd, Denmark.  
OPO 1,3-dioleoyl-2-palmitoyl-glycerol  
PA palmitic acid  
PBR packed-bed reactor(s)  
PC phosphatidylcholine  
PL(s) phospholipid(s)  
PLip phospholipase(s)  
POP 1,3-dipalmitoyl-2-oleoyl-glycerol  
POS 1-palmitoyl-2-oleoyl-3-stearoyl-rac-glycerol  
PPP tripalmitin  
PUFA polyunsaturated fatty acid(s)  
ROL *Rhizopus oryzae* lipase  
S short-chain fatty acid  
SFA saturated fatty acid(s)  
SL(s) structured lipid(s)

SPhenL(s) structured phenol lipid(s)  
 SPL(s) structured phospholipid(s)  
 StOSt 1,3-distearoyl-2-oleoyl-glycerol  
 TAG(s) triacylglycerol(s)

## Definition of Structured Lipids and Principal Types

Formulating foods for health, i.e. *functional foods*, having physiologically-active components to provide a health benefit beyond nutrition, enhancing both physical and mental health and well-being, is one of the top trends in the food industry (Hasler, 2000). In the field of oils and fats, these novel products are called Structured Lipids (SLs) or “Tailor-made” fats.

The SLs are modified lipids (or fats and oils) that do not exist in nature but present improved technological, functional and/or pharmaceutical properties. The SLs are currently defined as triacylglycerols (TAGs) or phospholipids (PLs) that have been (i) modified by the incorporation of new fatty acids (FAs), (ii) restructured to change the positions of FA or the FA profile, from the natural state, or (iii) synthesized to yield novel TAG (or phospholipids), either chemically or enzymatically (Osborn and Akoh, 2002).

Among the SLs obtained from TAG restructuring, the most important examples are: (i) low-calorie TAGs, (ii) human milk fat substitutes (HMFS), (iii) cocoa butter equivalents (CBE), (iv) TAGs rich in omega-3 polyunsaturated fatty acids (omega-3 PUFA), conjugated linoleic acid (CLA), gamma-linolenic acid (GLA) or in other interesting fatty acids or (v) *trans*-free plastic fats. The structurally modified phospholipids are usually enriched in specific FAs with health benefits (Lemaître-Delaunay et al., 1999).

In the last years, the definition of SLs has been enlarged to other modified lipids with nutritional or pharmaceutical applications, such as partial acylglycerols (monoacylglycerols and diacylglycerols) and structured phenolic lipids (Jala et al., 2012; Kim and Akoh, 2015). The structured phenol lipids (SPhenLs) are esters of a phenol acid or its ester with TAGs with antioxidant, anticarcinogenic, anti-inflammatory, anti-Alzheimer's disease effects and are potential UVA and UVB absorbing ingredients in cosmetics, due to the presence of phenolic acid (Jala et al., 2012).

These different types of SLs and respective properties have been described in several reviews (Willis et al., 1998; Gunstone, 1999; Osborn and Akoh, 2002; Gupta et al., 2003; Sakurai and Pokorný, 2003; Senanayake and Shahidi, 2005; Shahidi, 2006; Ferreira-Dias, 2010; Jala et al., 2012; Ferreira-Dias et al., 2013; Ferreira-Dias and Tecelão, 2014; Kim and Akoh, 2015).

## Biocatalysts Used for Structured Lipids Production

Some types of SLs (e.g. *trans*-free plastic fats) can be obtained using chemical catalysts, under high temperatures (higher than 100 °C). Since chemical catalysts are non-selective, they catalyze competing reactions and they act at random towards the acyl-positions in TAGs. Therefore, the yields are low, and the required products must be purified to remove side-products.

In the Food Industry, the replacement of inorganic catalysts by the enzymatic route has great benefits. SLs can be produced enzymatically using mainly lipases (EC 3.1.1.3., triacylglycerol acyl-hydrolase). Lipases catalyze the hydrolysis of esters at lipid/water interfaces, but when in organic media at low water activity, they can also catalyze esterification and interesterification reactions (Kazlauskas and Bornscheuer, 1998). The use of lipases as biocatalysts for the production of SLs has recognized advantages over the classical chemical catalysts, namely: (i) lipases act under milder conditions (temperature < 70 °C, atmospheric pressure), (ii) they present high selectivity (regio-, stereo-, typo-, and substrate selectivity), leading to a decrease in side products formation and to an easy product recovery and purification, (iii) the number of unit operations is smaller and (iv) the products can be labelled as “natural” since they result from a natural process. Also, (v) lipases are enzymes that do not need co-factors.

Lipases from microbial, plant or animal origin can be used as biocatalysts. Some lipases do not exhibit regioselectivity and they act at random in the acyl residues of TAGs (*sn*-1,2 or *sn*-3) like the chemical catalyst but under softer conditions (near-room temperature and normal pressure). The *sn*-1,3 regioselective lipases can only act at positions *sn*-1,3 of TAGs. Thus, the original fatty acids in the internal position (*sn*-2) of the acylglycerols are preserved, which is nutritionally desirable and not possible to attain by inorganic catalysis (Michalski et al., 2013). The use of this type of lipases is mandatory for the synthesis of SLs where the original FAs at *sn*-2 position must be maintained.

In SLs production, immobilized lipases have been used to lower the costs with the biocatalyst. These immobilized lipases in solid supports must present both high catalytic activity and operational stability, which allows using them in continuous bioreactors or reusing in consecutive batch reactions. However, the commercial immobilized lipases from microbial sources, which have been extensively used for SLs production, are expensive, limiting the economic viability of some processes and products (e.g. commodity fats for margarine manufacture).

Lipases can also be used as a catalyst for the synthesis of structured phospholipids, by acting on acyl ester bonds of the phospholipids, and structured phenol lipids.

Phospholipases A1 (EC 3.1.1.32) and A2 (EC 3.1.1.4) are enzymes, which specifically act on the first and second bond of phospholipids and can also be used as catalysts for the production of structured phospholipids (Kim and Akoh, 2015).

The search for alternative non-commercial enzymes and/or cheap immobilization supports and immobilization techniques, are routes to be explored to reduce production costs related with the biocatalyst. Plant lipases are interesting biocatalysts to explore because they can be obtained at lower cost, they are readily available, and present wide versatility and stability in organic media. Latexes with lipase activity obtained from *Carica papaya* and *Carica pentagona* (both tropical and subtropical plants) and *Euphorbia characias*, a wild plant growing in the Mediterranean area, are examples of plant biocatalysts (Villeneuve, 2003).

The cost of immobilization supports can also be reduced if agro-forestry and inert industrial wastes are used as immobilization supports.

## Reaction Systems for SLs Production

### Reactions

The most used reactions to synthesize SLs are as follows: acidolysis, interesterification (ester interchange), transesterification (alcoholysis) or direct esterification.

**Acidolysis:** is a reaction between an ester (a single TAG, oil or fat or phospholipid) and a free fatty acid (FFA), which can be compared with hydrolysis or alcoholysis, since the acid has a similar role of water or alcohol, respectively. This reaction is currently used to produce SLs modified in their FA composition, catalyzed by a *sn*-1,3-specific lipase, in non-aqueous media (Fig. 1).

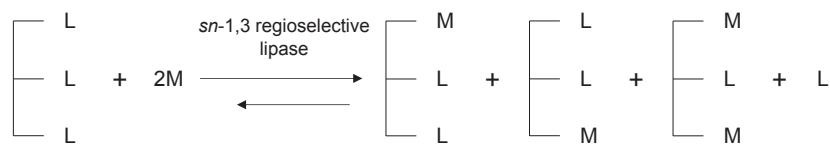
**Intesterification (ester interchange):** this reaction consists of a redistribution of the fatty acid moieties present in a TAG over its glycerol moieties. The interesterification of a blend of different TAGs will originate a mixture with novel TAG profile (Fig. 2).

**Transesterification (alcoholysis):** it consists of the reaction of an alcohol and a carboxylic acid ester to form a different carboxylic acid ester, i.e., one ester is transformed into another ester. When methanol, ethanol or glycerol are the alcohols used, this reaction is currently called as methanolysis, ethanolysis and glycerolysis, respectively.

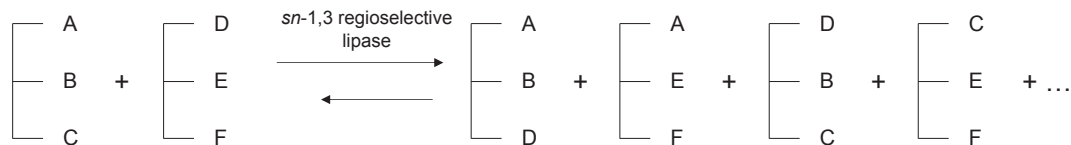
**Esterification:** it is the general name for a chemical reaction in which an alcohol and an acid react to form an ester, as the reaction product, and water.

### Reaction Mechanisms

The mechanisms of lipase-catalyzed acidolysis, alcoholysis and interesterification involve hydrolysis of ester bonds in TAGs followed by re-esterification (Xu, 2000). Thus, the optimization of interesterification reactions results from a balance between the rates of hydrolytic and esterification reactions. Enzymatic interesterification is a reversible reaction and equilibrium will be eventually reached. The product yield under reaction equilibrium is decided by the substrate ratio. Even when *sn*-1,3 regioselective lipases are used, the undesirable and unavoidable side reaction of acyl migration will occur, with the formation of undesirable TAG products (Kim and Akoh, 2005). Thus, acyl migration must be minimized mainly by controlling the following parameters: reaction temperature, biocatalyst load, type of immobilization support, water content and water activity, solvent type and reaction system (Xu, 2003).



**Figure 1** Schematic acidolysis of a long-chain TAG (LLL) with medium-chain (M) fatty acids catalyzed by a *sn*-1,3 regioselective lipase (the L at position *sn*-2 is maintained).



**Figure 2** Schematic interesterification (ester interchange) between TAGs with different fatty acid residues (A, ...F) catalyzed by a *sn*-1,3 regioselective lipase (the FAs at position *sn*-2 are maintained).

### Reaction Medium Composition

Currently, the production of SLs is carried out either in presence of an organic solvent or in solvent-free media. In these media, water must be present only in small amounts needed to maintain the catalytic activity of the biocatalyst but not in higher levels, to avoid lipid hydrolysis.

When fats of high melting point are used, an organic solvent can be used to dissolve them and, therefore, the reaction can take place at near-room temperature (saving-energy). Also, the use of an organic solvent immiscible with water facilitates the extraction and recovery of the final hydrophobic products (SLs) and promotes the balance of the reaction towards the desired direction (synthesis of SLs). However, the use of an organic solvent will increase (i) the complexity of the reaction medium, (ii) the operation costs and (iii) product recovery and purification. Also, the solvent used must be biocompatible (Laane et al., 1985) and food-grade.

As alternative, "solvent-free media" consist only of the substrates involved in the reaction. When these substrates are liquid at room temperature, lipases from mesophilic organisms can be used as catalysts. Conversely, when high melting point fats are used, the reaction must proceed at a temperature higher than that of the melting point of these substrates and thermostable biocatalysts are needed (Paula et al., 2015). Solvent-free media is a green option for SLs production systems, although a higher reaction temperature is required in some situations.

### Bioreactors

The synthesis of SLs by immobilized enzymes in solid carriers can be performed in batch or in continuous bioreactors, either in the presence of an organic solvent or in solvent-free media. At laboratory-scale, batch stirred tank reactors (BSTR) have been currently used. BSTR can be a good option if the support particles are not damaged by shear forces. This configuration is adequate to situations of substrate inhibition.

However, for process implementation in continuous mode, packed (PBR) or fluidized-bed (FBR) tubular bioreactors are preferred to completely-stirred tank reactors. Continuous PBR are the most adequate reactors to repress acyl-migration, due to shorter residence times needed (Xu et al., 1998; Xu, 2003). Fluidized-bed reactors allows for high mass transfer rates and low shear stress for the immobilized biocatalyst particles (Osório et al., 2005, 2006; Paula et al., 2015).

### SLs Modified in Their FA Composition: Characterization, Health Benefits and Production

The synthesis of this type of SLs is usually performed by acidolysis reaction between an ester (a single TAG, oil or fat or phospholipid) and a free fatty acid, catalyzed by a *sn*-1,3-regioselective lipase, in non-aqueous media. These SLs can also be obtained by ester-interchange between a single TAG, oil or fat and fatty acid alkyl esters containing the desired FA to be incorporated in the SLs.

#### Human Milk Fat Substitutes

The TAGs from Human Milk Fat (HMF) have a USU structure, *i.e.*, unsaturated fatty acids, U, at the external positions of the glycerol backbone, namely oleic acid, and saturated FAs (S) at the *sn*-2 position, predominantly palmitic acid. The 1,3-dioleoyl-2-palmitoyl-glycerol (OPO) is the major TAG in human milk fat. This structure is crucial for the efficient absorption of palmitic acid as mono-acylpalmitate. In infant formula, the use of TAGs from vegetable oils and cow's milk, both with a SUS structure, may lead to the formation of insoluble calcium soaps with saturated fatty acids released by the action of the *sn*-1,3 regioselective pancreatic lipase. This results in a deficient absorption of calcium and fatty acids by the infants (López-López et al., 2001). Kennedy et al. (1999) compared bone matrix development in breast-fed infants and in infants fed with standard formula or with a formula containing a SL with 50% of *sn*-2 palmitate (*sn*-2 formula). After 12 weeks, infants receiving the high *sn*-2 formula had a bone matrix development similar to the ones fed with human milk, and significantly higher than the ones fed with the standard formula. Moreover, infants fed with SL formula presented softer stools with a lower concentration of soap fatty acids.

The synthesis of HMF substitutes (HMFS) can be attained by acidolysis or interesterification with ethyl esters using *sn*-1,3 regioselective lipases as catalysts. Both reactions require the use of a TAG source rich in palmitic acid at the *sn*-2 position, namely high purity tripalmitin, palm stearin, palm oil, fractionated palm stearin, butterfat or lard. The use of tripalmitin has been restricted to lab-scale due to its high-cost and high melting point. Also, the use of lard may be limited due to ethical and religious issues. OPO is the target TAG in the synthesis of human milk fat substitutes.

In the acidolysis reaction, wide sources of FFA have been used, either as: (i) single mono- or polyunsaturated FA (namely oleic acid and DHA), (ii) blends from different vegetable oils (e.g. olive oil, rapeseed oil, sunflower oil, hazelnut oil), or (iii) sources of omega-3 PUFA (such as fish oils and, most recently, microalgae oil) (Soumanou et al., 2013; Ferreira-Dias and Tecelão, 2014).

In the interesterification reaction, blends of vegetable oils rich in long chain FA or methyl or ethyl esters, namely of oleic acid, have been used as acyl donor.

Also, multistep processes have been implemented to attain higher yields of TAGs resembling HMF, namely, a two-step process of an alcoholysis reaction followed by esterification (Soumanou et al., 2013).

Both acidolysis and interesterification reactions can be performed batchwise or in continuous mode (usually using packed-bed reactors) either in solvent or in solvent-free media.

The use of solvent-free media for HMFS production is preferred from economical and food safety perspectives. In solvent-free media, the use of thermostable lipases is required due to the high melting point of fats used as palmitic acid source, namely, tripalmitin.

Nowadays, commercial HMFS have been produced for incorporation in infant formula. "Betapol", developed by IOI Loders Croklaan (the Netherlands), is prepared by enzymatic acidolysis of fractionated palm oil with fatty acids from high-oleic sunflower oil. "InFat", sold by Advanced Lipids, is a structured TAG with a total palmitic acid content of at most 38% of the total fatty acid and a *sn*-2 position content of at least 60% of palmitic acid.

**Table 1** presents some examples of recently published studies on lipase-catalyzed HMFS production.

### Low Calorie and Dietetic Triacylglycerols

Low calorie and dietetic TAGs present one of the following structures: (i) SLS (short chain FAs at the external positions of glycerol backbone and long chain fatty acid at the *sn*-2 position), MLM (medium chain FAs at the external positions and long chain fatty acid at the *sn*-2 position) and MMM type SL.

MLM type SLs have been extensively studied as food source for people with fat malabsorption and other metabolism problems. The low caloric value of these TAGs (about 5 kcal/g) is due to the fact that medium chain fatty acids are more rapidly metabolized than long chain fatty acids, due to its higher solubility, being transported directly to the liver, thus providing a rapid source of energy.

One of the first uses of these SLs was in enteral (oral tube feeding) and parenteral (intravenous feeding) nutrition, as an alternative to physical mixtures of long-chain and medium-chain triacylglycerols (LCTAGs and MCTAGs). [Chen et al. \(2013\)](#) evaluated the effect of SLs as part of parenteral nutrition in elderly patients with severe sepsis. SLs emulsions proved to be safe, improving protein synthesis and contributing to a better prognosis. Several studies in mice have proved that SLs and physical mixtures with the same FA composition have different metabolic pathways due to the prevalence of PUFA at

**Table 1** Examples of lipase-catalyzed production of human milk fat substitutes (HMFS)

Product type	Reaction	Substrates	Biocatalyst	System type	Operation mode	Reference
HMFS rich in omega-3 PUFAs	Acidolysis	Microalgae TAG rich in PA at <i>sn</i> -2 position + FFA from microalgae oil rich in <i>n</i> -3 PUFA	Novozym 435, Lipozyme 435, Lipozyme TL-IM and Lipozyme RM IM	Solvent-free	Batch	<a href="#">He et al. (2017)</a>
1,3-dioleoyl-2-arachidonoylglycerol-rich structured lipids for infant formula	Acidolysis	ARA-rich Fungal oil + oleic acid	Lipozyme RM IM	Solvent-free	Batch	<a href="#">Abed et al. (2017)</a>
HMFS resembling TAG composition of HMF	Interesterification	Lard blending with selected oils	Lipozyme RM IM	Solvent-free	Continuous PBR	<a href="#">Zou et al. (2016)</a>
HMFS rich in PUFA, mainly omega-3 linolenic acid	Acidolysis	PPP + FFA from camelina oil	Immobilized non-commercial <i>Rhizopus oryzae</i> lipase and Lipozyme RM IM	Solvent-free	Batch	<a href="#">Faustino et al. (2016)</a>
HMFS	Acidolysis	Lard + FFA from a fish oil concentrate rich in DHA	Novozym 435, Lipozyme TL IM, Lipozyme RM IM and Immobilized non-commercial <i>Rhizopus oryzae</i> lipase	Solvent-free	Batch	<a href="#">Simões et al. (2014)</a>
HMFS	Acidolysis	PPP + oleic acid or omega-3 PUFA	<i>Carica papaya</i> lipase self-immobilized in papaya latex	Solvent-free	Batch	<a href="#">Tecalão et al. (2012)</a>
HMFS	Interesterification	Lard + soybean oil	Lipozyme TL IM	Solvent-free	Batch	<a href="#">Silva et al. (2009)</a>
HMFS	Interesterification	PPP + [coconut + safflower + soybean] oils	Lipozyme RM IM	<i>n</i> -hexane	Batch	<a href="#">Maduko et al. (2007)</a>
HMFS with omega-3 PUFA	Acidolysis	PPP + [hazelnut FA + omega-3 PUFA]	Lipozyme RM IM	<i>n</i> -hexane	Batch	<a href="#">Sahin et al. (2006)</a>
HMFS similar to Betapol	Acidolysis	Lard + soybean oil FA	Lipozyme RM IM	Solvent-free	Batch	<a href="#">Nielsen et al. (2006)</a>

**Table 2** Lipase-catalyzed production of dietetic SLs

Product type	Reaction	Substrates	Biocatalyst	System type	Reference
MLM rich in long-chain PUFA	Acidolysis	Grapeseed oil + C8:0 or C10:0	Non-commercial immobilized ROL and CPL self-immobilized in papaya latex	Solvent-free	Costa et al. (2018)
MLM	Acidolysis	Pumpkin seed oil + C10:0	Lipozyme TL IM	Solvent-free or <i>n</i> -hexane	Sousa et al. (2018)
MLM rich in omega-3 fatty acids	Direct esterification with glycerol; Two-step esterification; Acidolysis	Concentrated PUFA from sardine discards oil + C8:0	Novozym 435 Lipozyme RM IM	<i>n</i> -hexane	Morales-Medina et al. (2017)
MLM	Acidolysis	Avocado oil + C8:0	Lipozyme TL IM and Lipozyme RM IM	Solvent-free	Caballero et al. (2014)
MLM	Acidolysis	Olive oil + C8:0 or C10:0	Non-commercial immobilized <i>Yarrowia lipolytica</i> lipase 2	Solvent-free	Casas-Godoy et al. (2013)
MLM	Acidolysis	Olive oil + C8:0 or C10:0	Non-commercial immobilized ROL	Solvent-free	Nunes et al. (2012)
MLM rich in EPA, DHA and C10:0	Interesterification	Fish oil + C10:0 methyl ester	Lipozyme RM IM	Solvent-free	Feltes et al. (2009)
MLM	Acidolysis	Fish oil + medium-chain TAG Roasted sesame oil + C8:0	Lipozyme RM IM	Solvent-free	Kim and Akoh (2006)
SLS	Acidolysis	Rapeseed oil + C6:0	Lipozyme RM IM	Solvent-free	Zhou et al. (2001)
LSL	Acidolysis	Triacetin + C18:0	Chirazyme L-2	Solvent-free	Yang et al. (2001)

the *sn*-2 position. Faster absorption rates of the SLs were observed when compared to the other types of SLs (Lee et al., 2008; Straarup and Høy, 2000). Also, an increase in inflammatory response was observed in rats fed with SLs as total parenteral nutrition (Lin et al., 2009).

The anti-obesity effect of these SLs has been investigated. Mice fed with SLs gained less weight, had reduced amounts of adipose tissue and lower liver weight, the amount of fat excreted in the faeces increased, total-cholesterol and LDL-cholesterol levels decreased, while HDL-cholesterol levels in plasma increased (Cao et al., 2013; Moreira et al., 2017).

The production of MLM type SL is currently attained through acidolysis reaction between medium chain fatty acids, such as caprylic (C8:0) or capric (C10:0) acids, and different oils as source of long-chain fatty acids (e.g. olive, soybean, avocado, borago, corn and pine nut oils), catalyzed by *sn*-1,3 regioselective lipases.

The interesterification reactions between vegetable oils with long and medium fatty acids have also been performed to obtain a mixture of TAGs containing both M and L fatty acids, that is, MLM, MML, LML and LLM (Lee et al., 2012).

Several examples of this type of SLs obtained by acidolysis or interesterification reactions are presented in Table 2.

Structured lipids with low-calorie values have been commercialized namely SALATRIM (Nabisco, United States), Caprenin and Olestra (both manufactured by Procter and Gambler, United States).

### Cocoa Butter Equivalents

Cocoa butter (CB) is an important ingredient in chocolate and other confectionery industries. It is valued for its unique physico-chemical properties, which is given by its peculiar fatty acid composition and TAG profile. More than 70% of CB TAGs are symmetrical with oleic acid (O) at the *sn*-2 position. The three most important TAGs are 1,3-dipalmitoyl-2-oleoyl-glycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-rac-glycerol (POS), and 1,3-distearoyl-2-oleoyl-glycerol (StOSt) (Xu, 2000; Naik and Kumar, 2014).

Due to the high cost and fluctuations in supply and demand for cocoa butter, the industry is looking for CB alternatives. Cocoa butter equivalents (CBE) are SLs with TAG composition that is similar to cocoa butter and can be produced by (i) acidolysis using *sn*-1,3 regioselective lipases to incorporate palmitic and stearic acids into vegetable oils rich in oleic acid at the *sn*-2 position or by (ii) interesterification of fat blends containing similar fatty acid composition to CB.

Enzymatic modification of low-cost fats and oils have been successfully developed by Unilever and Fuji Oil to produce cocoa butter substitutes at a large scale via lipase-catalyzed reactions (Coleman and Macrae, 1977; Matsuo et al., 1981).

Several studies have been performed to obtain products similar to cocoa butter by (i) interesterification or (ii) acidolysis using commercial immobilized *sn*-1,3 regioselective lipases, such as Lipozyme RM IM and Lipozyme TL IM (Table 3).



**Table 3** Examples of lipase-catalyzed synthesis of cocoa butter equivalents (CBEs)

Product type	Reaction	Substrates	Biocatalyst	System type	Operation mode	Reference
CBE rich in StOst TAGs.	Acidolysis	High-oleic sunflower oil or high stearic high oleic sunflower oil + FAs (stearic acid, palmitic acid, myristic acid, arachidic acid)	Lipozyme RM IM	Solvent-free	Batch	Kadivar et al. (2016)
CBE	Acidolysis	Refined olive oil + mixture of palmitic-stearic acids	Lipozyme IM	<i>n</i> -hexane	Continuous PBR	Mohamed (2015)
CBE rich in POS TAGs	Interesterification	High-oleic sunflower oil + palmitic acids (ethyl palmitate ester) + stearic acids (ethyl stearate ester)	Lipozyme RM IM	Solvent-free	Continuous PBR	Kim et al. (2014)
CBE	Acidolysis	Refined olive pomace oil + with palmitic acid + stearic acid	Lipozyme IM	Solvent ( <i>n</i> -hexane)	Continuous PBR	Ciftci et al. (2009)
CBE	Transesterification	TAGS from <i>Pentadesma butyracea</i> + ethyl palmitate	Lipozyme TLIM	Solvent-free	Batch	Tchobo et al. (2009)
CBE	interesterification (reaction at high pressure)	Lard + tristearin	Lipozyme IM-20	Solvent-free	Batch	Liu et al. (2007)
CBE	interesterification (reaction at high pressure)	Camel hump fat + tristearin	Lipozyme TL IM	Solvent-free	Batch	Shekarchizadeh et al. (2009)

Currently, Fuji Oil holds the world market in the production of CBE by enzymatic synthesis. Other products on the market include Coberine® Cocoa Butter Equivalents developed by IOI Loders Croklaan (<http://europe.ioiloders.com/products/coberine/>).

### Triacylglycerols Rich in Specific Long-Chain Fatty Acids

SLs rich in specific long-chain fatty acids, some of them essential FA, with recognized health benefits, are synthesized for the purpose of obtaining nutraceutical or functional lipids. The most interesting fatty acids used to incorporate in these SLs are: linoleic acid (C18:2, omega-6), alpha-linolenic acid (C18:3, omega-3), arachidonic acid (C20:4; omega-6), eicosapentaenoic acid (EPA, C20:5 omega-3), docosahexaenoic acid (DHA, C22:6, omega-3), gamma-linolenic acid (GLA), and conjugated linoleic acid (CLA) (Ruiz-Rodriguez et al., 2010). SLs enriched in omega-3 polyunsaturated fatty acids (omega-3 PUFA), especially in EPA and DHA, have a great potential due to their benefits in human health, namely the prevention and treatment of heart diseases by increasing high density lipoprotein cholesterol levels in serum, and the reduction of inflammatory conditions (Simopoulos, 1998; Osborn and Akoh, 2002).

The incorporation of PUFA in food products more readily available for consumption than marine fat fish, such as vegetable oils, dressings and margarines, may be an interesting option to human diet.

The production of TAGs rich in specific long-chain fatty acids has been usually carried out by (i) acidolysis, (ii) interesterification of a single TAG, oil or fat with ethyl or methyl esters, or by (iii) direct interesterification, either in organic or in solvent-free media, as reported in several studies (Table 4). In the majority of these studies, commercial immobilized *sn*-1,3 regioselective lipases were used.

Different sources of oils, commercial or natural, were used to prepare these SLs namely: commercial concentrates of triacylglycerols rich in EPA and DHA, perilla and linseed oils, high-stearidonic soybean and echium oils and tuna or sardine oils or their free FA fractions (Table 4).

An example of commercial DHA-rich SLs manufactured by biocatalytic processes is Marinol D-40 from Stepan Lipid Nutrition. Marinol D-40 is a concentrate of natural fish oil with a high content of docosahexaenoic acid (DHA) in glyceride form. Concentration is done by means of an enzymatic process and molecular distillation ([https://www.stepan.com/uploadedFiles/Literature\\_and\\_Downloads/General\\_Lit/Product\\_Line\\_Information/MarinolBrochure.pdf](https://www.stepan.com/uploadedFiles/Literature_and_Downloads/General_Lit/Product_Line_Information/MarinolBrochure.pdf)).

### Structured Phospholipids

The principal reason for phospholipids (PLs) modification is to adapt PLs for specific applications by providing technical or physiological properties that the natural substance does not possess. PLs incorporated into lipoproteins of the blood stream are more efficient carriers than TAGs for PUFAs to several tissues and organs (i.e., brain, liver, lung, heart, etc.), including blood cells such as platelets and erythrocytes (Lemaître-Delaunay et al., 1999; Burri et al., 2012).

**Table 4**     Examples of lipase-catalyzed synthesis of TAGs rich in specific long-chain fatty acids in batch bioreactors

Product type	Reaction	Substrates	Biocatalyst	System type	Reference
Omega-3 FA-rich oil	Direct esterification	sardine oil FAs + Glycerol	Lipozyme IM	Solvent-free	Bispo et al. (2014)
Omega-3 FA-rich oil	Interesterification	Linseed oil + palm stearin	Lipozyme TL IM	Solvent-free	Farfan et al. (2013)
<i>n</i> -3 FA-rich oil	Interesterification	Grape seed oil + perilla oil	Lipozyme TL IM	Solvent-free	Yang et al. (2013)
Conjugated FA-rich oil	Acidolysis	Soybean oil + conjugated linoleic acid ethyl esters	Lipozyme RM IM	Solvent-free	Yang et al. (2012)
SL rich in omega 3 PUFA for table margarines	Interesterification	Palm stearin + Palm Kernel oil + "EPAX 4510 TG"	Lipase/acyltransferase on Accurel MP1000	Solvent -free	Osório et al. (2009)
LLL rich in C18:0	Acidolysis	Rice bran oil + C18:0	Lipozyme RM IM	Solvent ( <i>n</i> -hexane)	Chopra et al. (2008)
TAG rich in CLA	Acidolysis	Rice bran oil + CLA	Lipozyme TL IM	Solvent-free	Alim et al. (2008)
TAG rich in CLA and C:12	Acidolysis	Modified castor oil + C12:0	Lipozyme TL IM	Solvent-free	Villeneuve et al. (2007)
TAG rich in DHA and GLA	Acidolysis	Borage oil (source of GLA) + DHA	<i>Carica papaya</i> latex Novozym 435	Solvent ( <i>n</i> -hexane)	Senanayake and Shahidi (2002)

The molecular structure of PLs can be changed by either enzymatic or chemical means. The enzymatic processes permit the synthesis of new PL compounds for functional applications and human nutrition such as: *sn*-1 lysophospholipids (*sn*-1 Lyso-PLs); *sn*-2 lysophospholipids (*sn*-2 Lyso-PLs); *sn*-1 modified phosphatidylcholine (PC); *sn*-2 modified PC, which have not been possible to obtain by chemical routes.

Lyso-PLs are obtained by partial hydrolysis of the PL molecule with the removal of one fatty acid. When the hydrolysis of PLs is catalyzed by phospholipases A2, *sn*-2 Lyso-PL is obtained; if phospholipases A1 or *sn*-1,3 regioselective lipases are used, the final product is the *sn*-1 Lyso-PL (Guo et al., 2005; Kim and Akoh, 2015). The Lyso-PLs can also be synthesized by alcoholysis, esterification of glycerophosphorylcholine and thermodynamic transacylation of 2-acyllysophospholipids to 1-acyllysophospholipids (Guo et al., 2005).

Enzyme-catalyzed PLs modification to incorporate SFA, medium-chain fatty acids and/or PUFA to improve the absorption of these FAs by the humans has been carried out (Vikbjerg et al., 2007; Kim and Yoon, 2014; Zhao et al. 2014). These modifications have been currently performed, either in organic or in solvent-free media, by (i) acidolysis with FFA or (ii) interesterification between PLs and fatty acid esters (Table 5).

### Production of SLs Modified in the Original FA Position: Characterization, Health Benefits and Production

The production of TAGs modified in the original position of the fatty acids is usually performed by chemical-catalyzed interesterification, to change and improve certain physical properties of fat blends, namely the melting point, solid fat content and crystallization pattern, to obtain adequate fats for the margarine industry. In these SLs, the original fatty acid composition is maintained, and no *trans*-fatty acids are produced, conversely to what happens when the physical properties of fat blends are modified by hydrogenation (Erickson, 1995). The consumption of *trans* fatty acids has been associated with increased risk of coronary heart disease, due to increased low-density lipoprotein (LDL) cholesterol, and reduction in high density lipoprotein (HDL) cholesterol. Recently, the U.S. Food and Drug Administration (FDA) has banned the use of partially hydrogenated oils in processed foods, so the demand for *trans*-free solutions is increasing (<https://www.fda.gov/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/ucm449162.htm>).

The enzyme-catalyzed production of *trans* acid-free alternatives for margarines and shortenings, together with the incorporation of specific long-chain fatty acids (e.g. omega-3 PUFA) in these products, is an increasing market trend. The use of regioselective lipases as catalysts for the interesterification has the advantages previously presented. If a non-regioselective lipase is used, the final TAG profile is comparable to the product obtained with chemical catalysts; when a *sn*-1,3 regioselective lipase is used, the TAG profile is completely different because the original FA at *sn*-2 position is maintained, which is nutritionally desirable (Pires et al., 2008).

The margarine industry currently uses blends of high-saturated fats (e.g., palm stearin, palm kernel, palm or coconut fats) with polyunsaturated vegetable oils (e.g., canola, sunflower and soybean oils) to obtain plastic fats with intermediate physicochemical characteristics.

Several studies have been carried out to produce low-*trans* or *trans*-free plastic fats by interesterification, either in the presence of organic solvents or in solvent-free media, using commercial immobilized lipases (e.g. Novozym 435, Lipozyme RM IM, Lipozyme TL IM) or non-commercial lipases (e.g. *Candida parapsilosis* lipase/acyltransferase) (Table 6).

**Table 5** Examples of enzymatic synthesis of structurally modified phospholipids in batch bioreactors

Product type	Reaction	Substrates	Biocatalyst	System type	Reference
SPC	Acidolysis	phosphatidylcholine and free medium -chain FAs	Immobilized PLip A1	Solvent-free	Ochoa-Flores et al. (2017)
DHA-rich PC	Acidolysis	PLs and DHA-enriched FAs extracted from <i>Schizochytrium</i> sp	PLip A1	isooctane	Chen et al. (2017)
DHA/EPA-rich PC	Interesterification	PC and DHA/EPA rich ethyl esters	Immobilized PLip A1	Solvent-free	Li et al. (2016)
DHA/EPA-rich PC	Interesterification	Soy phosphatidylcholine + DHA ethyl ester and EPA ethyl ester	Immobilized PLip A1	Solvent-free	Li et al. (2014)
omega-3 FA-rich PC	Acidolysis	Soy phosphatidylcholine + fish oil FAs	Immobilized PLip A1	Solvent-free	Zhao et al. (2014)
MCFA-rich PC	Acidolysis	Phosphatidylcholine + C8:0	PLip A2 (free); <i>Mucor javanicus</i> lipase (free)	Solvent-free	Kim and Yoon (2014)
Lyso-PC	Hydrolysis	Sunflower lecithin	PLip A2	Solvent-free	Cabezas et al. (2011)

**Table 6** Examples of lipase-catalyzed synthesis of TAGs modified in the original position of the fatty acids

Product type	Reaction	Substrates	Biocatalyst	System type	Operation mode	Reference
<i>trans</i> -free SL for shortening	Interesterification	Coix seed oil, fully hydrogenated palm oil and <i>Cinnamomum camphora</i> seed oil	Lipozyme RM IM	Solvent free	Batch	Xu et al. (2018)
<i>trans</i> -free SL for confectionery and bakery products	Interesterification	high oleic sunflower oil + fully hydrogenated soybean oil	Lipozyme TL IM	Solvent free	Batch	Morselli-Ribeiro et al. (2017)
<i>trans</i> -free SL for margarine fats	Interesterification	camellia seed oil, palm stearin and coconut oil	Lipozyme TL IM	Solvent free	Batch	Ruan et al. (2014)
<i>trans</i> -free SL rich in omega 3 PUFA for table margarines	Interesterification (reaction under high pressure)	Palm stearin + Palm kernel oil + "EPAX 4510 TG"	Lipozyme TL IM	Solvent -free	Batch	Osório et al. (2008)
<i>trans</i> -free SL for margarines and shortenings	Interesterification	Palm stearin + Palm kernel oil + sunflower oil	Lipozyme TL IM	Solvent -free	Continuous PBR	Osório et al. (2006)
<i>trans</i> -free SL for margarines and shortenings	Interesterification	Palm stearin + soybean	Novozym 435	Solvent -free	Continuous FBR	Osório et al. (2005)

Commercial examples of SLs, obtained via enzymatic interesterification, include ADM's NovaLipid™ products (naturally stable oils and fats, trait-enhanced oils and fats, margarines and shortenings, and custom blends) (<https://www.adm.com/products-services/food/oils/novalipid>), and Crokvitol™ line of products (Crokvitol™ Stand, Crokvitol™ Allround, Crokvitol™ Vitality) from Lodders Croklaan. They were designed to provide an alternative to hydrogenated palm oil, low-*trans* or *trans*-free plastic fats for industrial margarine for bakery applications, standard table spreads and margarines and for healthy table spreads (<http://europe.ioiloders/products/>).

## Monoacylglycerols and Diacylglycerols

Traditionally, monoacylglycerols (MAGs) and diacylglycerols (DAGs) have been used as important emulsifiers in the food and pharmaceutical industries. However, MAGs and DAGs can also be considered as SLs with recognized health benefits (Taguchi et al., 2000). Partial acylglycerols have a positive effect in the reduction of obesity and of high TAG plasma levels. Also, *sn*-2-MAGs have beneficial effects for human health because they are most readily absorbed through the intestinal mucosa and are used directly for the resynthesis of new TAGs (Michalski et al., 2013). Furthermore, *sn*-2-MAGs are involved in the synthesis and degradation of endocannabinoids, which can regulate appetite, pain sensation, inflammation and lipid metabolism (Blankman et al., 2007; Panikashvili et al., 2006). In a study performed by Eom et al. (2010), the anti-obesity effect of DAGs was tested in

mice. A diet rich in the synthesized DAGs (20% w/w) reduced both body weight gain and plasma biochemical marks of obesity (total cholesterol, triacylglycerol and glucose levels) in these animals, when compared to a diet rich in triacylglycerols (TAGs) (20% w/w).

The industrial production of MAGs and DAGs has been usually carried out by chemical catalysis which requires the use of expensive and toxic solvents. As green alternative, MAGs and DAGs can be produced by lipase-catalyzed glycerolysis of a TAG, fat or oil in non-aqueous media (Ferreira-Dias et al., 2003; Liao et al., 2003). The use of non-selective or of a *sn*-1,3 regioselective lipase as biocatalysts will determine the yield and type of MAGs or DAGs obtained. With a *sn*-1,3 regioselective lipase, *sn*-2-monoacylglycerols (*sn*-2-MAG) or *sn*-1,3-DAGs are obtained; using a non-regioselective lipase, all types of MAG and DAG can be obtained. Partial acylglycerols can also be obtained by lipase-catalyzed esterification of glycerol with FFAs or by incomplete hydrolysis of TAGs.

With respect to DAG production, in late 1990s, specialty commercial SLs were developed with hypotriglyceridemic and anti-obesity effects. Commercial DAG oils such as Japan Enova oil (sold as Healthy Econa™ Cooking Oil) consists of 80% DAGs, being 70% of these DAGs of the *sn*-1,3 form (<https://pgio.info/project/enova-oil/>). In the human body, *sn*-1,3-DAGs are less efficiently utilized in the resynthesis of TAGs for chylomicron formation and secretion compared with typical TAGs. For the preparation of *sn*-1,3-DAGs, recent studies have used glycerolysis of plant oils as well as direct esterification of glycerol with free FAs catalyzed by commercial immobilized lipases (Dhara and Singhal, 2014; Wang et al., 2014; Mangas-Sánchez et al., 2015).

## Final Remarks

Structured lipids are important novel lipids with recognized technological, functional and health benefits. Some of these novel lipids can be synthesized either by chemical or enzyme-catalyzed reactions. However, some of them can only be obtained by enzyme catalysis, which is a green technology and presents great advantages with respect to the conventional inorganic routes. Also, the SLs obtained by reactions catalyzed by *sn*-1,3 regioselective lipases present superior bioactivity than those obtained by inorganic-catalyzed reactions. However, high prices of commercial immobilized lipases have been a constraint to the industrial implementation of some of these processes, such as the production of low-value commodity fats for the Food Industry. The enzyme preparations used must have both high catalytic activity and operational stability to be reused in batch or used in continuous bioreactors to make the process competitive. In addition, the implementation of low-cost sustainable enzymatic processes for SLs synthesis must address the following main issues: the use of solvent-free reaction media, the search for novel low-cost enzymes and the use of natural cheap oils and fats and/or oils extracted from agro-industry by-products, as raw materials to minimize costs.

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## Relevant Websites

<http://europe.ioiloders.com/products/coberine/> – Coberine® Cocoa Butter Equivalents.

[https://www.stepan.com/uploadedFiles/Literature\\_and\\_Downloads/General\\_Lit/Product\\_Line\\_Information/MarinolBrochure.pdf](https://www.stepan.com/uploadedFiles/Literature_and_Downloads/General_Lit/Product_Line_Information/MarinolBrochure.pdf) – Marinol®.

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<https://pgio.info/project/enova-oil/> – Enova Oil.

## Food for Brain Health: Flavonoids

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### Introduction

The brain is a metabolically active and peculiar organ that coordinates every activity of an organism. The brain is always in use, even in the state of sleep. As a result, brain health is important to the overall well-being of the entire organism. It is now an established scientific fact that nutrition plays a significant role in brain health. Diet has both structural and functional effects on the brain with diet quality and adequacy being directly linked to cognitive processes and emotions (Gómez-Pinilla, 2008; Sarris et al., 2015; Jacka et al., 2015; Zamroziewicz et al., 2016).

Available statistics paint a disturbing picture of the problem of neurologic diseases worldwide. The heavy impact of neurologic disorders and cerebrovascular disease is particularly evident in underdeveloped and developing countries (Chin and Vora, 2014; Silberberg et al., 2015). An estimated 795,000 strokes occur annually in the USA alone, which is the equivalent of one new stroke every 40 seconds. Although stroke mortality and morbidity have been decreasing over the past decade in the USA, stroke remains the second leading cause of death worldwide, with over two-thirds of stroke-related deaths occurring in the developing world (Wallin et al., 2015). The incidence of other neurologic diseases like epilepsy, dementia, Parkinsonism, Alzheimer's disease, brain neoplasms and multiple sclerosis is also a cause of worry as their global burden is on the increase and effective and reliable therapeutic remedies are presently unavailable (Wallin et al., 2015; Gaskin et al., 2016).

With increasing adoption of habits that promote poor mental health by many people and the predicted increase in global burden of neurologic diseases worldwide, it is imperative to unravel and adopt new approaches that can effectively address the menace of brain and nervous system disorders in addition to pharmacological based approaches. Lifestyle changes and nutritional medicine are gaining increasing prominence as veritable channels to achieving this goal (Sarris et al., 2015; Carroll, 2017).

Flavonoids are polyphenolic compounds which are widely distributed in the plant kingdom but particularly abundant in fruits, vegetables and teas, and having a broad spectrum of pharmacological properties such as cardiovascular, cerebrovascular and anti-cancer activities (Hoensch and Oertel, 2015; Varzakas et al., 2016; Watson and Preedy, 2010). Flavonoids are increasingly finding applications, not only in prophylaxis and therapy of diseases, but also as nutraceuticals and functional foods for improved health and well-being (Varzakas et al., 2016; Nasri et al., 2014). Physicochemical and pharmacodynamic properties of flavonoids meet demands imposed by the metabolic peculiarities of the brain enabling them to exert salubrious effects on brain and nervous system function through actions such as protection of neurons against injury induced by neurotoxins, suppression of neuroinflammation and excitotoxicity, and the potential to promote memory, learning and cognitive functions (Spencer, 2009; Muralidhara et al., 2015; Diniz et al., 2015).

### Metabolic Peculiarities and Functions of the Brain

The brain is a complex, heterogeneous organ in which many pathways for its input and output are organized in a somatotopic manner. Brain energy metabolism is dynamic, highly regulated and largely governed by moment-to-moment interactions among brain cells that process information from sensory and cognitive activities and to direct functions of the body. Cellular energy demands, consumption of oxygen and glucose, and blood flow are often described as "coupled" because these processes rise and fall together (Dienel, 2014).

### Brain Energy Metabolism

The energy requirement of the brain is high. Although the brain represents only 2% of the total body mass, about 20% of the oxygen and 25% of the glucose consumed by the human body are dedicated to cerebral functions. Maintenance and restoration of ion gradients dissipated by signaling processes such as postsynaptic and action potentials, as well as uptake and recycling of neurotransmitters, are the main processes contributing to the high brain energy needs (Bélanger et al., 2011; Alle et al., 2009). The overall energy use by human brain is estimated to be 21  $\mu\text{mol ATP/g/min}$ . Even during sleep there is only a relatively small decrease in cerebral metabolic rate which may be increased in rapid eye movement (REM) sleep (Clarke and Sokoloff, 1999).

Glucose and oxygen are the major, obligatory brain fuels and must be continuously supplied by blood to maintain brain functions. Glucose is transported into brain cells by specialized carriers and metabolized in the glycolytic, pentose-phosphate shunt, and TCA cycle pathways to generate ATP, which is primarily used for signaling processes. Oxygen is utilized in the brain almost entirely for the oxidation of carbohydrates (Dienel, 2014; McKenna et al., 2012). Glucose is catabolized by interrelated pathways to generate energy and synthesize neurotransmitters, redox compounds to manage oxidative stress, and essential compounds that cannot cross

the blood–brain barrier. Oxidative metabolism generates most of the ATP, but glycolysis is usually disproportionately up-regulated during activation, with release of lactate (Dienel, 2014).

### Lipid Composition of the Brain

The brain is the second most lipid-rich organ in the body after the adipose tissue (Betsholtz, 2015; Moutinho et al., 2016). Lipids represent up to 50% of its dry weight (Woods and Jackson, 2006; Fantini and Yahi, 2015) and up to 80% of the dry weight of myelin. The lipid composition of the whole brain of adult rats expressed in mmol/g of wet weight was estimated to be 89, 69 and 31 for glycerophospholipids, cholesterol, and sphingolipids, respectively (Fantini and Yahi, 2015; Schnaar et al., 2014). Brain lipids are not used for energy storage but as essential building blocks of cell membranes, which are extremely abundant in the brain, particularly in the vastly arborized neurons and astrocytes and in the myelin sheaths wrapped around axons by oligodendrocytes. How the brain builds and maintains this high lipid content has puzzled researchers because any transport of lipids from the blood to the brain has to pass the blood–brain barrier (BBB) which efficiently blocks the passage of lipoproteins and albumin, the main lipid carriers in the blood. However, emerging discoveries are pointing to the importance of transport proteins in lipid build-up in the brain (Betsholtz, 2015).

### The Blood Brain Barrier

The blood–brain barrier (BBB) is a diffusion barrier, which acts as a strict control point for the influx of compounds into the brain. It is a complex, dynamic system that involves biomechanical and biochemical signaling between the vascular system and the brain. Three cellular elements of the brain microvasculature compose the BBB–endothelial cells, astrocyte end-feet, and pericytes (PCs) (Ballabh et al., 2004; Hawkins et al., 2006; Obermeier et al., 2013; Wong et al., 2013). Every constituent cell type makes an indispensable contribution to the BBB's integrity and BBB disruption can cause or contribute to neurological disease (Obermeier et al., 2013). Although the BBB prevents harmful chemicals and bacteria from reaching the brain, it also blocks roughly 95% of medicine delivered orally or intravenously. As a result, doctors who treat patients with neurodegenerative diseases, such as Parkinson's, often have to inject drugs directly into the brain, an invasive approach that requires drilling into the skull. Minor successes are being recorded in getting intravenous drugs across the BBB with the help of ultrasound, in the form of nanoparticles, or other delivery systems but those methods can target only small areas and there are often other limitations such as toxicity (Brouillette, 2016; Yonehara and Roska, 2017; beeline).

### Brain Redox Homeostasis

The brain is more vulnerable to oxidative stress than any other organ. The brain requires higher amount of oxygen to meet metabolic demands compared to other organs. However, it possesses lower antioxidant capacity. The brain also contains a high content of polyunsaturated fatty acids (PUFAs). These factors, make it is very susceptible to free radical-mediated oxidative stress that affects brain health negatively (Kiple and Ornelas, 2000; Edem, 2002). Oxidative stress (OS), caused by the imbalance between the generation and detoxification of reactive oxygen and nitrogen species (ROS/RNS), plays an important role in brain aging, neurodegenerative diseases, and other related adverse conditions, such as ischemia. While ROS/RNS serve as signaling molecules at physiological levels, an excessive amount of these molecules leads to oxidative modification and, therefore, dysfunction of proteins, nucleic acids, and lipids (Salazar et al., 2009) which contribute to diverse pathologies.

The structural and functional complexity of the brain, its high lipid content, cerebral blood flow and oxygen utilization, coupled with the limitation imposed by the BBB makes it especially susceptible to oxidative stress and various dysfunctions which require that potential efficacious prophylactic and therapeutic agents possess suitable pharmacokinetic and pharmacodynamic characteristics. Flavonoids appear to satisfy many of these requirements.

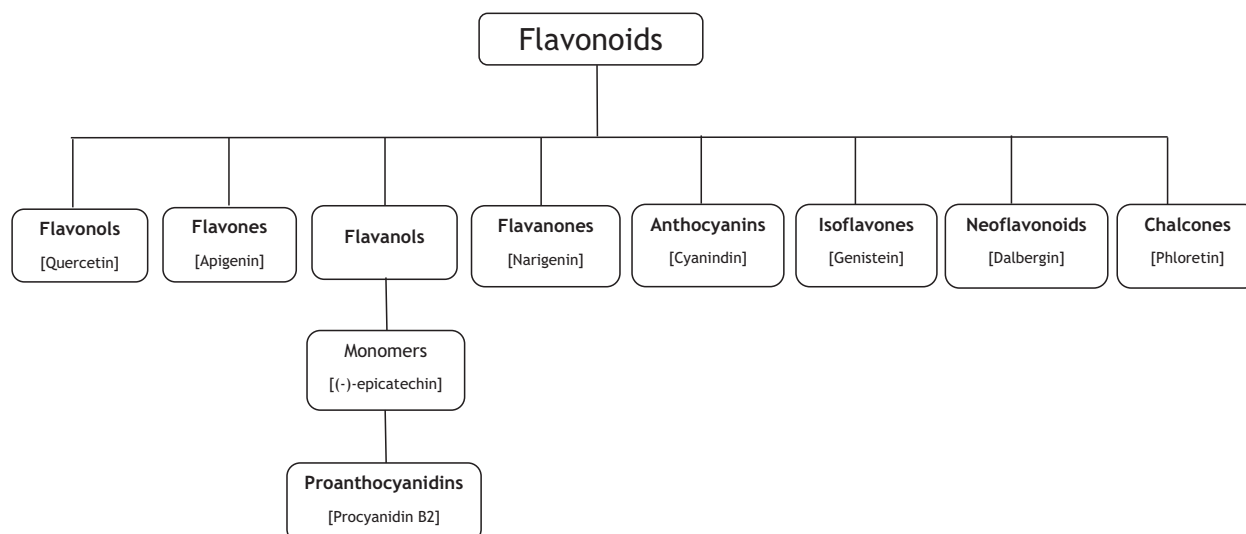
## Flavonoids

### Occurrence, Distribution and Classification

Flavonoids are a diverse group of bioactive phytochemicals with over 10,000 identified individual compounds. However, only very few of them have been investigated in detail (Pollastri and Tattini, 2011; Kozłowska and Szostak-Wegierek, 2014). They are a large group of polyphenolic compounds in plants and constitute one of the largest groups of plant secondary metabolites. Flavonoids are the most common and widely distributed group of plant phenolic compounds, occurring virtually in all plant parts, particularly the photosynthesizing plant cells. They are a major coloring component of flowering plants (Kumar and Pandey, 2013).

Flavonoids are synthesized through the shikimate/phenylpropanoid and acetate/malonate pathways and possess a characteristic C6–C3–C6 skeleton in which two aromatic rings are linked by a three-carbon bridge giving rise to a phenylchromane arrangement (Santos-Buelga and Feliciano, 2017).

Based on the degree of unsaturation, substitution pattern, hydroxylation pattern and variations in the chromane ring (Ring C), different flavonoid classes are distinguished: flavones, flavonols, flavanones, flavan-3-ols, anthocyanins, dihydroflavonols, and iso-flavones, as well as neoflavonoids and the biogenetic intermediate chalconoid forms (Fig. 1). While the vast majority of the



**Figure 1** Flavonoid classes and examples.

flavonoids have their Ring B attached to the C2 position of Ring C, some flavonoids such as isoflavones and neoflavonoids, whose Ring B is connected at the C3 and C4 position of Ring C, respectively, are also found in plants. Chalcones (flavan-opened chain compounds) though lacking the heterocyclic Ring C, or 5-membered C-ring derivatives aurones and auronols, and coumarins are still categorized as members of the flavonoid family (Catarino et al., 2016; Rodriguez-Mateos et al., 2014). In natural sources, flavonoids may occur in free forms (aglycones), as glycosylated or acylated derivatives, and as oligomeric and polymeric structures such as the flavan-3-ol-derived condensed tannins (or proanthocyanidins) (Santos-Buelga and Feliciano, 2017; Tsao, 2010).

Isoflavones are mostly found in the leguminous family of plants. Since beans, particularly soybean, are a major part of the diet in many cultures, isoflavones have a potentially great impact on human health. Genistein and daidzein are the two main isoflavones found in soy along with glycitein, biochanin A and formononetin (Mazur et al., 1998; Wang and Murphy, 1994). Neoflavonoids are not often found in food plants, but dalbergin is the most common and relatively widely distributed neoflavone in the plant kingdom (Garazd et al., 2003). The open-ring chalcones are found in fruits such as apples and hops or beers (Tsao et al., 2003; Zhao et al., 2005).

The position of the benzenoid substituent divides the flavonoid class into flavonoids (2-position), isoflavonoids (3-position) and neoflavonoids (4-position) (Catarino et al., 2016; Rodriguez-Mateos et al., 2014). Flavonols differ from flavanones by hydroxyl group at the 3-position and a C2–C3 double bond. Flavonoids are often hydroxylated in positions 3, 5, 7, 2, 3', 4', and 5'. Methyl ethers and acetyl esters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose, or arabinose (Kumar and Pandey, 2013). Biological activities of these compounds, including antioxidant activity, depend on both the structural difference and the glycosylation patterns (Tsao, 2010).

Flavanols can occur in different states such as monomers, oligomeric or polymeric forms, whereby the latter are referred to proanthocyanidins. Proanthocyanidins represent a unique class of secondary metabolites with high-molecular weight that despite their limited bioavailability, exhibit a wide range of sometimes astonishing antioxidant, vasorelaxant and other bioactivities (Xu et al., 2012; Ebrahimnejad et al., 2014). Generally, proanthocyanidins consist of successive monomeric units linked through carbon–carbon and ether linkages. Proanthocyanidins that comprise only (epi)catechin units, are called procyanidins, and are the most abundant type of proanthocyanidins in plants (Ebrahimnejad et al., 2014).

### Major Dietary Sources of Flavonoid Intake

Flavonoids are an integral part of human and animal diet and constitute the most abundant polyphenols in the human diet (Bawaked et al., 2017). The amounts of antioxidant flavonoids and polyphenols in plant-based foods of the human diet—in particular vegetables, fruits, tea, and wine—are generally much greater than the amounts of other antioxidants in these foods, such as vitamins C and E and carotenoids (Haytowitz et al., 2006; Lotito and Frei, 2006). Fruits and fruit juices are among the best sources of flavonoids in the human diet because of their high content in most fruits and the relatively large serving sizes. Vegetables are also very good sources of flavonoids.

Observational studies have repeatedly suggested a beneficial health effect of flavonoids intake and these class of polyphenolics may contribute more than any other, to the effect of plant-based diets on the risk of chronic diseases and mortality. Knowledge of population-based flavonoid intake pattern is important for the development of dietary recommendations. Additionally, the

identification of dietary patterns related to increased flavonoids consumption is relevant because of the potential impact of these bioactive compounds in disease prevention (Bawaked et al., 2017; Watzl, 2008).

Dietary flavonoid intake and sources vary according to cultural, regional or national diet patterns. Flavonoid sources and intake for different populations and population groups have been documented for some countries in the Americas, Europe, Oceania and Asia but no data currently exists for any African country. In the US, the major dietary sources of flavonoid intake consisted of tea, citrus fruit juices, berries, citrus fruit, wine, and apples (Kim et al., 2016). Among Spanish youth, the major dietary sources of total flavonoids intake were fruits (including fruit juices), vegetables, and chocolate products. Fruits were the major contributors. Vegetables, particularly spinach, onions, artichokes and lettuce as well as Cocoa powder and chocolate were also significant contributors to total flavonoid intake (Bawaked et al., 2017). The American and Spanish studies revealed that flavan-3-ols were the most abundant class of dietary flavonoids in children and adults (Kim et al., 2016). Tea was the major contributor to dietary flavonoid intake among Australians (Johannot and Somerset, 2006). Generally, fruits are the main dietary contributors to flavonoid intake in children, adolescents, and young adults (Bawaked et al., 2017).

In Mediterranean countries, fruits and wine are the principal sources for flavonoid intake in adults (Zamora-Ros et al., 2013). A study found that among Spanish adults, the richest sources of total flavonoid intake were apples (23.0%), red wine (21.0%), unspecified fruit (12.8%), oranges (9.3%), beans (4.9%), pears (4.0%), peaches (3.3%), chocolate (2.6%), and tea (2.2%) (Zamora-Ros et al., 2010). Specific foods that were major contributors (~60%) of flavonoid intake in the UK and Ireland include the following: grapes and oranges (41.6% UK, 34.9% Ireland); beer and wine (8.8% UK, 12.8% Ireland); apples and onions (6.8% UK, 6.5% Ireland); and tea (4.0% UK, 5.3% Ireland) (Beking and Vieira, 2011). In a study among Finnish adults, berries and berry products, including berry-based sweetened drinks, were the main anthocyanidin contributors. Bilberries (*Vaccinium myrtillus*) were the main food source for anthocyanidins. The most important contributors to the intake of flavanols, flavanones, and flavones were fruits (especially apple and citrus fruit) and tea. The main contributors to proanthocyanidins intake were apples, berries, tea, and chocolate (Ovaskainen et al., 2008). Tea was adjudged to be the main contributor to total flavonoid intake among middle aged and elderly Chinese men and women (Zhang et al., 2014). Fruits (35%), non-alcoholic beverages, Cocoa products and alcoholic beverages were estimated to be the main sources of flavonoids in a study on the dietary intake of 337 polyphenols in French adults (Pérez-Jiménez et al., 2011). Legumes were the most important source of dietary flavonoids in a study based on Brazilian National Dietary Survey (Corrêa et al., 2015). In another Brazilian study limited to adults and elderly adults of the state of Sao Paulo, major contributors of intakes of flavonoids were citrus fruits, mainly oranges (17.0%), and beans (14.3%) (Miranda et al., 2016). In a cross-sectional analysis aimed at estimating dietary intakes of all currently known individual polyphenols and total intake per class and subclass in the European Prospective Investigation into Cancer and Nutrition (EPIC) study, the main food sources of flavonoids were fruits in Mediterranean countries (45%), and tea in both non-MED countries (34%) and the UK health-conscious group (64%) (Zamora-Ros et al., 2016).

### Flavonoid-Rich Foods

Knowledge of flavonoid-rich foods is important because such may be unwittingly neglected even where available, in favor of popular ones that may not be as rich in flavonoids. Dietary sources of flavonoid intake in a population may not correlate with consumption of flavonoid-rich foods due to climatic, geographical and cultural factors and this could contribute to reduce scores for total flavonoid intake in such populations. Many commonly consumed foods are excellent sources of flavonoids. Apples, peas, grapes, berries, spinach, broccoli, onions, hot peppers, cherry tomatoes, sweet potato, black tea, coffee, red wine, dark chocolate and cocoa powder are rich sources of the flavanols, quercetin, myricetin and kaempferol. Dark chocolate, red wine, black tea, berries, grapes, pears and apples are additionally rich in epicatechin, catechin and epigallocatechin gallate. The anthocyanidins, cyanidin, malvidin, delphinidin and pelargonidin are relatively abundant in grapes, berries and red wine while the flavanones, naringenin, hesperetin and eriodictyol abound in citrus fruits (Kozłowska and Szostak-Wegierek, 2014; Lotito and Frei, 2006; Maccready et al., 2014; Bertoia et al., 2016; Hooper et al., 2008).

### Flavonoid Biokinetics and the Blood Brain Barrier

The bioavailability of flavonoids is low and this presents a limitation to their applicability as potent bioactive molecules. The limited bioavailability and consequently low plasma concentrations raise questions about the mechanisms by which they attain effective intracellular concentrations in the target tissues. For the brain, an additional impediment is presented by the BBB.

Variation in pathways affecting absorption, distribution, metabolism, and excretion (ADME) of flavonoids possibly influences exposure at the circulatory system, thus modifying disease risk in individuals. These factors include nuclear receptors (NRs), gene transcriptional regulation of drug-metabolizing enzymes (DMEs), and efflux transporters (ETs) (Liu et al., 2017).

All flavonoids are subject to phase I and II metabolism in the small intestine and liver during their absorption. Studies have detected several flavonoids, their aglycone forms and their metabolites such as O-methylated, sulphated, and glucuronidated conjugates in plasma of humans after ingestion of flavonoid-rich foods (Faria et al., 2012). The appearance of flavonoids in plasma provides evidence that these compounds are bioavailable to tissues in the periphery but cannot be used as evidence that they are also present in the brain and CNS (Faria et al., 2012).

The mechanisms of absorption of flavonoids have not been completely elucidated but some highlights could be deduced. Specific trans membrane transporter proteins and diffusion through the lipid portion of the membrane are involved. The

transporters are important in mediating the protective activity of flavonoids (Zibera et al., 2014). An important role has also been proposed for the mucus layer of the small intestine which modified previously held views. According to Gonzalez (Gonzales et al., 2016), hydrophobic flavonoid aglycones that reach the small intestines are unable to penetrate through the mucus layer and are thus pushed to the large intestines via peristalsis to be metabolized (i.e. into smaller phenolic acid derivatives) by intestinal bacteria. However, in the presence of dietary fat and bile, micelles form and serve as carriers of aglycones through the mucus layer. They are then released upon contact with the brush border where passive diffusion is likely to occur through the cells. Soluble flavonoid glycosides on the other hand, are able to penetrate through the mucus layer to reach the epithelium. Upon contact with the brush border,  $\beta$ -glucosidases such as the lactasephlorizin hydrolase (LPH) cleave-off the glucose moiety to release the aglycone, which could then passively diffuse through the cells. Since intestinal cells are unable to produce rhamnosidases, flavonoids that contain rhamnose moieties, such as rutin (quercetin-3-rutinoside) and hesperidin (hesperetin-7-rutinoside), remain intact in the small intestine and are thus pushed to the large intestine, where fermentation by intestinal bacteria occurs or the action of secreted bacterial rhamnosidases releases the aglycone. In the end, the aglycones that were released on the brush borders, or smaller phenolic acid derivatives that were produced after bacterial metabolism both in the small and large intestines, reach the epithelia, where phase I metabolism and phase II metabolism take place. It must be noted however, that while bacteria also exist in the small intestines, the rate of mucosal diffusion and intestinal uptake at that stage occurs rapidly and thus the effect of duodenal bacteria is not as pronounced as in the colon, where the absorption is slower and longer (Gonzales et al., 2016).

With respect to the brain, mounting evidence from several lines or investigations confirm that flavonoids and some of their metabolites are able to traverse the BBB (Faria et al., 2012, 2014; Ishisaka et al., 2011; Jäger and Saaby, 2011; Pogačnik et al., 2016). The major issue regarding the bioavailability of flavonoids to the brain concerns the mechanisms by which flavonoids (conjugated or unconjugated) are transported across the BBB. Lipophilicity-dependent and transporter-mediated uptake are involved but the precise nature and extent of uptake of flavonoids by the CNS are still poorly defined (Faria et al., 2012, 2014).

Several techniques have been employed to increase bioavailability of flavonoids by greatly increasing the aqueous solubility of the flavonoid aglycone. These include incorporation of flavonoids to borneol/methanol eutectic mixtures, micro-emulsions, polyvinylpyrrolidone dispersion, lecithin complexation, cyclodextrin complexation (Thilakarathna and Rupasinghe, 2013) and methylation (Koirala et al., 2016; Wen et al., 2017). Prenylation, while not specifically improving bioavailability, aids flavonoid tissue bioaccumulation by modulating cell uptake/efflux (Terao and Mukai, 2014). The formulation of flavonoid aglycones into nanocrystals has also been reported as an effective way of improving bioavailability (Li et al., 2013). Dietary factors have also been shown to greatly improve the bioavailability of flavonoid aglycones. For instance, it has been shown that dietary fat increases the bioavailability of quercetin aglycones (Azuma et al., 2002; Guo et al., 2013).

In addition, studies have shown that other dietary components may affect the access of flavonoids into the CNS. In a study by Ferri et al.,  $\alpha$ -tocopherol was reported to promote quercetin transport across the BBB (Ferri et al., 2015).

### Extraction Techniques for Flavonoids

Although there are general extraction methods for flavonoids, it is becoming increasingly realized that there is no extraction protocol that suits all types of flavonoids and matrices, and therefore sample preparation and extraction conditions usually need to be optimized depending on the nature of the sample, target analyses, and aim of the study (Santos-Buelga and Feliciano, 2017).

Extensive documentation of conventional and innovative procedures for the extraction of bioactive compounds in general (Houghton and Raman, 2012; Ong, 2004; Sarker et al., 2005; Wang and Weller, 2006; Colegate and Molyneux, 2007; Azmir et al., 2013; Rostagno and Prado, 2013; Mandal et al., 2015; Prado et al., 2015; Brglez Mojzer et al., 2016; Wu et al., 2017) and flavonoids in particular abounds in literature. Only an overview of the extraction techniques for flavonoids is intended in this section. Methods have also been developed for specific subclasses of flavonoids or individual flavonoids some of which are mentioned later on in this chapter. Preparation of samples for extraction may vary depending on the flavonoid-containing matrices involved, i.e. whether samples are solid, liquid, very hard, etc.

Traditionally, extraction of flavonoids from natural products has been carried out through simple direct solvent extraction and this is still the most widely used method. In these solvent-based methods, solvent type is a critical variable that influences the nature and composition of the extracted flavonoids. Due to their possible enzymatic degradation in fresh samples, the use of dry, lyophilized or frozen samples is advisable. To improve extraction efficiency, solid samples are usually pulverized. The choice of solvent is predicated on the type of flavonoid required with less polar flavonoids being extracted with chloroform, dichloromethane, diethyl ether, or ethyl acetate, while flavonoid glycosides and more polar aglycones are extracted with alcohols or alcohol–water mixtures (Marston and Hostettmann, 2005). Acidic, basic or enzymatic hydrolyses are frequently used to remove the sugar moieties from glycosides. The hydrolysis process could be to minimize degradation reactions of glycosides during prolonged extraction and to achieve complete release of aglycones (Stalikas et al., 2010).

Powdered plant material can also be extracted in a Soxhlet apparatus, first with highly non polar solvents, hexane for example, to remove lipids and then with ethyl acetate or ethanol to obtain phenolics. This approach is not suitable for heat-sensitive compounds. A convenient and frequently used procedure is sequential solvent extraction. A first step, with dichloromethane, for example, will extract flavonoid aglycones and less polar material. A subsequent step with an alcohol will extract flavonoid glycosides and polar constituents (Marston and Hostettmann, 2005). Other techniques \*revolve around obtaining a crude extract from samples using aqueous alcohol mixtures (Stobiecki and Kachlicki, 2006) and then fractioning the crude preparation into a series



of solvents which would include one with appropriate polarity to harvest the flavonoids or, the flavonoid fraction could be obtained from the crude extract preparation through procedures including hydrolysis and precipitation. A lot of procedures have been developed to obtain flavonoids and other polyphenols from specific sources (Choudhary et al., 2011; Furer et al., 2015; Kandikattu et al., 2015; Falode et al., n.d; Ajayi et al., 2017; Zhang et al., 2017).

Relatively modern techniques which improve extraction efficiency and yield over the traditional maceration and Soxhlet methods have gained increased prominence in the last couple of decades. These include Pressurized Liquid Extraction, Microwave Assisted Extraction (MAE), Ultrasound Assisted Extraction and Supercritical Fluid Extraction. Additional advantages of these new methods include lower solvent consumption, reduced quantity of starting material, milder conditions of extraction and reduced extraction time.

Other innovative extraction techniques used to extract dietary flavonoids include Pulsed-electric field extraction (PEF) and enzyme assisted extraction (EAE) (Azmir et al., 2013). These methods are often optimized and adapted for targeted extraction of flavonoids from specific sources. In addition, novel extraction methods are commonly developed and have multiplied in recent times.

Following extraction, quantitation, fractionation, purification, separation and detection of the flavonoids are often carried through chromatographic and spectroscopic analyses. In contemporary times, several hyphenated techniques including HPLC-DAD, HPLC/MS/MS, GC-MS, LC-MS, LC-MS/ESI and LC-NMR-MS are used to combine separation and resolution/identification of individual flavonoids from extracts.

## Flavonoids and Brain Health

A basic and prominent characteristic of flavonoids is the possession of strong antioxidant ability shown either directly by scavenging free radicals or, indirectly, by augmenting cellular antioxidant defenses (Ferri et al., 2015). In the face of recurrent failures in the development of clinically effective drugs against neurologic dysfunctions, experimental, clinical and epidemiologic evidences substantiate the cognitive enhancing, neurobehavioral modulating and neuroprotective efficacy of dietary flavonoids. Consumption of flavonoid-rich foods has been linked to reduction in the incidences of neurodegenerative diseases and other neurologic pathologies. It has also been amply shown that even when used as therapeutic interventions, dietary flavonoids still confer neuroprotection against neurologic dysfunctions. These observations support the concept of flavonoids as food for the brain. Flavonoids exert a multiplicity of neuroprotective actions within the brain, including a potential to protect neurons against injury induced by neurotoxins, an ability to suppress neuroinflammation, and the potential to promote memory (Vauzour et al., 2013).

Reports of neuroprotective property of single dietary flavonoid compounds as well as flavonoid-rich/total flavonoid fractions of food and medicinal plants in *in vitro*, *in vivo* and other models of neurologic diseases abound in scientific literature (Vauzour et al., 2008; Hwang et al., 2012; Zeng et al., 2013; Nakayama et al., 2011; Hosseinzadeh et al., 2017; Ren et al., 2016; Choi et al., 2013; Keddy et al., 2012; Dok-Go et al., 2003; Dajas et al., 2003; Jobaliya and Benzeroual, 2011; Farooqui, 2017; Zhu et al., 2007; Nabavi et al., 2016; Sun et al., 2014; Seo et al., 2014; Pu et al., 2007; Spencer and Crozier, 2012; Bei et al., 2009; Solanki et al., 2016; Wang et al., 2017). Variations between pharmacological effects of isolated flavonoids or actions of the same molecules in complex mixtures which may be due to metabolism, bioavailability or final brain concentrations have been observed (Dajas, 2012; Dajas et al., 2015; Zhou et al., 2008). This appears consistent with the notion that the consumption of flavonoid-rich foods throughout life holds the potential to limit neurodegeneration, decrease neuroinflammation, and prevent or reverse age-dependent losses in cognitive performance (Vauzour et al., 2013). It also underpins the fact that while discovering therapeutically efficacious agents is the major goal of neuroprotective drug development, prophylactic approaches based on dietary lifestyle modification should also be emphasized (Akinmoladun et al., 2015a).

In the following paragraphs, some reports revealing the neuroprotective action of individual flavonoids and flavonoid mixtures/extracts are highlighted.

## Neuropharmacology of Some Individual Flavonoid Compounds

Quercetin, a ubiquitous flavonoid present in beverages, food and plants has been shown to have an important role in the prevention of neurodegenerative and cerebrovascular diseases. Quercetin demonstrated neuroprotection in models of cerebral ischemia, Parkinson's disease, Alzheimer's disease, epilepsy and Huntington's disease (Dajas et al., 2015; Chakraborty et al., 2014; Nassiri-Asl et al., 2013a; Sabogal-Guáqueta et al., 2015; Sriraksa et al., 2012; Akinmoladun et al., 2015b; Elumalai and Lakshmi, 2016). Specifically, treatment with 50 mg/kg i. p. quercetin for days showed protective effects in a 3-nitropropionic-induced experimental Huntington's disease in rats (Sriraksa et al., 2012). The same dose, also administered i. p. attenuated pentylentetrazole-induced seizure severity in kindled rats (Akinmoladun et al., 2015b) while 100, 200 and 300 mg/kg of the flavonoid administered orally for 14 days cognitive-enhancing effect in 6-hydroxydopamine-induced PD (Sriraksa et al., 2012), and 20 mg/kg administered i. p. 30 min before ischaemia afforded protection in global ischemia/reperfusion induced brain injury in rats (Nassiri-Asl et al., 2013a). Quercetin, and not caffeine was reported as a major neuroprotective component in coffee (Lee et al., 2016). In neuronal culture, quercetin increases survival against oxidative insults. Antioxidation appears to be a necessary but not sufficient condition for its neuroprotective action. Modulation of intracellular signaling and transcription factors, increasing the expression of antioxidant and pro-survival proteins and modulating inflammation, appear as important for neuronal protection. Quercetin also regulates

the activity of kinases, changing the phosphorylation state of target molecules, resulting in modulation of cellular function and gene expression. Co-treatment with orally administered quercetin at 10 and 20 mg/kg for 45 days ameliorated manganese-induced neurotoxicity through mechanisms associated with restoration of acetyl cholinesterase activity, augmentation of redox status and inhibition of lipid peroxidation in brain of rats (Adedara et al., 2017).

The flavonol, rutin is a vital nutritional component of food stuff that is abundantly found in sources such as passion flower, buckwheat, tea, and apple. It is a common dietary flavonoid that has been much studied by the scientific community. Hundreds of products containing rutin are currently marketed in the USA (Gullón et al., 2017; Ganeshpurkar and Saluja, 2017). Post-treatment of female Sprague-Dawley rats with rutin at 50 and 100 mg/kg, i.p., for three days significantly reduced the levels of reactive oxygen species, malondialdehyde, NLRP3, ASC, caspase-1, IL-1 $\beta$ , IL-18, and tumor necrosis factor- $\alpha$  and improved locomotor recovery in rats with spinal cord injury (Wu et al., 2016). Rutin promoted microglial proliferation and induced microglial polarization to the M2 profile when cells are stimulated with a lipopolysaccharide, which reveals its potential in the treatment or prevention of neurodegenerative disorders (Bispo da Silva et al., 2017). Rutin showed anti-epilepsy potential (Nieoczym et al., 2014; Nassiri-Asl et al., 2013b), protective effect in cerebral ischemia/reperfusion injury, and was able to promote significant recovery of sensor motor loss, which correlated with reduction of neurodegeneration in the periphery of cortical injury (Rodrigues et al., 2013). Post-treatment with rutin at 100 mg/kg, i. p. for 3 weeks also ameliorated the destructive effects of A $\beta$  on memory through increased extracellular signal-regulated protein kinase 1 (ERK1), cAMP response element-binding protein (CREB) and brain-derived neurotrophic factor (BDNF) gene expression in the hippocampus of rats (Moghbelinejad et al., 2014). Rutin (50 mg/kg i. p.) administered 30 min after injury, conferred protection in a rat model of subarachnoid hemorrhage possibly through inhibiting RAGE–NF- $\kappa$ B mediated inflammation signaling pathway (Hao et al., 2016). Supplementation of diets of rats with 0.75% rutin for 20 days was protective against toxicant-induced hippocampal injury (Koda et al., 2009). At 100 mg/kg and 200 mg/kg, rutin afforded protection against acrylamide-induced neurotoxicity (Motamedshariaty et al., 2014). Other neuropharmacological effects attributed to rutin include sedative, anticonvulsant, anti-Alzheimer, analgesic and antidepressant effects (Ganeshpurkar and Saluja, 2017).

Apigenin, found in several dietary plant foods such as parsley, celery, thyme, celeriac, chamomile, onions, lemon balm, and oranges demonstrated potent anti-inflammatory and antioxidant activities in various neurodegenerative diseases and epilepsy. Apigenin at 10 and 20 mg/kg, i.p., post administered to male SD rats was also found to ameliorate oxidative stress and neuronal apoptosis in early brain injury following subarachnoid hemorrhage, which is a serious condition with high rates of mortality and morbidity in the world. In addition, oral pre-treatment with apigenin at 100 mg/kg ameliorated traumatic brain injury in rats (Nabavi et al., 2015a; Kanazawa et al., 2006; van Gijn et al., 2007; Han et al., 2017). Apigenin was potent in increasing the life span, dopamine content, and reducing the oxidative stress as well as apoptosis in transgenic *Drosophila* model of PD (Siddique and Jyoti, 2017). Also, apigenin (10  $\mu$ M) improved neuron formation and strengthened the connections between brain cells, *in vitro*, indicating potential for the treatment of diseases like schizophrenia, depression, Alzheimer's and Parkinson's (Souza et al., 2015a).

The flavone, diosmin is generally utilized as an antioxidant in food supplements and by pharmaceutical companies. It is considered a vascular-protection cure for chronic venous insufficiency and can protect the brain by enhancing the activity of antioxidant enzymes and thus reduce the inflammation induced by cerebral ischemia. Pre-treatment with diosmin showed protective effects against traumatic brain injury-induced memory and long-term potentiation impairment possibly through a decrement in the hippocampal concentration of the pro-inflammatory cytokine, TNF- $\alpha$  (Benavente-García and Castillo, 2008; Silambarasan and Raja, 2012; Liu et al., 2014; Mirshekar et al., 2017). Oral pre-treatment of mice with 50 and 100 mg/kg diosmin for 6 days protected against cerebral ischemia (Liu et al., 2014).

Luteolin (3',4',5,7-tetrahydroxy flavone) is an important flavone, which occurs in several plant species including carrot, broccoli, pepper, thyme, and celery, olive oil, lettuce, chocolate, rooibos tea, pomegranate and cucumber (López-Lázaro, 2009). Luteolin, luteolin containing extracts and combinations of luteolin with other phytochemicals have been reported to have beneficial effects in models of epilepsy, autism spectrum disorders, Alzheimer's disease, Parkinson's disease, traumatic brain injury and multiple sclerosis (Kritas et al., 2013; Tambe et al., 2017; Choi et al., 2014; Nabavi et al., 2015b). A single dose i. p. pre-treatment with 10 and 20 mg/kg luteolin was in an acute model of epilepsy in mice (Liu et al., 2014). Mechanistic insights into the neuropharmacological activities of luteolin were provided in experiments, which showed that luteolin inhibited cytokine expression, nuclear factor kappa B (NF $\kappa$ B) signaling, and TLR4 signalling at micromolar concentrations in immune cells, including mast cells. Luteolin was also shown to attenuate microglial activation and mediate BDNF-like behaviour both *in-vitro* and *in-vivo*. Luteolin also reduced the mRNA expression of numerous genes up regulated in response to exogenous 6-OHDA, including BIM (a pro-apoptotic BH3-only member of the Bcl-2 family, required for initiation of apoptosis induced by endoplasmic reticulum [ER] stress), the p53 target genes, GADD45a and PUMA, and TRB3 (a proapoptotic gene that is upregulated in response to a variety of stresses, including ER stress, nutrient deprivation, and hypoxia). Luteolin can induce unfolded protein response, leading to decreases in phospho-eIF2 $\alpha$ , ATF4, GRP78 and CHOP (which are upregulated in response to ER stress). In addition, luteolin can also inhibit the Keap1-Nrf2-ARE pathway, leading to decreases in the expression of heme oxygenase-1 (HO-1) and glutamate cysteine ligase (GCL) (Nabavi et al., 2015b; Lee et al., 2009; Weng et al., 2015; Patil et al., 2014; Lin et al., 2010; Zhang et al., 2013a). Post-treatment with luteolin (4 mg/kg), injected through the tail vein, reduced infarct area and inhibited neuronal cell death in cerebral ischemia/reperfusion injury in rats (Zhang et al., 2013a).

Chrysin is a hydroxylated flavone derivative mainly found in honey, propolis and many plant species (Nabavi et al., 2015c). Chrysin treatment significantly inhibited the release of nitric oxide (NO) and proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) in lipopolysaccharide (LPS)-stimulated microglia. Additionally, expressions of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) were also significantly inhibited by chrysin. Also,

chrysin was shown to inhibit the activations of c-Jun N-terminal kinase (JNK) and nuclear factor- $\kappa$ B (NF- $\kappa$ B). These observations point to neuroprotective potential in neuropathologies like Alzheimer's and Parkinson's disease, and stroke (Nabavi et al., 2015c; Gresa-Arribas et al., 2010; Ha et al., 2010; Madeira et al., 2015; Yao et al., 2014). Pre-treatment of male mice with 75 mg/kg chrysin administered by oral gavage for 7 days protected against cerebral/ischemia reperfusion injury by ameliorating increase in glial cell numbers and proinflammatory cytokine secretion as well as inhibiting up-regulation of NF- $\kappa$ B, COX-2 and iNOS (Yao et al., 2014). Chrysin or chrysin-rich extracts have demonstrated antioxidant and neurotrophic effects, antidepressant effects, anti parkinsonism, anti-epileptic effect and protective effect in spinal cord injury. These effects are mediated through mechanisms that include modulation of brain-derived neurotrophic factor (BDNF) and nerve growth factor production, increased nuclear factor-erythroid 2-related factor 2 protein expression and transcriptional activity, inhibition of nuclear factor- $\kappa$ B activation and downregulation of inducible nitric oxide synthase expression, and reduced expression of bax, bcl-2 and caspase-3 (Souza et al., 2015b; Filho et al., 2015; Zhang et al., 2015; Singh et al., 2012; Kandhare et al., 2014). Specifically, pre-treatment with chrysin at 1 or 10 mg/kg (p.o.) for 60 days prevented age-related cognitive decline via attenuation of oxidative stress and modulation of BDNF levels in aged mouse brain (Souza et al., 2015b). At 20 mg/kg (p.o.) for 28 days, chrysin showed antidepressant effect in female mice subjected to chronic unpredictable mild stress by up-regulating BDNF and NGF levels and ameliorating oxidative stress (Filho et al., 2015) while at 20 and 40 mg/kg (p.o.) pre-treatment for 28 days, chrysin showed neuroprotection in experimental spinal cord injury in male rats through antioxidant and anti-apoptotic mechanisms (Kandhare et al., 2014).

Hesperidin and its aglycone, hesperetin are two citrus flavonoids with widely reported *in vivo* and *in vitro* neuropharmacological properties. Hesperidin and hesperetin have demonstrated neuroprotective potentials via antioxidant and other mechanisms. The antioxidant activity of Hsd was not limited to radical scavenging activity, but included augmentation of the antioxidant cellular defenses via the ERK/Nrf2 signaling pathway (Elavarasan et al., 2012). Hsd and Hst enhanced learning and memory through elevation of brain-derived neurotrophic factor (BDNF) and other mechanisms. Hesperidin (100 mg/kg, i. p.) administered for 10 days attenuated oxidative stress and neuronal damage caused by global ischemia (Oztanir et al., 2014). Hsd showed antidepressant activities via some mechanisms differing from those of conventional antidepressant drugs. At 0.1, 0.3 and 1 mg/kg (i.p.) for 21 days, hesperidin exerted antidepressant-like effects through decrease in hippocampal NOx and increased BDNF levels in adult male Swiss mice (Donato et al., 2014). Along with other flavanones, they protected PC-12 cells against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity. They also inhibited amyloid  $\beta$ -induced autophagy and improved glucose utilization in Neuro-2A cells, which suggests potential efficacy against Alzheimer's disease. The two flavonoids also protected neurons from excitotoxic damage in primary cultured rat cortical cells. Rotenone-disrupted mitochondrial membrane potential, increased reactive oxygen species generation, depleted glutathione, enhanced activities of enzymatic antioxidants, up-regulated Bax, cytochrome c, and caspases 3 and 9, and down-regulated Bcl-2 protein expression were attenuated by Hsd indicating its potential application in the treatment of Parkinson's disease. Hsd showed anti-seizure and cognitive-enhancing effects in pentylenetetrazole (PTZ)-induced neurotoxicity, and NO/cGMP pathway and BDNF modulation-dependent anti-depressant effect (Donato et al., 2014; Tamilselvam et al., 2013).

Kaempferol is a natural flavonol, found in fruits vegetables and herbs, including grapes, tomatoes, broccoli, tea, and *Ginkgo biloba* leaves (Shields, 2017). Equimolar kaempferol-3-O-rutinoside (10 mg/kg) and kaempferol 3-O-glucoside (7.5 mg/kg) administered through tail vein injection at the beginning of reperfusion prevented ischemic brain injury and neuroinflammation by inhibition of STAT3 and NF- $\kappa$ B activation. This showed that kaempferol has therapeutic potential in neuroinflammation-related diseases, such as ischemic stroke (Yu et al., 2013). The neuroprotective effect of 50 and 100 mg/kg (p.o.) kaempferol administered to male mice for 14 days in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse model of Parkinson's disease has been reported. Kaempferol improved motor coordination, raised striatal dopamine and its metabolite levels, increased SOD and GSH-PX activity, reduced the content of MDA and prevented the loss of TH-positive neurons induced by MPTP (Li and Pu, 2011).

Catechin is present in many dietary products, plants, fruits (such as apples, blueberries, gooseberries, grape seeds, kiwi, strawberries), green tea, red wine, beer, cacao liquor, chocolate, cocoa, etc (Zanwar et al., 2014). Catechins possess pleiotropic neuroprotective actions within the brain and have a potential to protect neurons against injury, an ability to inhibit oxidative stress, and the potential to promote learning, memory, and other cognitive functions. These effects appear to be related to the interactions of catechins with key signaling cascades in the brain, leading to an inhibition of apoptosis and to a promotion of neurogenesis, neuronal survival, and synaptic plasticity. Catechins-elicited increased levels of neurotrophins, namely, the brain-derived neurotrophic factor (BDNF) and antiapoptotic proteins, such as B cell lymphoma-2 (Bcl-2), stimulate synaptic plasticity, neurogenesis, and improved neuronal survival. Pre-treatment of male Wistar rats with 20 mg/kg (p.o.) of catechin hydrate for 21 days protected the brain from damage caused by focal cerebral ischemia by ameliorating redox imbalance and limiting inflammatory response possibly through down regulation of NF- $\kappa$ B expression (Ashafaq et al., 2012). These pleiotropic properties of catechins suggest that green tea can be helpful in delaying the harmful effects of aging in the brain and preventing neurodegenerative diseases (Andrade and Assunção, 2015).

Naringin at 80 mg/kg (p.o.) for two weeks demonstrated neuroprotective effect against 3-Nitropropionic acid (3-NP)-induced neurodegeneration through decreased expressions of pro-apoptotic markers like Bad and Bax and inhibition of 3-NP-induced decrease in Bcl-2 mRNA expression in male Wistar rats (Gopinath et al., 2011). Its aglycone, naringenin at 10, 20 and 50 mg/kg administered by oral gavage to male ICR mice displayed potent antidepressant-like property via the central serotonergic and noradrenergic systems suggesting its therapeutic potential in central nervous system disorders especially depression where monoaminergic systems are involved (Yi et al., 2010).

The citrus flavonoid 3,5,6,7,8,3',4'-heptamethoxyflavone (HMF) showed protective effect on hyperactivity induced by MK-801 in the Y-maze test and open field test suggesting that HMF has the ability to relieve MK-801-induced schizophrenia positive symptom-like behavior (Okuyama et al., 2013).

Fisetin, found in various fruits and vegetables, such as strawberry, apple, persimmon, grape, onion, and cucumber (Khan et al., 2013), is emerging as a potential efficacious agent for Parkinson's and Alzheimer's disease (Nabavi et al., 2016; Patel et al., 2012; Ahmad et al., 2017; Prakash and Sudhandiran, 2015). Fisetin post-administration at 20 mg/kg (i.p.) for two weeks corrected the A $\beta$ 1–42 intracerebroventricular injection-induced cognitive/synaptic dysfunctions, neuroinflammation and neurodegeneration in adult mice. It decreased the accumulation of A $\beta$ , BACE-1 expression, and hyperphosphorylation of tau protein at serine 413. Fisetin also markedly reversed A $\beta$ 1–42-induced synaptic dysfunction by increasing the levels of both presynaptic (SYN and SNAP-25) and postsynaptic proteins (PSD-95), SNAP-23, p-GluR1 (Ser 845), p-CREB (Ser 133) and p-CAMKII (Thr 286). Fisetin significantly activated p-PI3K, p-Akt (Ser 473), and p-GSK3 $\beta$  (Ser 9) expression in A $\beta$ 1–42-treated mice (Yi et al., 2010). Fisetin (15 mg/kg, p. o.) showed potential anti AD activity when co-administered with aluminum chloride for 4 weeks through regulation of neuronal apoptosis (Prakash and Sudhandiran, 2015).

The potential efficacy of rutin, quercetin, apigenin, chrysin, kaempferol, and 3',4'-dihydroxyflavone in the inhibition of tumor growth and migration of central nervous systems tumors has been reported. The flavonoids, exhibited the capacity to induce differentiation and inhibit the migratory and invasive activity in human glioblastoma cell cultures, as well as induce ultrastructural modifications indicative of death by apoptosis suggesting that these flavonoids can be considered positive candidates in aid of treatment of malignant gliomas (Santos et al., 2015).

### Neuroprotective Profile of Flavonoid Extracts From Dietary Sources

There is a copious mention of the neuroprotective property of dietary flavonoid extracts and extracts containing flavonoids in addition to other phytochemicals in scientific literature. Due to the relative ease of preparing these extracts, there are lots of studies investigating their bio-efficacy in neurological diseases with reported beneficial outcomes in majority of cases. The mechanisms involved in the neuroprotective action of these extracts include oxidative stress amelioration, antiexcitotoxicity, anti-inflammatory responses, modulation of the activity of cholinesterases, prevention of dysfunction of mitochondrial enzyme complexes such as complex I, regulation of cell death pathways and cell signaling mechanisms. There are positive and negative sides to the use of extracts. Extracts could show a more potent activity than individual compounds due to synergistic interactions amongst constituent compounds. On the other end, inactive chemical entities could mask the bioactivity of one or more compounds in the extract leading to reduced activity of the extract.

Flavonoid extracts of *Rosa laevigata* Michx fruit, *Radix Ilicis pubescentis*, *Scutellaria baicalensis* Georgi, *Garcinia kola*, and *Cyperus rotundus*, grape seed procyanidin extract, among others demonstrated neuroprotection in brain ischemia/reperfusion injury. This pointed to the potential of the extracts in the management of ischemic stroke (Akinmoladun et al., 2015b; Zhang et al., 2006, 2013b; Yan et al., 2017; Tang et al., 2004; Sunil et al., 2011; Kong et al., 2017). For example, pretreatment of male SD rats with 50, 100 and 200 mg/kg flavonoid-rich extract from *Rosa laevigata* Michx, intragastrically, for 7 days protected against cerebral ischemia–reperfusion injury through suppression of apoptosis and inflammation (Zhang et al., 2013b). In addition, a meta-analysis of prospective cohort studies by Tang and colleagues showed that higher dietary flavonoid intake may moderately lower the risk of stroke (Tang et al., 2016).

The anti-aging and protective effect of flavonoid extracts against neurodegenerative diseases is a research area of current interest. The anti-aging effects of A-type proanthocyanidins (PACs)-rich cranberry concentrate and B-type PACs-rich grape seed extract was reported (Jiao et al., 2017). Both extracts exerted anti-aging effects probably via regulating *in vivo* redox state.

Ethyl acetate extract from persimmon leaves consisting of flavonoids and triterpenoids displayed potent protective effect on cognitive deficits induced by A $\beta$  when administered intragastrically to male SD rats at 200 and 400 mg/kg for 30 days. This effect appears to be associated with the regulation of the antioxidative defense system and the mechanism of mitochondrial-mediated apoptosis (Huang et al., 2016). Black tea extract rich in flavonoids protected against aluminium induced Alzheimer's disease (Mathiyazahan et al., 2015). Also, flavonoid extracts from sausage tree demonstrated protective potential in Alzheimer's disease (Falode et al., n.d.).

Flavonoid extracts from dietary sources have been shown to confer neuroprotection in Parkinson's disease. These extracts are from sources such as green tea, *Ginkobiloba*, Safflower, grapes, cocoa, purple basil, etc (Ren et al., 2016; Hang et al., 2016; Bensalem et al., 2015).

Acetyl cholinesterase inhibitors are used clinically to counteract various pathologies. The use of reversible acetyl cholinesterase inhibitors has attracted attention, in particular for the treatment of Alzheimer-type dementia (Bivar et al., 2012). Individual flavonoid compounds as well as flavonoid extracts have been shown to possess anticholinesterase activity alluding to their neuroprotective properties (Bivar et al., 2012; Hacöbekiroğlu and Kolak, 2015; Sevindik et al., 2015; Dzoyem and Eloff, 2015; Cao et al., 2015; Akkol et al., 2012; Khan et al., 2009).

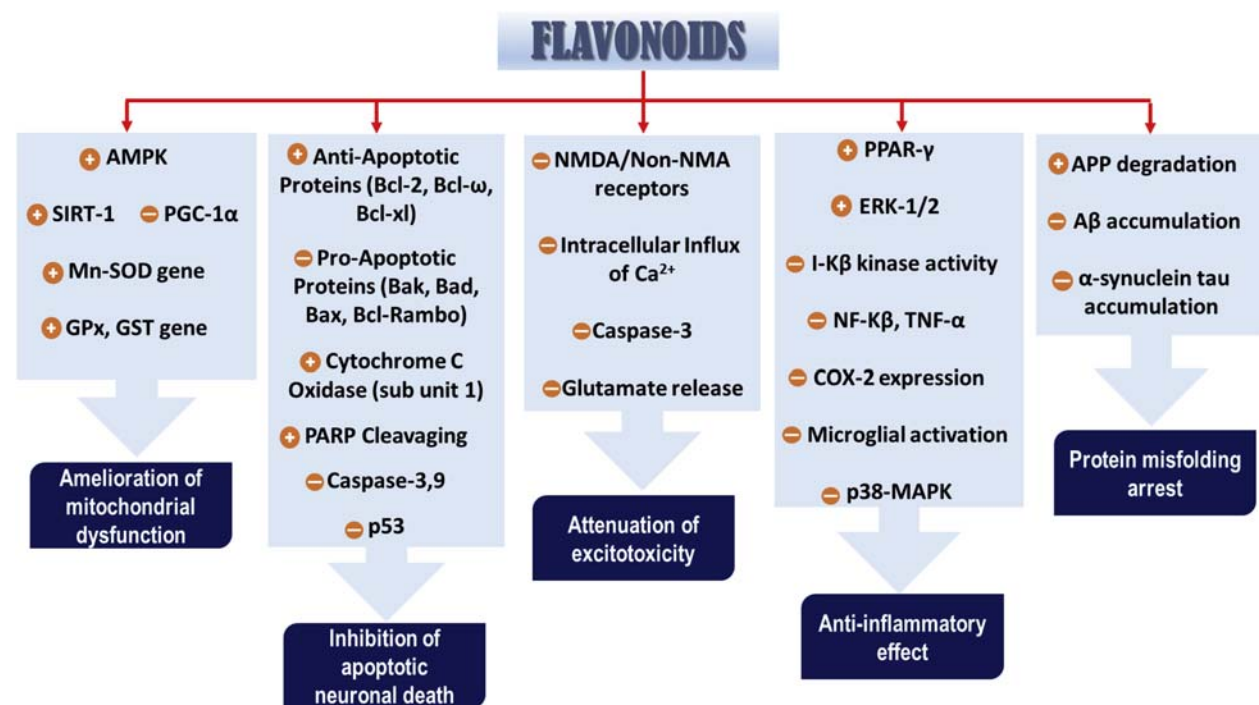
Flavonoid extracts have also shown promising activity in epilepsy (Citraro et al., 2016; Singh et al., 2014; Taiwe et al., 2016; Chindo et al., 2015), cognitive and memory enhancement (Kirisattayakul et al., 2017; Cho et al., 2013; Kandikattu et al., 2017; Smach et al., 2015) and environmental toxicant-induced neurotoxicity (Gupta et al., 2015; Abdou and Wahby, 2016; Igado et al., 2012; Abarikwu et al., 2011).



### Common Mechanistic Routes in Flavonoid Neuroprotection

Flavonoids principally address neuroprotective effects throughout the modulation of signal transduction cascades or effects on gene expression (Ferri et al., 2015). It has been argued that the classical hydrogen-donating antioxidant activity for which flavonoids are historically known, cannot account for the bioactivity of flavonoids in the brain, where they are found at only very low concentrations. It has become apparent that flavonoids are able to exert neuroprotective actions (at low concentration) via their interactions with critical neuronal intracellular signalling pathways pivotal in controlling neuronal survival and differentiation, long-term potentiation (LTP) and memory (Spencer, 2009). As depicted in Fig. 2, these effects appear to be underpinned by their interaction with critical protein and lipid kinase signaling cascades in the brain leading to an inhibition of apoptosis triggered by neurotoxic species and to a promotion of neuronal survival and synaptic plasticity (Vauzour et al., 2013).

Flavonoids affect the synthesis of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). They interact with and modulate signaling pathways involved in neuroprotection such as protein kinase C (PKC) and PI3 kinase. During activation of glial cells in neuroinflammatory states, various transcription factors including NF- $\kappa$ B, activator protein-1 (AP-1), and the signal transducer and activator of transcription-1 (STAT-1) have been shown to be involved in proinflammatory responses in astrocytes and microglia (Sun et al., 2014; Seo et al., 2014; Pu et al., 2007; Spencer and Crozier, 2012; Bei et al., 2009), which can contribute to neuronal death and flavonoids act to suppress this pathway. Flavonoids also act on the ERK pathway. ERK activation often leads to the activation of the cAMP response element-binding protein (CREB), a transcription factor which regulates the expression of a number of important genes, including brain-derived neurotrophic factor (BDNF), which is linked with the control of synaptic plasticity and long-term memory. CREB and BDNF expressions have also been shown to lead to the activation of the PI3 kinase/Akt signaling pathway through binding of BDNF to TrkB receptors. Activation of Akt/PKB, which is under the control of a lipid kinase, PI3K by flavonoids afford neuroprotection by inhibiting proteins associated with cell death such as apoptosis signal-regulating kinase 1 (ASK1), Bad, caspase-9, and caspase-3 (Vauzour et al., 2008, 2013).



**Figure 2** Scheme illustrating several mechanistic routes of neuroprotection by flavonoids. Flavonoids and their physiological metabolites are capable of inducing neuronal and glial signalling pathways crucial in inducing synaptic plasticity, though at nanomolar concentrations. Flavonoids ameliorates mitochondrial dysfunction by activating AMPK which in turns activates SIRT-1 and inhibits PGC-1 $\alpha$  leading to adequate expression of Mn-SOD, GPx and GST genes. By activation and inhibition of anti-apoptotic and pro-apoptotic proteins respectively, flavonoids and their physiological metabolites possesses the ability to inhibit apoptotic neuronal death. Flavonoids attenuates excitotoxicity by greatly inhibiting NMDA receptors, intracellular Ca<sup>2+</sup> influx, caspase-3 expression and glutamate release in the pre-synaptic cleft. Also, flavonoids can act as inflammatory suppressor through a series of cascade that synergistically inhibit NF- $\kappa$ B signaling pathway and microglial activation. Finally, flavonoids can correct protein misfolding by activation of APP degradation which prevents eventual accumulation of misfolded proteins. AMPK => AMP activated protein kinase; SIRT-1 => Sirtuin protein family-1; PGC-1  $\alpha$  => peroxisome proliferator-activated receptor-gamma co-activator 1 alpha; PARP => poly (ADP-ribose) polymerase; NMDA => N-methyl-D-aspartate; PPAR- $\gamma$  => peroxisome proliferator-activated receptor-gamma; Erk-1/2 => extracellular signal related kinase-1,2; IKK => inhibitory kappa-b kinase; TNF- $\alpha$  => Tumor necrotic factor-  $\alpha$ ; COX-2 => cyclooxygenase-2; P38-MAPK => p38 mitogen-activated protein kinase; APP => Amyloid precursor protein; A $\beta$  => Amyloid-  $\beta$ .

## Conclusion and Future Perspectives

The multitarget nature of flavonoids, their ability to cross the BBB and their relative safety in the CNS make them suitable candidates for promotion of brain health. Their wide availability in diets and nutraceuticals are advantageous and will preclude the regimented condition involved in therapeutic interventions with orthodox drugs. The barriers posed by low bioavailability of flavonoids in the CNS due to the BBB are already being addressed by methods including designed synergistic interactions with other natural products and the use of other special delivery systems.

Much is still to be done before optimum benefits of neuroprotection can accrue from dietary consumption of flavonoids. Factors that contribute to individual differences in disposition of flavonoids must be delineated. More controlled pharmacokinetic and pharmacodynamics investigations are needed including studies to evaluate effects of genotypes on flavonoid disposition. Also, a framework for standardized pharmacological and toxicological assessment of flavonoids and flavonoid-based nutraceuticals should be established.

As human lifespan increases due to advances in medical care, cases of neurodegenerative, neurologic and other age-related diseases are also bound to increase. In addition, the unchecked increase in environmental pollution in many countries will contribute to increasing cases of neurotoxicity and poor mental health. Lifestyle adjustment focusing on consumption of dietary flavonoids will do a lot to safeguard human health. How early the consumption of flavonoid should start is another area requiring careful evaluation.

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## Food for Liver Health: Probiotics

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### Glossary

**Anthropometric** Refers to the measurement of the human individual.

**Autoimmune** Condition in which the immune system mistakenly attacks the body.

**Dysbiosis** Condition of having imbalances in the microbial communities either in or on the body.

**Endotoxemia** The presence of toxins in the blood.

**Homeostasis** Tendency towards a relatively stable equilibrium between interdependent physiological processes.

**Metabolism** Chemical processes that occur within a living organism in order to maintain life.

**Microbiota** Ecological community of commensal and pathogenic microorganisms.

**Probiotic** Denoting a substance that stimulates the growth of microorganisms, especially those with beneficial properties.

**Symbiosis** Any type of a close and long-term biological interaction between two different biological organisms.

### Nomenclature

ALT alanine aminotransferase

CFU colony forming unit

GST glutathione S-transferase

HE hepatic encephalopathy

LPS lipopolysaccharide

HVPG hepatic venous pressure gradient

NAFLD non-alcoholic fatty liver disease

NASH nonalcoholic steatohepatitis

RCTs randomized clinical trials

VHB viral hepatitis B

VSL#3 a probiotic cocktail which includes *Streptococcus thermophilus*, *Lactobacillus (acidophilus, delbrueckii, casei and plantarum)* as well as *Bifidobacterium*

## Probiotics Mechanisms of Action

The expression “probiotics” is defined as products containing one or more types of live microorganisms that, when administered in adequate amounts, provide a favorable result to the host by altering the intestinal microbiota (Patel and Denning, 2013; Schrezenmeier and de Vrese, 2001; Sanders et al., 2016). Probiotics may be useful in health-related benefits due to their potential ability to decrease the population of pathogenic bacteria through acidification of the intestinal lumen and by the production of antimicrobial substances that reduce inflammation and activate the host's immune system. Probiotics also regenerate intestinal microbiota equilibrium between host and pathogenic bacteria by competition for nutrients, as well as prevent bacterial translocation to preserve the intestinal barrier integrity and lower toxic products that come from the food or the microbiota itself (Nakamura et al., 2010).

The appropriate dose of probiotic changes depends on the strain; nevertheless, the majority is in a range of 1–10 billion CFU/dose. Even so, it is difficult to determine an accurate treatment, which should be established by well-controlled clinical trials focused on health benefits (Merenstein and Salminen, 2017). *Lactobacilli* and *Bifidobacteria* have been used in either animal or human models as probiotics in the treatment of different liver disorders. The results show that there is an improvement not only in hepatic function tests but also a decrement in endotoxemia and inflammatory responses has been observed (Abdou et al., 2016).

Indeed, some species such as *Bacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus* and *Escherichia coli* are also used together with the yeast *Saccharomyces boulardii* as probiotics (Guarner et al., 2017). Moreover, the benefits provided by probiotics seem to depend on bacterial strains (Isaacs and Herfarth, 2008), which are classified by gender, species, subspecies (if applicable) and an alphanumeric designation. Therefore, it is essential to accurately recognize the strains using new molecular technologies that include:

determining the presence of genetic elements, fermentation of sugars and detection of products obtained from fermentation (Fijan, 2014).

The intestinal microbiota, composed by viruses, fungi, parasites as well as non-pathogenic bacteria, occupy the gastrointestinal tract from the mouth to the colon (Doulberis et al., 2017), partly regulating the host metabolism (Al-Muzafar and Amin, 2017); this symbiosis represents a complex ecosystem that plays a vital role in the body's homeostasis (Tsilingiri et al., 2012).

In humans, *Bacteroidetes* and *Firmicutes* represent the predominant phyla (90%) and to a lesser extent *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria* and *Cyanobacteria* (Miura and Ohnishi, 2014; Paoletta et al., 2014). Starting with the mouth, which contains approximately more than 500 distinct bacteria (D'Argenio and Salvatore, 2015), the complexity and diversity of the intestinal microbiota increases distally reaching up to  $10^{12}$  per gram in the colon (Berg, 1996). Environmental factors, such as age, body mass index (BMI), health status, the diet, gender, and others may have an impact on the bowel bacteria settlement (Bashiardes et al., 2016; Power et al., 2014). Also, the amount of anaerobic versus aerobic bacteria varies, depending on the mucosa layer, where the anaerobes are closer to the epithelium and aerobic are superficially close to the lumen (Shanahan, 2002). However, there are data implying that human's microbiome singularity can be under the host's genetic influence (Van de Merwe et al., 1983).

Some of the functions carried out by the regular gut microbiota are opposite those of pathogenic species in addition to differences in the substances they produce (bacteriocins), pH modification, and stimulation of mucin secretions, all of which contribute to the maintenance of the host defense (Aguirre Valadez et al., 2016). The regular microbiota also functions as an assistant for internal and external stimuli by producing various nutrients and metabolites that are transported to the liver through the intestine-liver axis by means of the venous portal system, where the liver plays a crucial defense role (Usami et al., 2015). Changes in these factors could modify the number of microorganisms available to maintain the intestinal balance that leads to the storage of energy in the form of lipids, bringing adverse effects on the metabolism and increasing obesity, which would later develop liver diseases (Miura and Ohnishi, 2014).

Different liver diseases such as non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease and viral hepatitis may have diverse intestinal microbiota mechanisms to influence liver fibrosis as well as progression of the disease to cirrhosis and subsequently to hepatocarcinoma (Usami et al., 2015).

## Nonalcoholic Fatty Liver Disease

Obesity and accumulation of liver fat are intimately associated to a modification in the composition of the gut microbiota. Therefore, it is thought that probiotics could play a significant role in the treatment of patients with NAFLD and nonalcoholic steatohepatitis (NASH) by reducing bacterial translocation through the intestine and the balance of pathogenic bacteria (Bashiardes et al., 2016; Qamar, 2015). Therefore, it is suggested that different strains of probiotics can be used as a therapeutic option in humans as it has been observed in various animal models (Ma et al., 2013).

*L. acidophilus* did not improve the steatosis or intestinal permeability, but *B. longum* probiotic attenuated hepatic fat accumulation (Xu et al., 2012a). On the other hand, *Clostridium butyricum*, a butyrate-producing probiotic, decreased hepatic inflammatory indexes, insulin resistance, triglycerides content and endotoxin level.

VSL#3, a probiotic cocktail which includes *Streptococcus thermophilus*, *Lactobacillus (acidophilus, delbrueckii, casei and plantarum)* as well as *Bifidobacterium*, was administered during 4 weeks to mice with NAFLD. The results showed a reduction in liver function tests, hepatic inflammation, serum lipid levels, as well as insulin resistance and reversed hepatic steatosis (Kirpich et al., 2015; Ma et al., 2008). Also, another model of mice that were given VSL#3 and a diet deficient in methionine and choline (promotes NASH development without being associated with obesity or the metabolic syndrome), had less hepatic fibrosis (Velayudham et al., 2009). Another study conducted in rats with a diet deficient in choline found a decrease in the expression of tight junction proteins and an increase in antioxidant enzymes, which enhanced suppression of oxidative stress in the liver (Endo et al., 2013).

In other studies with mice that received a diet rich in fats and sucrose, intestinal microbiota dysbiosis occurred due to changes in oxidant stress, inflammation markers, short chain fatty acids and lipopolysaccharide (LPS); however, the intake of probiotics reduced these adverse effects as well as hepatic steatosis while dysbiosis was improved (Haque, 2016).

So far there are few human studies on the efficiency of probiotics in NAFLD or NASH prevention or treatment. Loguercio et al. studied patients diagnosed with NAFLD, who were treated with VSL # 3 for 3 months and patients with NASH who were administered a mixture of symbiotic *Lactobacillus*, *L. casei*, *L. plantarum*, *L. lactis*, *L. bifidus*, *L. rhamnosus*, *L. salivarius*, *L. bulgaricus*, *L. breve*, *L. bulgaricus*, fructooligosaccharides and antioxidants for 60 days (Loguercio et al., 2002, 2005). Both studies showed the beneficial effects of probiotics, indicating a decrease in oxidative stress as well as liver damage and inflammatory levels. Novel double-blind, randomized clinical trials (RCTs) also evaluated the employment of probiotics in liver disease, which showed a decrease in liver function levels. However, there was no difference in anthropometric and cardiovascular risk factors (Aller et al., 2011).

An RCT with obese children and nonalcoholic fatty liver studied the effect of *Lactobacillus rhamnosus* GG and found a significant decrease in alanine aminotransferase (ALT) values and concentrations of polysaccharides (Vajro et al., 2011). Another study with children with the same characteristics, who were supplemented with VSL#3 for four months, the same benefit in liver function tests and inflammation markers was observed (Alisi et al., 2014). A recent meta-analysis (Ma et al., 2013) demonstrates the improvement in inflammation markers and liver function tests of patients with NAFLD, suggesting that the clinical benefits of probiotics is a real hypothesis for this condition.

Most studies in animal and human models demonstrate that probiotics reduce proinflammatory cytokines and liver enzymes, which confirms the benefits of different probiotics strains in the treatment of NAFLD/NASH. The evidence in animal models is clear, but more studies are needed in humans with larger sample sizes and population diversity to propose the use of probiotics in different patients with liver disease.

## Viral Hepatitis

It is known that hepatitis viruses, particularly B and C, may cause long-term hepatocellular damage. As in other liver diseases, the plasma level of endotoxin increases in these patients due to changes in the intestinal microbiota. High levels of proinflammatory molecules cause cell death and worsen liver disease. Lu et al. (2008) studied patients with severe symptomatic hepatitis, dividing individuals into different research groups, controls and treatments, who then received an unspecified probiotic preparation. Proinflammatory molecules concentrations decreased, while anti-inflammatory molecules increased. Moreover, clinical symptoms improved in 84% of the treated group. On the other hand, it has been seen that immune regulation through the production of interferon could also be modified with probiotics (at least in vitro) (Sugimura et al., 2013; Weiss et al., 2011).

In a research study with three groups: apparently healthy patients, infected with hepatitis B virus and with cirrhosis caused by viral infection, changes in fecal *Bifidobacterium* species were measured. The results showed that patients with cirrhosis had a lower amount of *B. longum*, *B. dentium* and *B. pseudocatenulatum/catenulatum* compared with patients who were only infected with viral hepatitis B (VHB) and in turn with the control patients (Xu et al., 2012b). The effects of probiotics in VHB with vaccines have also been studied. Soh et al. administered probiotics to 0–1 month-old infants vaccinated against VHB and a booster of the VHB plus the combined diphtheria pertussis tetanus vaccine at six months of age. The results showed a better surface antibody response when compared to babies that did not receive the probiotic (Soh et al., 2010). Therefore, this study concluded that probiotics could stimulate the immune responses of babies through specific antibodies, although more studies are required to adequately evaluate the benefits of probiotic therapy.

Moreover when the probiotic VSL#3 was administered during 4 months, 20 patients with chronic hepatitis related to virus C and 16 patients who progressed to cirrhosis due to viral infection type C had reduced levels of liver damage; however, the  $\gamma$ -glutamyl transferase (enzyme that shows liver cell death) only developed in the first group (Loguercio et al., 2005).

All these findings sustain the possible use of probiotics in patients infected with the hepatitis virus who present an altered microbiota, serving as a guide for future interventions.

## Cirrhosis

One RCT of patients with cirrhosis and large esophageal varices without hemorrhage classified the study subjects into three groups. The first were patients treated with propranolol and placebo, the second received propranolol and norfloxacin (400 mg) and the third received propranolol and VSL#3 (900 billion/day) with all treatments administered for two months. Supplementation with the probiotic improved the rate of response to the hepatic venous pressure gradient (HVPG) when compared to adjuvant antibiotics and the placebo group (Gupta et al., 2013).

In cirrhosis, probiotics restore the phagocytic faculty of neutrophils, decreasing the bowel wall permeability, bacterial translocation, and endotoxemia in both animal models and clinical trials, in addition to limiting oxidative stress and inflammatory damage in the liver. In fact, VSL#3 lowered oxidative/nitrosative stress parameters in patients with alcoholic liver cirrhosis (Doulberis et al., 2017; Fukui, 2015).

A systematic review by Barjesh et al. demonstrate that probiotics are employed for the prevention of hepatic encephalopathy (HE), a medical term assigned to describe a neuropsychiatric abnormality caused by brain and nervous system toxicity secondary to liver failure mainly produced when the patient has cirrhosis. A significantly lower plasma ammonia concentration was found in patients treated with three months of probiotics, resulting in a representative health improvement, where the absolute risk reduction was 23.8% (Sharma and Singh, 2016).

In the work by Radha K. et al., the use of VSL#3, a probiotic combination of eight strains of *Lactobacilli*, *Bifidobacteria* and *Streptococcus*, for HE secondary prophylaxis, significantly decreased hospitalization over a 6-month period, with reference to the importance of concomitant encephalopathy and inflammatory markers. On the other hand, there was a progress in the health-related quality of life and the pumped heat electrical storage parameters. Also, hepatic function and biochemical measurements of systemic hemodynamics improved, without significant adverse effects (Dhiman et al., 2014).

## Hepatocellular Carcinoma

In a study in which an in vitro and in vivo model was used, Endo et al. demonstrated the efficacy of *C. butyricum*, showing how the whole spectrum of NAFLD to NASH improves, including some mechanisms involved in hepatocarcinogenesis, such as the reduction in the placental glutathione S-transferase (GST) expression (Endo et al., 2013). As well, *Bifidobacterium* and *Lactobacillus* could modify the pathogenic T Helper 17 cells that are involved in the immunogenic mechanisms of liver cancer (Sung et al., 2012).

Analogous data in a murine model supplemented with *L. rhamnosus* GG, showed a decrease in proteins responsible for cell cycle progression after exposure to aflatoxin (Kumar et al., 2011). Interesting clinical data showed how probiotics (*L. rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *Shermanii*) reduced the effective biological dose of aflatoxin exposure (El-Nezami et al., 2006).

There is limited information on epidemiology that suggests the role played by nutrients as a risk factor for hepatocellular carcinoma. However, changes in the microbiota as a result of our eating habits could be responsible pathogenic mechanisms; however, the role of probiotics in this clinical scenario is still limited (Mandair et al., 2014).

## Conclusion

Some probiotics produce several intestinal benefits, such as reduction of inflammation, which in turn influence host immunity, restore the regular gut microbiota, but also prevent bacteria and toxins translocation by maintaining the intestinal barrier integrity. Therefore, since the liver receives a constant flow of toxic molecules, the use of probiotics represents an alternative therapeutic approach. Thus, probiotics could play a fundamental role in the treatment of liver diseases such as NAFLD, NASH and even advanced conditions like fibrosis or hepatocellular carcinoma.

Currently, there are numerous probiotic foods and strains available on the market; however, the question that still arises is how to determine the real potential of microorganisms. Thus, to maintain or improve overall health, safety assessments must be demonstrated to develop a promising probiotic with specific characteristics that offer desirable opportunities for successful commercialization. On the other hand, more RCTs are needed to determine the therapeutic doses and the appropriate probiotic for each liver disease.

In most chronic liver diseases, the main effect of probiotics lies in the resolution of the endotoxemia and the proinflammatory mechanisms. However, evidence of the probiotics effect on cirrhosis and hepatocellular cancer remains weak due to the complexity of their natural history and comorbidities. In summary, modifications of the intestinal microbiota and its metabolites may promote liver damage. But it can be prevented or modified by a healthy diet, the use of probiotics, as well as exercise.

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## Food for Oxidative Stress Relief: Polyphenols

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### Introduction

Oxygen is required for a myriad of activities in the body and lack of thereof, appropriate form or imbalance may preclude proper functioning and be lethal to vital processes and organs in the body. However, the oxidizing potential of oxygen has been implicated in free radical production and resulting oxidative stress. Uncontrolled oxidative stress has been associated with carcinogenesis, DNA damage, cardiovascular disease, cancer, inflammation and other disorders and diseases (Cobley et al., 2018; Rogers and Moorthy, 2018). These diseases and disorders account for millions of morbidity and mortality globally annually. As such, several strategies have been devised to mitigate the incidence and prevalence of these major public health burden. Certain compounds, including polyphenols have been shown to be effective against oxidative stress. The protective role of polyphenols has been linked to their antioxidant properties and impact on other regulatory pathways. Food-source polyphenols from fruits, vegetables, spices, and oils such as artichokes, berries, grapes, capers, cilantro, red onions, tea, coffee are rich in anthocyanins, quercetin and other polyphenolic compounds, and are gaining popularity among consumers as alternative to pharmaceutical remedies (Meshkini, 2018; Rogers and Moorthy, 2018). The use of pharmaceutical drugs to attenuate these pathologies are riddled with negative side effects and longterm safety concerns. Polyphenols function against oxidative stress by various mechanisms. In anti-atherosclerosis, polyphenols regulate lipid metabolism, vascular, endothelial and platelet function (Meshkini, 2018; Santhakumar et al., 2018). The anti-inflammatory role of polyphenols in diseases such as diabetes and obesity include inhibitory effect on sources of reactive oxygen species (ROS) as well as antioxidant activity (Rochette et al., 2014). Other mechanisms include metal sequestration, modulating cellular signaling processes, and expression of metabolic and antioxidant genes (Han et al., 2007; Priftis et al., 2018; Rafei et al., 2018; Rogers and Moorthy, 2018).

### Oxidative Stress

A class of important biochemical substances that is necessary for life is free radicals, which play a key role in physiological processes such as signal transduction, cell division, elimination of damaged cells, and defense against infections in living body systems (Valko et al., 2006; Seifried et al., 2007; Winterbourn, 2015). As the name suggests, free radicals are highly reactive species with unpaired electrons and often produced from oxygen (reactive oxygen species/ROS), nitrogen (reactive nitrogen species/RNS), and metal ions (Valko et al., 2006; Phaniendra et al., 2015). The production of free radicals is inevitable, since organisms are constantly exposed to these substances from external sources such as atmospheric pollutants, energy radiations (UV, X-ray, gamma rays, etc.), or via internal generation during normal metabolic processes of organelles such as mitochondria and peroxisomes (Winterbourn, 2015). However, as with most ingested substances in life, it is the dosage that makes the poison. The presence of high levels of free radicals in the body results in negative consequences in a phenomenon called oxidative stress, which could lead to the damage of sensitive cellular macromolecules such as proteins, DNA and lipids (Liochev, 2013). Living organisms counteract the harmful effects of free radicals by producing a cascade of antioxidant defense systems, which keep the concentration of these radicals under control. Failure in the antioxidant system leads to oxidative stress, which has been identified as a risk factor in the pathological development of several age-related illnesses such as cancers, inflammations and neurodegenerative disorders (Jomova and Valko, 2012; Liochev, 2013; Singh et al., 2015). Table 1 gives a list of some common reactive species implicated in oxidative stress.

### Strategies to Control Oxidative Stress

Natural and synthetic antioxidants have been used to control the harmful effects of reactive species. Antioxidants do so by preventing the formation of excess ROS or by inhibiting the reactions of ROS with other biological molecules (Finkel and Holbrook, 2000; Seifried et al., 2007; Maharjan et al., 2016). Antioxidant defense involves a number of enzymatic and non-enzymatic strategies (Giustarini et al., 2009; Halliwell and Gutteridge, 2015). Some examples of antioxidant enzymes are superoxide dismutase (SOD), catalase, thioredoxin reductase, and glutathione peroxidase (GPx). Non-enzymatic antioxidant systems include glutathione (GSH), and phytochemicals such as tocopherols, carotenes, ascorbates, ubiquinol, isoflavones, flavanoids and polyphenols (Giustarini et al., 2009; Halliwell and Gutteridge, 2015).



**Table 1** Types and description of some common reactive species implicated in oxidative stress

Group	Name	Chemical symbol
Reactive oxygen species	Superoxide	$O_2^{\bullet-}$
	Hydroxyl	$OH^{\bullet}$
	Alkoxy radical	$RO^{\bullet}$
	Peroxy radical	$ROO^{\bullet}$
	Hydrogen peroxide	$H_2O_2$
	Singlet oxygen	$^1O_2$
	Ozone	$O_3$
	Organic peroxide	$ROOH$
	Hypochlorous acid	$HOCl$
	Hypobromous acid	$HOBr$
Reactive nitrogen species	Nitric oxide	$NO^{\bullet}$
	Nitrogen dioxide	$NO_2^{\bullet}$
	Peroxynitrite	$ONOO^-$
	Nitrosyl cation	$NO^+$
	Nitrosyl anion	$NO^-$
	Dinitrogen trioxide	$N_2O_3$
	Dinitrogen tetraoxide	$N_2O_4$
	Nitrous acid	$HNO_2$
	Peroxynitrous acid	$ONOOH$
	Nitryl chloride	$NO_2Cl$
Metal ions	Iron	Fe
	Copper	Cu
	Chromium	$Cr^{+5}$ and $Cr^{+6}$
	Cobalt	$Co^{2+}$
	Vanadium	$V^{5+}$
	Peroxo vanadyl radicals	$V(IV)-OO^{\bullet}$
	Vanadyl hydroperoxide	$V(IV)-OH^{\bullet}$
	Cadmium	Cd
	Arsenic	$As_2O_3$
	Dimethylarsinic peroxy radical	$(CH_3)_2AsOO^{\bullet}$
	Dimethylarsinic radical	$(CH_3)_2As^{\bullet}$
	Nickel	Ni

Adapted from Valko et al., 2006, Lobo et al., 2010, Phaniendra et al., 2015.

## Role of Polyphenols in Controlling Oxidative Stress

Polyphenols are one of the most abundant groups of plant metabolites recognized for their ability to control oxidative stress (Sabu et al., 2002; Katiyar et al., 2001; Pignatelli et al., 2006; Morillas-Ruiz et al., 2006).

### Types and Sources of Polyphenols

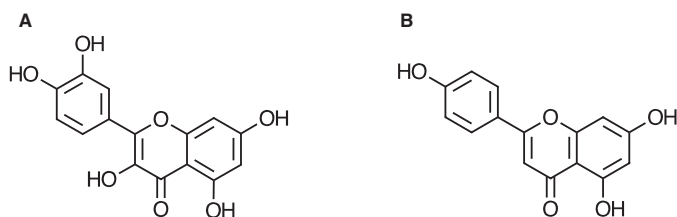
Polyphenols are mainly plant-derived. They comprise a wide variety of molecules that have a polyphenol structure. They also have molecules with one phenol ring, such as phenolic acids and phenolic alcohols (D'Archivio et al., 2007; Holland et al., 2017). The main groups of polyphenols are flavonoids, phenolic acids, phenylethanoid, stilbenes and lignans (D'Archivio et al., 2007).

#### Flavonoids

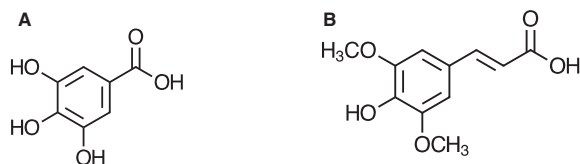
Flavonoids are divided into six subclasses, including flavonols, flavones, flavanones, isoflavones, anthocyanidins and flavanols (D'Archivio et al., 2007; Holland et al., 2017). Important sources of flavonoids are soybeans, hazelnuts, oranges, celery, oregano, grapefruit, green tea, peaches, vinegar, apples, almonds and blueberries (Tangney and Rasmussen, 2013). The chemical structures of two flavonoids are presented in Fig. 1.

#### Phenolic Acids

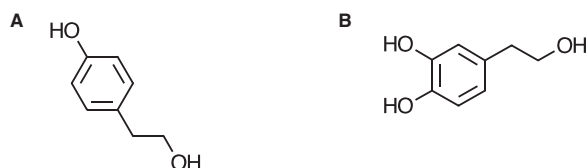
Phenolic acids are divided into two classes, namely hydroxybenzoic and hydroxycinnamic acids (Holland et al., 2017). Examples of hydroxybenzoic acid are gallic, vanillic and protocatechuic acids, while those of hydroxycinnamic acid include coumaric, caffeic, ferulic, sinapic and quinic acids (Holland et al., 2017). Dietary sources of phenolic acids include grape, flaxseed, plum, red wine, black tea, rosemary, green olives, chokecherries and tomatoes (Liu, 2013, D'Archivio et al., 2007; Tangney and Rasmussen, 2013). The chemical structures of gallic acid and sinapic acid are shown below (Fig. 2).



**Figure 1** Chemical structures of (A) quercetin and (B) apigenin.



**Figure 2** Chemical structures of (A) gallic acid and (B) sinapic acid.



**Figure 3** Chemical structures of (A) tyrosol and (B) hydroxytyrosol.

### Phenylethanoid

Three prominent types of polyphenols in this group are tyrosol, hydroxytyrosol and oleuropein. They are found mainly in olive leaf and oil (Holland et al., 2017). Tyrosol and hydroxytyrosol (Fig. 3) have also been reported to be present in argan oil (Rueda et al., 2016).

### Lignans

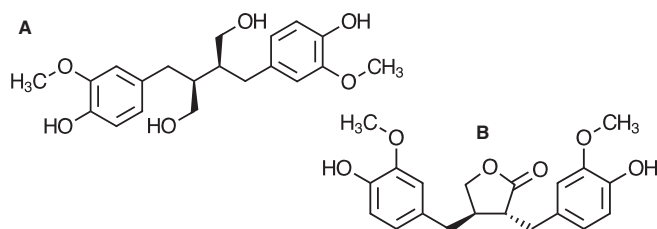
Lignans are a group of fiber-associated polyphenols that are mostly present in nature in the free form or glycosidically linked to different carbohydrates (D'Archivio et al., 2007). Secoisolariciresinol and matairesinol (Fig. 4) are the major types of lignans that have been found in food products such as flaxseed, sesame seed, rye bran and flour, broccoli, garlic, strawberry, black and green tea (Willför et al., 2006).

### Stilbenes

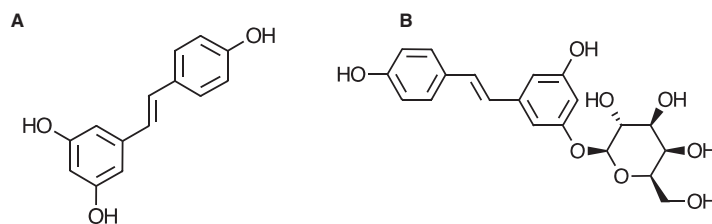
Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is the most widely known stilbene. Polydatin or pieced is another example, but it is a glucoside of resveratrol in which the glucoside group bonded in position C-3 substitutes a hydroxyl group (Lanzilli et al., 2012) (Fig. 5). Plant sources of these stilbenes are grapes, berries and peanuts (D'Archivio et al., 2007).

### Polyphenols as Antioxidants

Since oxidation plays a major role in several disease pathways, the potent antioxidant activity of many polyphenols *in vitro*, can be linked to their ability to prevent some diseases. The abilities of polyphenol to protect against cardiovascular and neurodegenerative diseases are as a consequence of their potent antioxidant and radical scavenging activities (Holland et al., 2017). These properties help them quench ROS and RNS, thus blocking the onset of cardiovascular diseases (Quinones et al., 2013). Polyphenols inhibit



**Figure 4** Chemical structures of (A) secoisolariciresinol and (B) matairesinol.



**Figure 5** Chemical structures of (A) resveratrol and (B) polydatin.

the oxidation of low density lipoprotein (LDL) *in vitro* and *in vivo* (Urquiaga and Leighton, 2000). For instance, procyanidin was found to reduce the levels of oxidized LDLs in diabetic patients (Brito et al., 2009). Also, red wine polyphenols have been reported to reduce the susceptibility of LDL to oxidation (Urquiaga and Leighton, 2000). Polyphenols such as procyanidin B<sub>4</sub>, catechin, and gallic acid were found to prevent oxidative damage of cellular DNA (Fan and Lou, 2004), which has been implicated in age-related development of cancer (Bjelland and Seeberg, 2003; Urquiaga and Leighton, 2000). Polyphenol-rich foods and beverages may also increase plasma antioxidant capacity (Pandey and Rizvi, 2009).

### Methods for the Extraction of Polyphenols

To facilitate the utilization of polyphenols as nutraceuticals or functional food ingredients, an extraction step is often required. The extract may be further processed or formulated with other ingredients to improve their biological properties and storage stability. The extraction of polyphenols is also useful as a strategy for adding value to polyphenol-rich plant material wastes. This is an important value-added strategy because some of these wastes (e.g. olive pomace and waste waters) are produced in very huge quantities (4 million tons and 3 million cubic meters per year, respectively) and contain very high levels of phytochemicals that can negatively impact soil quality as well as plant, microbial and aquatic life when these wastes are deposited into the environment without treatment without treatment (Dermeche et al., 2013; Muktadirul et al., 2013).

To obtain polyphenols from plant waste materials, four key steps are followed, namely: (a) preparation of plant matrix, (b) extraction of polyphenols using solvents and fluids, (c) fractionation of polyphenols with the aid of resins, and (d) conjugation/modification of polyphenols to improve their activity, storage stability, and delivery efficiency.

Preparation of plant matrix involves steps such as milling of matrix to reduce particle size, drying, removal of lipids, and disassociation of polyphenols in complex with other molecules such as carbohydrate and proteins (Khoddami et al., 2013). The purpose is to render the polyphenols in the plant matrix more accessible to extraction agents to be employed in later stages. The extraction of polyphenols is achieved using solvents and fluids at subcritical and supercritical states. The chemical diversity of polyphenols precludes the suitability of a single solvent in removing all polyphenols found in a plant matrix. As such, parameters such as polarities of the solute and solvent are taken into consideration. For food application, the safety or potential toxicity of the solvent is also taken into consideration. Examples of solvents/plant matrix systems that have been used for the extraction of polyphenols include water/sorghum leaves (Agbangnan et al., 2012), aqueous ethanol/wheat bran (Verma et al., 2008), ethanol/olive mill wastewater (Lafka et al., 2011; Azaizeh et al., 2012), or ethyl acetate/olive mill wastewater (El-Abbassi et al., 2012; Kalogerakis et al., 2013). Use of solvents present certain demerits: some are toxic and cannot be used to extract polyphenols for food applications, while others have a negative impact on the structural integrity and therefore, biological properties of the polyphenol extracts. But overall, the use of solvents for the extraction of polyphenols offers several advantages such as, relatively low cost, ease of scalability, ability to recycle solvents, possibility of improving polyphenol yield and purity by using solvent mixtures, feasibility of use on all plant matrixes, and compatibility with energy treatments such as heating or microwave technology to improve extraction efficiency.

Pressurized fluids (e.g. supercritical fluids, subcritical water, and high hydrostatic water) are alternative extractants that offer advantages (excellent food compatibility; low extraction time; high extraction yields, product quality and purity) over organic solvents, although they are usually accompanied with higher processing and investment costs. 'Green' and inert compounds such as water and carbon dioxide are converted into the supercritical states, resulting in a compound with superior properties (e.g. density, diffusivity, dielectric constant, etc.) that make them better extraction agents (da Silva et al., 2016). Supercritical fluid extraction has been used to obtain polyphenol extracts from rocket salad (Solana et al., 2016), peach (Kazan et al., 2014), olive oil mill wastes (Lafka et al., 2011), red dragon fruit (Fathordoobady et al., 2016), etc. Polyphenols from pomegranate seed residues (He et al., 2012), flaxseed meal sticks (Kanmaz, 2014), olive fruit dreg (Yu et al., 2015), citrus peels (Casquete et al., 2014), grape pomace (Kangliang et al., 2017), etc. have also been obtained by using sub-critical water and high hydrostatic water extraction techniques.

After extraction, polyphenols can be subjected to further separation to give pure fractions by harnessing their adsorption behavior to solid phase resins such as activated carbon (Yangui and Abderrabba, 2018), ion exchange resins (Bertin et al., 2011), and molecularly imprinted polymers (Puoci et al., 2012). The purified polyphenol fractions can be further conjugated to other biomolecules such as lipids to modify (and often enhance) their physicochemical and biofunctional properties (Holland et al., 2017; Akanbi and Barrow, 2018).

## Effect of Polyphenols on Oxidative Stress

Several studies have shown the ability of polyphenols to prevent or attenuate the damage caused by oxidative stress *in vivo* and *in vitro*. A comparison of the results within test is complicated by the variability in amount, type, and dose of polyphenolic compounds used as well as the duration of the study. Some of these will be discussed in this section.

### In Vitro and Cell Culture Tests

Several *in vitro* studies have shown the protective effect polyphenols in attenuating oxidative stress. Acetone extracts of almond green hulls prevented the propagation of free radical reactions and exerted an inhibitory effect on platelet aggregation (Meshkini, 2018). Black-sticky rice was shown to have higher antioxidant activity than red rice and also DNA-protective characteristics in human mononuclear leukocytes in hydrogen peroxide-induced environments (Warin et al., 2010). Polyphenols in red and purple rice extracts were demonstrated to inhibit adipocyte differentiation by significantly reducing lipid accumulation and down-regulating expression of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) in C3H10T1/2 adipocyte (Callcott et al., 2018). Foods containing polyphenolic compounds such as resveratrol, quercetin, catechin, cyanidin, and kuroman in protected against palmitate-induced nitroso-oxidative stress, mitochondrial impairment and endoplasmic reticulum stress (Rafiei et al., 2018). These factors have been shown to be involved in the progression of steatosis and apoptosis. Ethanolic extracts of grape pomace and coffee showed different composition and amount of polyphenols and exhibited different antioxidant mechanisms (Priftis et al., 2018). Using C2C12 cells, the grape pomace extract with low concentration of polyphenols scavenged free radicals, whilst coffee extract induced the expression of metabolic and antioxidant genes (Priftis et al., 2018).

### In Vivo Tests

Grape fruit flavonoids have been found to provide protection against oxidative stress *in vivo*. These grape polyphenols protected serum, plasma proteins and LDL against oxidation in healthy adults (O'Byrne et al., 2002). In another study, grape polyphenols helped reduce oxidative stress and lowered plasma lipids in pre- and postmenopausal women (Zern et al., 2005). Authors found that the major polyphenols responsible for these were flavans, anthocyanins, quercetin, myricetin, kaempferol and resveratrol. The 4 week study found a significant reduction in the plasma triglyceride concentrations, plasma LDL cholesterol and apolipoproteins B and E in a total of 42 pre- and postmenopausal women (Zern et al., 2005). Polyphenols in red wine synergistically inhibited oxidative stress in 20 healthy subjects (Pignatelli et al., 2006). After drinking 300 mL of red wine for 15 days, there was a significant decrease in PGF-2 $\alpha$ -III, a marker of oxidative stress in the subjects. Their level of plasma polyphenols also increased significantly (Pignatelli et al., 2006). Green tea epigallocatechin gallate suppressed tumorigenesis in a xenograft mouse model by directly targeting the proliferation of cells expressing peptidyl prolyl *cis/trans* isomerase (Urusova et al., 2011).

## Conclusion

The use of polyphenols as antioxidants in food has received greater acceptance in recent years, because they are natural alternatives to the synthetic commercial antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxy anisole (BHA) and tert-butylhydroquinone (TBHQ). Polyphenols have also been widely reported to be more effective antioxidants than ascorbic acid (vitamin C) and tocopherols (vitamin E) (Rice-Evans et al., 1997; Urquiaga and Leighton, 2000; Akanbi and Barrow, 2018). A number of polyphenols have been used as antioxidants in edible oils rich in omega-3 and omega-6 fatty acids (Akanbi and Barrow, 2018; Zhang et al., 2017; Wang et al., 2018). Hydroxytyrosol, procyanidins, ferulic, caffeic and tannic acids are some of the polyphenolic compounds that have been used to retard lipid oxidation in fish mince and other muscle-based food products (Maqsood et al., 2014; Holland et al., 2017).

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# Health-Promoting Fermented Foods

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## Introduction: Overview of Fermented Foods

Fermentation of food substrates is an ancient practice, often described as a cheap, energy efficient, low-technology, and one of the oldest forms of traditional food processing and preservation techniques, usually carried out to introduce a variety of diets into food preparations (Ferri et al., 2016; Guyot, 2012; Kebede et al., 2007; Simango, 1997). Most food materials are highly perishable in their raw states (e.g. milk, cassava, fruits and vegetables, edible bamboo shoots etc.), which make them prone to spoilage attack, and in some cases, inedible and surplus after harvesting. Hence, they are fermented by natural inoculation and biochemical activities of microorganisms, which help to achieve post-harvest preservation, prevent physiological deterioration and losses, make inedible ones edible, with improved shelf-life, food safety, as well as ensuring availability of foods all year round (Patra et al., 2016; Rolle and Satin, 2002). Generally, these microorganisms are mostly from the raw food materials, fermentation vessels and utensils, processors, contaminants and other environmental microflora, selected to actively dominate and ferment the substrates through competitive adaptation and direct competition for available nutrients (Franz et al., 2014; Tamang, 1998).

A diversity of fermented foods and beverages obtained from food substrates, such as cereal grains, legumes and pulses, roots and tubers, fruits, vegetable leaves and edible bamboo shoots, milk and dairy products, meat and seafoods, miscellaneous food commodities (e.g. tea leaves, cocoa, sugar cane juice, oil palm sap etc.) is abundantly available in different parts of the world (Tamang et al., 2016a). Divergent microbial strains of bacteria (*Bacillus*, lactic acid bacteria, *Acetobacter*, Micrococcaceae), yeasts and mycelia or filamentous moulds have been reported to be principally responsible for the biotransformation of these food materials, resulting in the production of either acidic, alcoholic or alkaline-fermented food products (Steinkraus, 1997; Tamang, 1998). During the process of fermentation, microorganisms become exposed to food substrates, which leads to utilization of the nutrient contents. The nutrients and organic chemical compounds are used as carbon, nitrogen, electron and energy sources, through various enzymatic and biochemical reactions, which bring about desirable functional changes along with the production of metabolites that impart functional benefits. Beneficially, microbial fermentation enhances nutrient enrichment and bioavailability, development of attractive flavour, taste, aroma and texture, in addition to improved digestibility of carbohydrates and proteins, as well as bio-preservative effects (Blandino et al., 2003; Nout, 2009; van Boekel et al., 2010). Apart from the production of pleasant and acceptable quality food substances preferred by consumers (compared to their respective raw food materials), food fermentation also supports prolonged shelf-life of final food products (Caplice and Fitzgerald, 1999; Holzapfel, 1997). Other benefits include detoxification and reduction in undesirable toxic components and anti-nutritional factors, food fortification with essential amino acids and fatty acids, vitamins, minerals and antioxidants, stimulation of health promoting functions, value-added advantage and new products development (Oboh, 2006; Ouoba et al., 2003; Teniola and Odunfa, 2001).

Among these advantages, the consumption of fermented foods and beverages for their health-promoting properties, especially in disease prevention and improvement of human health has long been recognized by consumers. This is in consonant with early comments by the Russian Scientist, Élie Metchnikoff who suggested that the prolonged life span of the Bulgarian peasants resulted from the consumption of fermented dairy foods, such as yoghurt, sour milk and *kefir*, which contain lactic acid bacteria (LAB) (Metchnikoff, 1907). Metchnikoff's observation has since then stirred up consumers' consciousness and awareness in the consumption of foods with health-promoting values, beyond the purpose of nutrition and other basic benefits. Thus, food fermentation has received increasing research interests and attention, especially in food science, human nutrition and applied microbiology (Saarela et al., 2002; Salmerón, 2017). Multiple studies and evidence-based investigations have shown that fermented foods carrying both large populations of live microbial cultures and their metabolites, or either of these, may impart health beneficial functions (Barla et al., 2016; Kim et al., 2016; Singh et al., 2014; Tamang et al., 2016b). Hence, the scope of this chapter provides a comprehensive account and current information on health-promoting fermented foods and beverages around the world. Such benefits include reduction in serum and blood cholesterol, production of exopolysaccharides (EPSs) and bioactive compounds, production of anti-microbial compounds against potential pathogenic microorganisms, anti-mutagenic, anti-carcinogenic, anti-tumour effects and fibrinolytic activities. Other health effects include amelioration of metabolic and physiological disorders, improvement in cognitive brain functioning, enhanced probiotic properties, etc. In addition, highlights of the different types of fermented foods, and the predominant microorganisms associated with them will be discussed.

## Diversity of Fermented Foods and Beverages Around the World

Fermented foods and beverages are classified on the basis of raw food materials used in producing them, whether alkaline, acidic, alcoholic, or both acidic and alcoholic as well as the predominant group of microorganisms (Table 1).

**Table 1** Some selected fermented foods and beverages around the world, based on divergent raw food substrates

Products	Food substrates	Nature	Predominant microorganisms	Country of origin
<i>mawé</i>	maize	acidic dough	<i>Lactobacillus fermentum</i> , <i>Saccharomyces cerevisiae</i>	Benin Republic
<i>tchoukoutou</i>	sorghum	alcoholic opaque beer	<i>Sac. cerevisiae</i> , <i>Candida krusei</i> , <i>Lac. fermentum</i>	Benin Republic
<i>dosa</i>	rice and black gram	acidic and slightly alcoholic batter	<i>Leuconostoc mesenteroides</i> , <i>Lac. fermentum</i> , <i>Bacillus amyloliquefaciens</i> , <i>Sac. cerevisiae</i> , <i>Debaryomyces hansenii</i>	India, Sri Lanka, Malaysia, Singapore
<i>pozol</i>	maize	mildly acidic dough	<i>Lac. fermentum</i> , <i>Lac. plantarum</i> , <i>Lac. casei</i>	Mexico
sourdough	rye, wheat	mildly acidic, leavened bread	<i>Lac. sanfranciscensis</i> , <i>Lac. alimentarius</i> , <i>Lac. buchneri</i>	United States of America, Europe, Australia
<i>iru</i>	African locust bean	alkaline condiment	<i>Bac. subtilis</i> , <i>Bac. amyloliquefaciens</i> , <i>Bac. cereus</i>	Nigeria
<i>kinema</i>	soybean	alkaline condiment	<i>Bac. subtilis</i> , <i>Bac. licheniformis</i> , <i>Bac. cereus</i>	India
<i>natto</i>	soybean	alkaline condiment	<i>Bac. subtilis</i> ( <i>natto</i> )	Japan
<i>fufu</i>	cassava	acidic dough	<i>Lac. plantarum</i> , <i>Lac. cellobiosus</i> , <i>Bacillus</i> species	West Africa
<i>tarubá</i>	cassava	acidic beverage	<i>Lac. plantarum</i> , <i>Lac. brevis</i> , <i>Leu. mesenteroides</i> , <i>Pichia exigua</i> , <i>Can. tropicalis</i>	Brazil
sauerkraut	cabbage	acidic, sour salad	<i>Leu. mesenteroides</i> , <i>Pediococcus pentosaceus</i> , <i>Lac. plantarum</i> , <i>Lac. brevis</i>	Europe, Canada, United States of America, Australia
table olives	olive	acidic, side dish salad	<i>Lac. plantarum</i> , <i>Lac. pentosus</i> , <i>Leu. mesenteroides</i> , <i>Ped. pentosaceus</i> ,	United States of America, Spain, Portugal, Peru, Chile
<i>kimchi</i>	cabbage, green onion, hot pepper	acidic, mildly sour, side dish	<i>Leu. mesenteroides</i> , <i>Leu. citreum</i> , <i>Leu. kimchi</i> , <i>Lac. plantarum</i> , <i>Weissella cibaria</i>	Korea
<i>kefir</i>	milk	acidic, mildly alcoholic fermented milk	<i>Lac. kefiranofaciens</i> , <i>Lac. brevis</i> , <i>Streptococcus thermophilus</i> , <i>Lac. plantarum</i> , <i>Lac. casei</i> , <i>Can. kefir</i> , <i>Sac. cerevisiae</i>	Russia, Europe, Middle East, North Africa
<i>dahi</i>	milk	acidic viscous curd	<i>Lac. alimentarius</i> , <i>Lac. paracasei</i> , <i>Lac. acidophilus</i> , <i>Lac. helveticus</i>	India, Nepal, Sri Lanka, Bangladesh, Pakistan
<i>amasi</i>	milk	acidic, sour, with thick consistency	<i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Lac. lactis</i> subsp. <i>cremoris</i> , <i>Leuconostoc</i> spp.	South Africa, Zimbabwe
<i>alheira</i>	pork or beef	dry/semi-dry sausage	<i>Lac. plantarum</i> , <i>Lac. paraplantarum</i> , <i>Lac. brevis</i> , <i>Lac. sakei</i>	Portugal
<i>ngari</i>	fish	mildly acidic fermented fish	<i>Lac. plantarum</i> , <i>Lac. pobuzihii</i> , <i>Lac. coryniformis</i> , <i>Bac. subtilis</i> , <i>Staphylococcus carnosus</i>	India
<i>tej</i>	honey	sweet, effervescent and cloudy alcoholic	<i>Sac. cerevisiae</i> , <i>Deb. phaffii</i> , <i>Kluyveromyces bulgaricus</i>	Ethiopia
<i>kombucha</i>	tea	fermented tea drink	<i>Sac. cerevisiae</i> , <i>Acetobacter aceti</i> , <i>Gluconobacter oxydans</i>	China, India

Adapted and modified from Tamang et al. (2016a).

## Cereal Fermented Foods

Non-alcoholic fermented cereal foods produced from maize (*Zea mays* L.) in Africa include *doklu* (Côte d'Ivoire), *kenkey* (Ghana), *amawewu* (or *mahewu*) and *incwancwa* (South Africa), *mawè* (Benin Republic), *ogi* (Nigeria), *poto-poto* (Congo), *togwa* (Tanzania), *ikii* and *uji* (Kenya), *munkoyo* and *chibwantu* (Zambia), as well as *tobwa*, *mutwiwa* and *ilambazi lokubilisa* (Zimbabwe). *Ben-saalga* and *dégué* (Burkina Faso), *koko* or *akasa*, *koko* sour water and *fura* (Ghana), and *kunun-zaki* (Nigeria) are made from pearl millets [*Penisetum glaucum* (L.) R. Br.], while *mangisi* (Zimbabwe) is from finger millet (*Eleusine coracana* Gaertn.). Fermented sorghum [*Sorghum bicolor* (L.) Moench] foods include *obushera* (or *bushera*) (Uganda), *gowè* (Benin Republic), *hussuwa* and *kisra* (Sudan), *bogobe* (Botswana), *obiolor* and *ogi-baba* (Nigeria) (Assohoun-Djeni et al., 2016; Gadaga et al., 1999; Odunfa and Adeyele, 1985; Owusu-Kwarteng et al., 2012; Schoustra et al., 2013). Major fermented cereal alcoholic beverages are *pito*, *dolo*, *tchapalo*, *burukutu*, *tchoukoutou*, *busaa*, kaffir beer, *muramba*, *pombe*, *impeke*, *malwa*, *merissa*, *amgba*, *bouza*, *umbugug*, *doro/uthwala*, *tella* (Holzapfel, 1997; Jespersen, 2003). Fermented baked snacks and pancakes also produced from cereals include *injera*, *kisra* and *masa*. Common fermented cereal foods in Asia are *dosa* (pancake), *idli* (pudding), *hamei* and *xaj-pitha* (rice wines, India), *congee* and *suanzhou* (acidic gruel, China), *chhang* (barley beer, India), *jalebi*, *koozh* (Indian porridge), *adhirasam* (rice doughnut), *hopper* (steamed baked, Sri Lanka), *puto* (Philippines), *sake* (also *saké*, Japanese rice wine) and *dhokla* (dough) (Jeyaram et al., 2008; Qin et al., 2016). Bread, sourdough bread, *San Francisco* bread, rye bread, beer, *boza* (Turkish sour drink), *bagni* (millet alcoholic drink, Russia), *hulumur* (sorghum beverage drink, Turkey), *kvass* (a non-alcoholic beverage similar to *boza*), *perkarnaya* (Russia), *pumpernickel* (Switzerland), and Mexican *pozol* and *atole agrio* comprise the various cereal fermented foods in Western and Eastern countries. Products such as *champús* and *chicha de jora* (both mild alcoholic beverages), and *masa agria* (maize dough) are found in South America (Chaves-Lopez et al., 2016; Elizaquível et al., 2015; Väkeväinen et al., 2018; Ventimiglia et al., 2015).

## Fermented Legume Protein-Rich Seeds

Traditional fermented protein-rich, legume-based foods are widely consumed in many African and Asian countries. In East and Southeast Asia, soybean [*Glycine max* (L.) Merr.] seeds are fermented by *Bacillus subtilis* to produce varieties of alkaline fermented food condiments, such as Japanese *natto*, Indian *kinema*, *hawaijar*, *bekang*, *tungrymbai*, *peruyaan* and *aakhone*, Thailand *thua nao*, Korean *cheonggukjang*, and Chinese *douchi* and *yandou* (Sanjuka and Rai, 2016). The major biochemical change is protein hydrolysis due to high proteinase activity of *Bac. subtilis*, which results in rapid production of polypeptides, amino acids, ammonia, and polyglutamic acid (PGA) in addition to other volatile compounds that contribute to the product's characteristic pungent smell and ammoniacal flavour (Leejeerajumnean et al., 2001; Odunfa, 1985). A similar food flavouring agent in Africa from soybean is *soy-dad-dawa*, but *iru* (also known as *daddawa*), *soumbala*, *afitin*, *nététou*, *kinda*, *oso*, *kawal*, *cabuk*, *bikalga*, *dawadawa botso*, *datou*, *mbuja*, *furundu*, *maari* and *tayohounta* are produced using non-soybean seeds, while cassava leaves form the raw material for the production of *ntoba mbodi* (Adewumi et al., 2014; Parkouda et al., 2009; Vouidibio Mbozo et al., 2017). Other fermented soybean foods in Asia where filamentous and mycelia moulds like *Aspergillus oryzae*, *Rhizopus oligosporus*, *Rhi. oryzae*, *Rhi. microsporus*, *Mucor sufu*, *Muc. wutungkiao*, *Muc. plumbens*, *Actinomucor taiwanensis*, *Act. elegans* and *Absidia corymbifera* dominate the fermentation process or with the participation of *Bacillus* species, include Indonesian *tempe*, Chinese *sufu* (also *fu-ru* or *tofu*) and *soy sauce*, Korean *meju*, *doenjang*, *ganjang* (a soy sauce), *doenjang-meju* and *gochujang*, Japanese *miso* and *shoyu* (Han et al., 2001; Jung et al., 2014; Nout and Kiers, 2005).

## Fermented Starchy Roots and Tuber Products

Cassava, yam, cocoyam and potatoes constitute starchy root and tuber crops that provide carbohydrate, an energy source in the diets of millions of people, especially in sub-Saharan (Cock, 1982; O'Hair, 1990). In Africa, cassava (*Manihot esculenta* Crantz) is the most abundant and importantly consumed root crop despite the presence of linamarin and lotaustralin, which are toxic cyanogenic glucosides (Aryee et al., 2006; Kimaryo et al., 2000). Processing by fermentation, involving *Lactobacillus plantarum*, *Lac. fermentum*, *Lac. pentosus*, *Lac. brevis*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Weissella paramesenteroides*, *Wei. cibaria*, *Wei. confusa* and *Pediococcus pentosaceus*, primarily brings about detoxification and biochemical activities of the cassava tubers. This enhances acidification and imparts organoleptic properties, leading to the production of varieties of edible fermented food products, such as *gari*, *agbelima*, *akyeke*, *attiéké*, *fufu*, *kiwunde*, *lafun* (or *kokonte*), *chikwangue*, *cingwada*, *kocho*, *ikivunde*, *imikembe*, *inyange*, and *ubuswage*, among others (Adesulu-Dahunsi et al., 2017; Amoa-Awua et al., 1997; Kostinek et al., 2007; Obilie et al., 2004). Cassava fermentation for *gari* production is a solid-state natural inoculation, unlike *fufu*, *attiéké*, *lafun* and *agbelima* where submerged fermentation is employed (Coulin et al., 2006; Oyewole, 2001). Other microorganisms, including *Bacillus* species and yeasts have also been reported to partake in cassava fermentation. *Bac. subtilis* was found to produce amylases that were involved in the initial breakdown of cassava starch into simple sugars that are then fermented by LAB (Amoa-Awua and Jakobsen, 1995). *Saccharomyces cerevisiae*, *Pichia scutulata*, *Kluyveromyces marxianus*, *Hanseniaspora guilliermondii*, *Candida tropicalis*, *Can. glabrata* and *Can. krusei* are the yeasts species isolated during traditional *gari* and *lafun* production in West Africa (Oguntoyinbo, 2008; Wilfrid Padonou et al., 2009). Cassava is also processed by solid-state fermentation for the production of *tarubá*, an indigenous beverage by the Amerindian tribes in Brazil (Ramos et al., 2015).

## Fermented Fruits and Vegetables

Lactic acid fermentation of fruit and vegetable foods is traditionally carried out in most parts of Europe, United States of America and Asian sub-continent for the purpose of preservation against spoilage and rotting. Other reasons include prevention of post-harvest

losses, shelf-life extension, improvement in nutrient composition, detoxification and reductions in levels of anti-nutritional components (e.g. glucosinolates in cabbage and oleuropein in olive), in order to make them edible and available during the off season (Gail-Eller and Gierschner, 1984; Ross et al., 2002; Sánchez et al., 2000a). Fermentation processing of heads of white cabbage (*Brassica oleracea* var. *capitata* L.), cucumber (*Cucumis sativus* L.) and olive (*Olea europaea* L.) for the production of sauerkraut ('sour herb' or 'sour cabbage' as known in Germany), pickles and table olives respectively, is commonly practised in Western countries. However, Korean kimchi from Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) is the most popular fermented vegetable side dish food in Asia (Oguntoyinbo et al., 2016a; Patra et al., 2016). Fermented vegetable ethnic foods that are also common in Asia are gundruk, sinki, khalpi, sunki, pak-sian-dong, tursu, suan-tsai, salgam, kanji, jiang-gua, hardaliye, dhamuoi, dakguadong, goyang, phak-gard-dong and gherkins, while the edible fermented bamboo shoots include mesu, soidon, soibum, soijim, ekung, herring, naw-mai-dong, inziangsang, doubanjiang and pao cai (Altay et al., 2013; Anandharaj et al., 2015; Tamang et al., 2008; Tamang and Tamang, 2009). In Africa however, very few vegetable leaves like those from cowpea [*Vigna unguiculata* (L.) Walp.] and African kale [*Brassica carinata* A. Braun] are subjected to fermentation before consumption (Kasangi et al., 2010; Oguntoyinbo et al., 2016b). Spontaneous fermentation of fruits of capper berries (*Capparis spinosa* L.), sweet cherry (*Prunus avium* L.) and 'Almagro' egg plant (*Solanum melongena* var. *esculetum* L.) are equally found in the Mediterranean, where they form part of various cuisines (Perez Pulido et al., 2005; Sánchez et al., 2000b).

### Fermented Milk and Dairy Products

Among other fermented food substrates is milk, which is highly perishable with a very short shelf-life because it contains major classes of nutrients. The high nutrient density makes milk a suitable medium for microbial contamination and colonization by autochthonous, spoilage and pathogenic microorganisms. For preservation and digestibility purposes, milk is naturally fermented at ambient temperature, or with starter cultures in the raw form or after pasteurization (Jans et al., 2017). Yoghurt is a fermented dairy food that is produced commercially from pasteurized milk using strains of *Lac. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* starter cultures. While *Str. thermophilus* is responsible for the metabolism of lactose sugar to produce lactic acid, which enhances milk acidification, *Lac. delbrueckii* subsp. *bulgaricus* contributes to aroma and flavour production (Innocente et al., 2016). Cheese is another commercial fermented milk food, with different types depending on the origin of production, ripened or unripened, soft or hard. During cheese manufacturing, the acidic environment created by LAB, neutralizes the negative charge ion of the milk casein, for precipitation and coagulation, forming a gel cheese curd at isoelectric point of pH around 4.6. The major cheese microbiota includes *Lactococcus lactis* subsp. *lactis*, *Lac. lactis* subsp. *cremoris*, *Lac. lactis* subsp. *lactis* var. *diacetylactis*, *Leu. mesenteroides* subsp. *cremoris*, *Lac. helveticus*, *Lac. casei*, *Lac. plantarum*, *Lac. salivarius*, *Enterococcus faecium*, *Ent. durans*, *Propionibacterium freudenreichii*, *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluveromyces marxianus*, *Geotrichum candidum*, *Penicillium roqueforti*, *Pen. camemberti*, *Pen. glaucum* and *Staphylococcus* species (Quigley et al., 2011).

Naturally fermented milk (NFM) products are likewise available in different parts of the world apart from cheese and yoghurt. Examples include kefir (fermented milk with kefir grain), koumiss (in Russia), sethemi, amasi/wakakora, mukaka, hodzeko, mbanik, kule naoto, mursik, amabere, amaruranu, suusac, raib, zabady (like plain yoghurt), urubu, amateregua, amavuta, jben, leban, rob, gariss, fenè, mabisi, dhanaan, makamo, nunu, nyarmie, kindirmo, maishanu, arera and pendidam (from different parts of Africa), laban (Lebanon), doogh (Iran), aryan (Turkey), and kurut (China) (Akabanda et al., 2013; Franz et al., 2014; Jans et al., 2017). Others are lāngfil, film-jölk, viili, tettemelk, ymer and talouspiimä in the Scandinavian, and dahi (yoghurt-like), chhurpi, somar, chhu, khachu, philu, shrikhand, philu and shyow in India (Duboc and Mollet, 2001; Ghatani and Tamang, 2017). Fermented curds similar to cottage cheese include datsi, warankashi/woagashi, oscypek, batzos, rigouta, ergo, ititu, ayib, while traditional fermented butters are kibe, neterkibe, omashikwa, klila and chhash.

### Fermented Meat and Seafood Products

Meat and fish products are highly nutritious, and perhaps the richest source of protein foods, when compared to plants, because of their amino acid quantity and quality, which may be lacking in other protein sources (Lücke, 2000). Fresh cut meat and fish are susceptible to microbial contamination and spoilage because of their relatively high moisture contents and water activity ( $a_w$ ) (Adams, 2010; Gram and Huss, 1996). They are usually processed by cooking, smoking, drying, canning and grilling before consumption. Fermentation of meat and seafood is also carried out, especially in Southern and Central Europe, United States of America, including Asia and some parts of Africa. Fermented sausage (also called 'salami' in Italy) is the most popular and the microbial ecology indicates the important presence and technological roles of two main groups of bacteria, LAB (*Lac. sakei*, *Lac. curvatus*, *Lac. paracasei*, *Lac. plantarum*, *Lac. pentosus*, *Lac. buchneri*, *Lac. brevis*, *Lac. rhamnosus*, *Lac. alimentarius*, *Lac. farciminis*, *Ped. pentosaceus*, *Ped. acidilactici*, *Leu. mesenteroides*, *Leu. pseudomesenteroides*, *Leu. carnosum*, *Leu. gelidum*), and Gram-positive coagulase-negative cocci (CNS: *Staphylococcus xylosum*, *Sta. carnosus* subsp. *carnosus*, *Sta. pasteurii*, *Sta. warneri*, *Sta. saprophyticus*, *Sta. epidermidis*, *Sta. equorum*, *Sta. simulans*, *Sta. sciuri*, *Sta. succinus*, *Kocuria varians*) (Greppe et al., 2015; Rantsiou et al., 2005; Villani et al., 2007). *Can. famata*, *Deb. hansenii*, *Willopsis saturnus*, *Pen. nalgiovense*, *Pen. chrysogenum* and *Pen. camemberti* have also been found to be present. Other sausage-like fermented meat products are alheira in Portugal, sucuk in Turkey, nham in Thailand, nem chua in Vietnam, Taiwanese ham, wakalim in Ethiopia, jamma, arjia and karyong in India (Albano et al., 2008; Bacha et al., 2010; Kesmen et al., 2012; La Anh, 2015; Oki et al., 2011; Tu et al., 2010).

Fermented fish are numerous and predominantly produced in South and Southeast Asian regions, with a few of them in Africa and some other parts of the world. They include salted fermented fish sauce (e.g. budu, kecap ikan/bakasang, nam-pla, ngan pyaye, nuoc



mam, patis, yu-lu, shottsuru, ishiru, jeotkuk, garos), and fish pastes (bagoong, belacan, hentak, jaadi, kapi, kung chao, mehiawah, ngapi, nar-ezushi, pla ra, trassi, tungtap (Jung et al., 2013; Thapa et al., 2004). Fermented fish foods that are neither sauce nor paste, with or without salt, and sometimes sun-dried include jeotgal, hákarl, surströmming, rakÖrret, fessiekh, ngari, pedah, balao balao, guedj, bonome, shiokara, pekasam, kung som (Devi et al., 2015; Guan et al., 2011; Sanchart et al., 2017).

### Alcoholic Drinks and Other Miscellaneous Fermented Foods

Non-cereal alcoholic beverages and miscellaneous fermented food products are available in various parts of the world, and are produced from different food raw materials. For example, wine made from grape juice/must, is consumed throughout the world, and fermentation occurs through a divergent community species and strains of yeasts, although predominantly by *Sac. cerevisiae* strains (Perrone et al., 2013). LAB, mostly *Oenococcus oeni*, is responsible for secondary or malolactic fermentation (MLF) that involves conversion/decarboxylation of dicarboxylic L-malic acid found in grape juice to monocarboxylic L-lactic acid (Liu, 2002; Osborne and Edwards, 2005). Technologically, *Oen. oeni* population in wines usually cause reductions in pH below 3.5, resulting in a softer-tasting wine; ensures microbial stability, and evolution of various sensory changes, due to production of a number of secondary metabolites (Dicks and Endo, 2009; Swiegers et al., 2005). *Oen. oeni* has been described as the preferred starter culture for MLF, whereas other wine-related LAB are known to cause spoilage in wine. This is because *Oen. oeni* has higher tolerance to wine conditions, such as pH < 3.5 and ethanol concentration > 10% and is less prone to off-flavour production (Versari et al., 1999).

Traditional wines, beers and alcoholic drinks, prepared from carbohydrate-rich substrates and indigenous fruits are particularly popular in Africa, South America and some other parts of the world. These include palm wine from palm sap/juice, *tej* (honey wine in Ethiopia), *mbege*, *urwarwa* and *isongo* (banana beer in Tanzania and Burundi), *marula*, *murara*, *mutandavira*, *masau* and *mudetemwa* fruit wines and beer in Zimbabwe, *basi* and *cachaça* (fermented sugar cane juices), *kanji* and *aloja* (alcoholic beverages from carrot and carob beans respectively), *caxiri*, *pulque* etc (Aloys and Angeline, 2009; Escalante et al., 2008; Gadaga et al., 1999; Mulaw and Tesfaye, 2017; Nyanga et al., 2013; Santos et al., 2012). Apart from black tea that is popularly consumed around the world, traditional fermented tea products such as *miang*, *fuzhuan brick*, *puer* and *kombucha* are also found in Asia. Other miscellaneous fermented products are coffee and cocoa beans in chocolate manufacturing, *nata de coco* and *nata de piña*.

## Health-Promoting Effects of Fermented Foods and Beverages

### Production of Antimicrobial Compounds Against Pathogenic Microorganisms

In starchy food materials, LAB species typically secrete  $\alpha$ -amylase enzyme, which hydrolyzes complex carbohydrates into fermentable sugars such as glucose and maltose. Upon sugar metabolism, LAB produce lactic acid as the principal metabolite but bacteriocins, a group of antimicrobial proteins or peptide compounds that inhibit closely related bacterial genera and other unrelated microbial species are also released. The production of lactic acid causes pH reduction to below 4.2 in acidic fermented foods and beverages, facilitating the inhibition and/or elimination of the onset and growth of food spoilage microorganisms and food-borne pathogens, which are known causative agents of several food-borne diseases and human illnesses (Giraffa, 2004; Holzapfel, 1997). Lactic acid-fermented food products containing viable bacterial cultures and metabolites, and their potential health benefits in diseases control have been previously identified.

Odugbemi et al. (1991) demonstrated the effective control of enteropathogenic *Escherichia coli* (EPEC), *Salmonella typhi* and *Sal. paratyphi* in *ogi*, a lactic acid cereal gruel used for infant feeding in West Africa. Mbugua and Njenga (1992) reported the antimicrobial activities of LAB against diarrhoea-causing bacteria – *Sal. typhi*, EPEC and *Shigella dysenteriae* in *uji*. Other studies on *mahewu*, *kenkey*, *ikii*, *bushera* and *togwa*, revealed the inhibition of the proliferation of food-borne pathogens and disease-causing bacteria such as *Campylobacter jejuni*, *Shi. flexneri*, EPEC and coliforms (Kalui et al., 2009; Kingamkono et al., 1998; Mensah et al., 1988, 1991; Muyanja et al., 2003; Nout et al., 1989; Simango and Rukure, 1991). An optimized *ogi*, 'DogiK', prepared with lactobacilli strain starter cultures, was developed for the control of infantile diarrhoeal disease in Nigeria (Olukoya et al., 1994). In a related study, Adebolu et al. (2007) confirmed the anti-diarrhoeal potential of *ogi* liquor harbouring *Lactobacillus* species, with respect to *Shi. dysenteriae*, *Sal. typhimurium*, *Esc. coli*, *Sta. aureus* and *Enterobacter* spp. Bacteriocinogenic *Lac. plantarum* strains from *ben-saalga* was found to exhibit broad spectra antimicrobial properties against food-borne pathogenic strains of *Bac. cereus*, *Ent. faecalis*, *Listeria innocua*, *Lis. monocytogenes*, *Sta. aureus* and *Sal. enterica* (Omar et al., 2006). In like manner, bacteriocin-producing *Lac. plantarum*, *Ent. faecium* and *Leu. lactis* in *boza* demonstrated bactericidal effects regarding *Esc. coli*, *Klebsiella pneumoniae*, *Listeria* spp. *Pseudomonas aeruginosa* and *Staphylococcus* spp. (Todorov, 2010).

There are various reports on the health-promoting functions of LAB in fermented milk foods, fruits and vegetables. Yoghurt and acidophilus-fermented milk containing *Lac. acidophilus* were effectively used in the treatment of gastrointestinal tract (GIT) disorders, including colitis, diarrhoea and constipation (Sanders, 1993). Olasupo et al. (1999), Ghrairi et al. (2004) and Mitra et al. (2010) isolated a nisin Z *Lac. lactis* strain from *wara* (short form of *warakanshi*), *rigouta* and *dahi* respectively, which possessed anti-listerial characteristics, by inhibiting *Lis. innocua*, *Lis. monocytogenes*, as well as *Clostridium butyricum*, *Clo. perfringens*, *Bac. cereus* and *Sta. aureus*. Another *Lac. lactis* strain from goat cheese whey in Brazil likewise showed anti-listerial properties (Chaves de Lima et al., 2017). LAB present in Iranian, Turkish and Algerian fermented dairy foods produced bacteriocin and bacteriocin-like inhibitory substances (BLIS) against *Lis. monocytogenes*, *Lis. innocua*, *Sal. enteritidis*, *Sta. aureus*, *Ent. faecalis*, *Bac. cereus*, *Sta. epidermidis*, *Esc.*

*coli* and *Yersinia enterocolitica* (Aslim et al., 2005; Iranmanesh et al., 2014; Mezaini and Bouras, 2013). Bacteriocin-producing LAB screened from *kurut*, a Chinese traditional fermented *yak* milk was antagonistic towards *Sta. aureus* and *Esc. aerogenes* (Luo et al., 2011). *Lac. lactis* subsp. *lactis* biovar. *diacetylactis* strain was used alone as starter culture or in combination with *Can. kefir*, to produce traditional fermented milk in Zimbabwe; this retarded the growth and survival of *Esc. coli* and *Sal. enteritidis* strains originating from human clinical samples (Mufandaedza et al., 2006). Similarly, *Lac. lactis* subsp. *lactis* bacteriocin was applied *in situ* for the biological control of *Lis. monocytogenes* in *jben* (Benkerroum et al., 2000). A crude extract containing antimicrobial peptide of milk fermented with *Lac. plantarum* 26, displayed antagonistic characteristic towards food-borne pathogens, particularly *Lis. innocua* (Aguilar-Toalá et al., 2017). Pediocin, a bacteriocin, produced by a strain of *Pediococcus* in *kimchi*, was characterized to have bactericidal effects, and was resistant to *Micrococcus luteus*, *Clo. perfringens*, *Lis. monocytogenes*, *Sta. aureus*, *Esc. coli*, *Shi. flexneri* and *Sal. typhimurium* (Kwon et al., 2002). Lee et al. (2009) investigated the growth inhibitory effects of LAB species from *kimchi* on food-borne pathogens, and found strong antimicrobial activities against *Lis. monocytogenes*, *Sta. aureus*, *Esc. coli* and *Sal. typhimurium*. Fruits and vegetables fermented by lactobacilli strains in India and Romania showed antimicrobial activities against *Lis. monocytogenes*, *Esc. coli* (including ESBL strains), *Sta. aureus* and multi-drug resistant *Sta. aureus* (MRSA) strains (Grosu-Tudor and Zamfir, 2013; Patel et al., 2014).

An intervention programme on hospitalized children suffering from acute diarrhoea was conducted in New Delhi, India. The consumption of *dahi*, containing *Lac. lactis*, *Lac. lactis* subsp. *cremoris* and *Leu. mesenteroides* subsp. *cremoris* starter strains, significantly reduced the mean duration of diarrhoea in a randomized, double-blind study (Agarwal and Bhasin, 2002). *In vitro* screening of enterococci from *dahi* showed antimicrobial inhibition of food-borne pathogens, such as *Lis. monocytogenes*, *Sal. typhi*, *Sta. aureus* and *Shi. dysenteriae* (Gupta and Malik, 2007). Historically, the Maasai nomadic communities in Kenya have been consuming *kule naoto*, for the treatment of diarrhoea and constipation (Mathara et al., 2004).

In addition to the antibacterial activities of LAB in fermented foods, their antifungal properties have also been documented. Mould growth and mycotoxin production in food substances pose serious health risks and concerns to the consumers (Batish et al., 1997). Aflatoxins (AFs) and ochratoxins (OTs) are among the most potent mycotoxins reported so far with carcinogenic, mutagenic, teratogenic, neurotoxic, nephrotoxic, immunosuppressive and estrogenic effects when consumed, even at low concentrations (Bennett and Klich, 2003; IARC, 1993; Nwagu and Ire, 2011). Studies have shown the potentials of LAB as bio-protective cultures, in controlling or preventing mould growth and development, and their mycotoxins production in various fermented food matrices, thereby conferring significant health benefits on them.

Roger et al. (2015) confirmed the inhibition of the growth of *Asp. flavus* and its aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) metabolism by divergent LAB strains in *kutukutu*, a fermented maize dough in northern Cameroon. Carboxylic acids synthesized by *Lac. plantarum* FST1.7 and *Lac. brevis* R2Δ starter strains during wort fermentation were antagonistic against spores of mycotoxin-producing *Fusarium culmorum* (Peyer et al., 2016). Strains of *Lac. brevis* from *katak* (a yoghurt-like drink) in Bulgaria showed broad spectrum antifungal activities, suppressing the growth of carcinogenic *Asp. niger*, *Asp. awamori* and *Pen. claviforme*, and partially inhibiting mycelial growth and conidia germination of *Asp. flavus* (Tropcheva et al., 2014). Hassan and Bullerman (2008) earlier reported the anti-mycotoxigenic potentials of *Lac. paracasei* isolated from sourdough bread culture, against several species of *Aspergillus*, *Penicillium* and *Fusarium*. *Lac. brevis*, *Lac. plantarum* and *Lac. sanfranciscensis* strains used as starter cultures for cocoa fermentation, exhibited antifungal properties in the control of ochratoxinogenous *Asp. ochraceus*, *Asp. niger* and *Asp. carbonarius* (Essia Ngang et al., 2015). LAB present in naturally fermented *amahewu* and other fermented maize meal, potentially reduced AFB<sub>1</sub>, fumonisin B<sub>1</sub> (FB<sub>1</sub>) and zearalenone (ZEA) to undetectable levels (Chelule et al., 2010; Mokoena et al., 2005). The ability of some lactobacilli species, originating from fermented dairy foods, to bind AFB<sub>1</sub> was assessed. Specifically, *Lac. amylovorus* and *Lac. rhamnosus* strains bind more than 50% AFB<sub>1</sub> throughout a 72-h incubation period (Peltonen et al., 2001). Generally, the anti-mycotic compounds of LAB that make them active against mycotoxigenic moulds, include lactic acid, indole lactic acid, phenolic acid, phenyllactic acid, 4-hydroxy-phenyllactic acid, 3-(R)-hydroxydecanoic acid, 3-hydroxy-5-cis-dodecenoic acid, 3-(R)-hydroxydodecanoic acid, 3-(R)-hydroxytetradecanoic acid, cyclo (L-Phe-trans-4-OH-L-Pro), cyclo (L-Phe-L-Pro) and 3-hydroxylated fatty acids (Crowley et al., 2013; Haskard et al., 2001; Lavermicocca et al., 2000; Sjögren et al., 2003).

The production of antimicrobial compounds against pathogenic microorganisms, which cause various human diseases and other health related issues, has also been reported in fermented food products that are not lactic acid fermented. One example is the alkaline pH-fermented protein-rich legume foods that are widely consumed in West Africa sub-region and Southeast Asia. A number of traditional alkaline-fermented food condiments in West Africa like *bikalgá*, *maari*, *okpehe* and *soumbala* have been found to possess health beneficial functions. They contain vegetative cells of *Bac. subtilis*, *Bac. pumilus*, *Bac. amyloliquefaciens* ssp. *plantarum*, *Bac. subtilis* subsp. *subtilis* and *Bac. licheniformis* that are capable of producing inhibitory peptide and antibiotic compounds. These substances such as iturin, fengycin, surfactin, difficidin, macrolactin, bacillaene, bacilysin, subtilisin, subtilosin A, subtilin, sublan-cin and ericin, showed broad spectrum antagonistic properties towards *Mic. luteus*, *Sta. aureus*, *Bac. cereus*, *Ent. faecium*, *Lis. monocytogenes*, *Esc. coli*, *Sal. typhimurium*, *Shi. dysenteriae*, *Yer. enterocolitica*, *Asp. ochraceus* and *Shi. flexneri* (Compaoré et al., 2013a; b; Kaboré et al., 2012; Oguntoyinbo et al., 2007; Ouoba et al., 2007). *Bac. natto* TK-1 and *Bacillus* strains from Japan, Korea and Thai fermented soybean foods, *natto*, *chungkookjang* and *thua-nao* respectively, produced heterogenous and bio-surfactant lipopeptides against *Bac. cereus*, *Lis. monocytogenes* *Ent. faecalis*, *Sal. typhimurium*, *Esc. coli* and *Sta. aureus*, including inhibition of *Asp. flavus* growth and significant detoxification of AFB<sub>1</sub> and ochratoxin A (OTA) by more than 70% (Cao et al., 2009a; Lee et al., 2016; Petchkongkaew et al., 2008). *Bac. subtilis* HJ18–4 isolated from buckwheat *sokseongjang*, a traditional Korean fermented soybean food, produced an antimicrobial peptide against *Bac. cereus*, causing the down-regulation of expression of diarrhoeal and enterotoxin genes (Eom et al., 2014).



Two strains of *Ent. faecium*, LMG 19827 and 19828 identified in Malaysian *tempe* produced enterocins that inhibited *Lis. monocytogenes* growth (Moreno et al., 2002). The antimicrobial potentials of Indonesian *tempe* was earlier demonstrated by its protective effects against diarrhoeal EPEC and enterotoxigenic *E. coli* (ETEC), with health beneficial functions in diarrhoea prevention, control and management among children (Karyadi and Lukito, 2000; Kiers et al., 2003; Kiers et al., 2002). Bacteriocin-producing *Bac. coagulans* was found in *ngari*, which inhibited *Bac. cereus*, *Sta. aureus* and *Mic. luteus* (Abdhal et al., 2015). A peptide antibiotic, polyxin, from *Paenibacillus polymyxa*, isolated from Argentinean fermented sausage, was previously found to be antagonistic to *Esc. coli*, *Sal. newport*, *Serratia marcescens*, *Sta. aureus*, *Kle. pneumoniae*, *Bac. thuringiensis israeliensis* and *Bac. cereus* (Piuri and Ruzal, 1998). Another *Pae. polymyxa* strain isolated from *kimchi*, co-produced a lantibiotic (polymyxin E1) and an antimicrobial peptide (paenibacillin) that were active against *Clo. sporogenes*, *Sta. aureus* and *Listeria* spp. (He et al., 2007).

### Bioactive Compounds Synthesis

Different biologically active compounds, synthesized during food fermentations, either as metabolites of wild-type microbial strains/starter cultures or as substances released from the hydrolysis of organic components of food substrates, are widely associated with various functional health-promoting benefits. These bioactive compounds usually have antimicrobial (already discussed above), antihypertensive, antioxidant, anti-diabetic, anti-mutagenic, anti-cancer, anti-tumour effects and fibrinolytic activities.

#### Antihypertensive

Angiotensin converting enzyme-inhibitory (ACE-I) peptides are among the bioactive peptides formed during food fermentation by the action of proteolytic enzymes (i.e. proteases) on the native proteins present in many protein-based food substrates. They are not digested by the GIT digestive enzymes (e.g. trypsin, pepsin and chymotrypsin), and inhibit the enzyme responsible for converting angiotensin I to angiotensin II, a potent vasoconstrictor that causes re-absorption of water and sodium ions, thereby affecting the electrolyte balance, volume and blood pressure (BP) (Hartmann and Meisel, 2007; Rai et al., 2017). The inhibition of angiotensin converting enzyme (ACE) by ACE-I peptides is suggested to be made possible by the presence of hydrophobic (aromatic or branched side chain: Tyr, Phe, Trp, Ala, Ile, Val and Met) and positively charged amino acids (Arg and Lys), including Pro at the C terminal of ACE-I peptides, which show affinity for ACE protein (Haque and Chand, 2008; He et al., 2012; Rai et al., 2017). In addition to converting angiotensin I to angiotensin II, ACE also inactivates bradykinin and kallidin, two important vasodilators, which leads to increased BP, and risk of hypertension, including other cardiovascular diseases (CVD), strokes, etc (Sanjuka and Rai, 2016). Investigations on ACE-I peptides production are mostly on fermented milk products and legumes, and there are reports of their ACE inhibition, to cause vasodilator effects, which lowers BP. They are thus gaining wide popularity as antihypertensive agents in prophylactic medicine.

An *in vitro* spectrophotometric analysis is most commonly used for the evaluation of ACE-I activities. Hippuryl-His-Leu (HHL) serves as the substrate, which is hydrolysed by ACE to produce hippuric acid and His-Leu. ACE-I peptides in the water-soluble fraction, produced by bacterial cell wall proteinase enzymes system, prevents this reaction from taking place. ACE-I peptide activity is then expressed as the percentage of ACE inhibition or as the minimum concentration of peptide to inhibit 50% of ACE activity, the IC<sub>50</sub> (Hernández-Ledesma et al., 2011). Several LAB strains have been screened for high proteinase and ACE-I activities of dipeptides, tripeptides and oligopeptides liberated from milk proteins,  $\alpha$ <sub>s1</sub>-casein and  $\beta$ -casein, as a strategy for the development of fermented milk foods with antihypertensive properties. Empirical studies on different LAB dairy and non-dairy starter cultures, alone or in combination with yeasts species, including wild-type strains, to ferment milk, for yoghurt, cheese and other traditional fermented milk products, as well as their specific ACE-I peptide sequences, properties and IC<sub>50</sub> values after fermentation and during storage, have been documented (Beltrán-Barrientos et al., 2016; Rai et al., 2017). Li et al. (2017) characterized the ACE-I peptides in milk fermented with *Lac. casei* strains. More than half of the strains produced fermented milks with ACE-I activity of over 60%, and maximum Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) concentration of  $6.60 \pm 0.25$   $\mu$ mol/L. Goat and camel milk fermented by *Lac. plantarum* 69 and *Lac. rhamnosus* MTCC 5945 (NS4), respectively had ACE-I activities up to 78.09% and 91.62%, under optimum fermentation conditions (Chen et al., 2018; Solanki and Hati, 2018).

To validate the *in vitro* antihypertensive potential of ACE-I peptides, animal models, using spontaneously hypertensive rats (SHR) and clinical trials of human subjects are conducted, measuring reduction or drop in systolic blood pressure (SBP) or diastolic blood pressure (DBP), after oral or intravenous/intra-peritoneal administration. Earlier works on fermented milk products, like *calpis* sour milk in Japan, fermented with *Lac. helveticus* and *Sac. cerevisiae*, and containing tripeptides VPP and IPP, showed hypotensive effects and decrease in ACE tissue activity of SHR (Nakamura et al., 1995; Nakamura et al., 1996). Milk fermented with *Lac. lactis* strains NRRL B-50571 or NRRL B-50572 had similar reductions in SBP and DBP of SHR, in comparison with captopril administration (Rodríguez-Figueroa et al., 2013). A single oral dose of *Lac. helveticus* H9 in fermented milk significantly lowered the systolic, diastolic and mean blood pressure of SHR (Chen et al., 2014). Increase in frequency unit of Gamalost cheese consumption, rich in ACE-I peptides, among Norwegian population, corresponded to a reduction in SBP of 0.72 mm Hg (Nilsen et al., 2014). Beltrán-Barrientos et al. (2018) examined the BP-lowering effect of milk fermented by *Lac. lactis* NRRL B-50571 in a double blind, randomized controlled, clinical trial of pre-hypertensive patients, administered daily for 5 wk, and observed reductions in SPB and DBP, in addition to triglyceride, total cholesterol and low density lipoprotein in blood serum. Other *in vivo* studies involving SHR and human subjects on both short- and long-term antihypertensive effects of fermented milk peptides are available in Beltrán-Barrientos et al. (2016) and Rai et al. (2017) reviews.

Similarly, protein-rich legume fermented seeds, particularly soybean, containing glycinin and  $\beta$ -conglycinin protein fractions, have also been reported to possess ACE-I peptides. But while information is available on the antihypertensive activities of different fermented soybean foods consumed in Asia, little or none is known about closely related fermented foods in Africa. For instance, an antihypertensive peptide identified in *natto*, fermented with *Bac. subtilis natto* O9516, showed *in vitro* ACE-I activity, and *in vivo* reduction of SBP within 5 h of single dose oral administration in SHR (Ibe et al., 2009). Extracts of *tofu* composed of Ile-Phe-Leu and Trp-Leu peptide sequences were resistant to GIT digestive enzyme treatments and had good ACE-I activity (Kuba et al., 2003). Toshiro et al. (2004) reported *chunggugjang* soy product to possess antihypertensive peptides, which when administered in volunteer human subjects reduced SBP and DBP by 15 mm Hg and 8 mm Hg, respectively after 2 h. ACE-I activity was recorded in *suifu*, fermented with fungal strain, which correlated with peptide content, and increased during fermentation and maturation (Ma et al., 2013). Pigeon pea seeds [*Cajanus cajan* (L.) Millsp.] fermented with a strain of proteolytic *Asp. niger*, produced an ACE-I octapeptide Val-Val-Ser-Leu-Ser-Ile-Pro-Arg, which had competitive inhibition against *in vitro* ACE activity (Nawaz et al., 2017). Fermented soymilk products with strains of *Lac. casei*, *Lac. acidophilus*, *Lac. bulgaricus*, *Ent. faecium* and *Bifidobacterium longum* have been shown to possess ACE-I properties (Martinez-Villaluenga et al., 2012; Tsai et al., 2008). In addition to milk and legume, fermented fish sauce from salmon, sardine, anchovy, blue mussel and oyster in Asia were reported to contain ACE-I peptides. A purified peptide from fermented blue mussel significantly reduced BP in SHR by oral administration (Je et al., 2005).

Gamma-aminobutyric acid (GABA) is another peptide compound with hypotensive activity, in addition to other physiological functions such as relaxation, sleep enhancement (opioid), anti-depression, enhanced immunity, anti-diabetic, anti-cancer and anti-obesity. GABA also possesses anti-inflammatory, pro-neurotransmitter, menopausal syndrome relief, activation of liver and kidney function, amelioration of oxidative stress, as well as treatments of Parkinson's disease, seizures, Alzheimer's disease, stiff-man syndrome and schizophrenia (Wong et al., 2003). It is a non-protein four-carbon free amino acid (FAA), synthesized by the irreversible  $\alpha$ -decarboxylation of L-glutamic acid or its salts, i.e. monosodium glutamate (MSG), catalysed by glutamic acid decarboxylase (an enzyme found in bacteria, moulds and yeasts), in the presence of pyridoxal 5' phosphate cofactor (Shelp et al., 1999). Evidence of GABA hypotensive effect on SHR was established in *Lac. plantarum*-fermented skim milk diet, where SBP and DBP were significantly decreased (Liu et al., 2011). *Lac. plantarum* produced 77.4 mg/kg of GABA in an enriched functional fermented milk food; this increased in concentration to 144.5 mg/kg, in combination with other LAB strains, and was recommended for mild hypertensive condition (Nejati et al., 2013). GABA concentration of 10–12 mg in 100 mL of milk fermented by *Lac. casei* strain Shirota and *Lac. lactis* YIT 2027, significantly decreased BP when fed two or four weeks in a randomized, placebo-controlled trial with mild hypertensive patients as participants (Inoue et al., 2003). *Lac. lactis* ssp. *lactis* improved the GABA content of cheese (16 mg of GABA/50 g cheese), which decreased BP by 3.5 mm Hg in human subjects (Pouliot-Mathieu et al., 2013). GABA and nattokinase in *Bac. subtilis* B060-fermented beans significantly lowered SBP and DBP in SHR and Wistar-Kyoto rats (Suwanmanon and Hsieh, 2014).

### Antioxidant

Free radicals (i.e. atoms or molecules with an unpaired electron) and reactive oxygen species (ROS), such as superoxide anion radicals ( $O_2^{\cdot-}$ ), hydroxyl radicals ( $HO^{\cdot}$ ), hydrogen peroxide ( $H_2O_2$ ) and singlet oxygen ( $^1O_2$ ) are frequently generated in the human body during various metabolic processes and environmental stresses, besides those consumed in oxidized edible fats and oils. These free radicals play significant roles in cell signalling, apoptosis, gene expression and ion transportation (Lü et al., 2010). However, oxidative stress occurs when these molecules are produced in excess and/or there is lack of cellular defences against them, leading to oxidation of proteins and lipids, DNA mutation, cell and tissue disruption, permanent damage, and eventually death, as well as oxidative modification of low density lipoproteins (LDL) (Hu et al., 2004). Consequent upon this is the development of a number of degenerative diseases e.g. CVD (atherosclerosis), cancer, tumour growth, diabetes, arthritis, increase in blood cholesterol level, Alzheimer's and Parkinson's diseases (Afonso et al., 2007). Though not enough, the human system has non-enzymatic, i.e. reduced glutathione (GSH) and enzymatic antioxidants in the form of superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT), as defence and repair mechanisms against oxidative damages (Miller and Britigan, 1997). To alleviate oxidative stress, hydrolysed antioxidative peptides, FAA, free polyphenols (intermediates of  $\beta$ -glucosidase hydrolysis of polyphenols), genistein and daidzein (isoflavones), malvidin and delphinidin (flavonoids) and aglycones, which are naturally enhanced in fermented foods, can chelate metal ion, scavenge free radicals (by a way of proton or  $H^+$  donation) and quench singlet oxygen (Mathew and Abraham, 2006). For peptides, the radical scavenging activity (RSA) is supported by the side chain groups of the amino acids residues, i.e. imidazole, indole and phenol in His, Trp and Tyr, respectively (Guo et al., 2009). Therefore, these bioactive compounds can serve anti-cancer, anti-tumour, anti-mutagenic and anti-diabetic purposes.

Fermentation of buckwheat, wheat gram, barley and rye with *Lac. rhamnosus* and *Sac. cerevisiae*, compared to their unfermented equivalents, led to increase in total phenolic content (TPC), and antioxidant activities (AOA) as assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity, ferric ion-reducing antioxidant power (FRAP) and thiobarbituric acid (TBA) methods (Đorđević et al., 2010). Ethanolic extract of wheat *koji* prepared with *Asp. oryzae* and *Asp. awamori nakazawa* greatly increased the TPC and free RSA (Bhanja et al., 2009). Fermentation of adlay, chestnut, lotus seed and walnut cereal grains by food-grade *Bac. subtilis* and *Lac. plantarum* increased the phenolic and flavonoid contents of the methanolic extracts, with a stronger DPPH radical scavenging and FRAP activities (Wang et al., 2014). Solid-state fermentation (SSF) of wheat improved the water-soluble TPC and antioxidant property. There was a 14-fold improvement in TPC in *Asp. oryzae*-fermented wheat, as well as 6.6 and 5.0-fold enhancements of DPPH and ABTS radical scavenging, respectively in *Rhi. oryzae*-fermented wheat (Dey and Kuhad, 2014). The

TPC, total flavonoids and AOA were significantly enhanced in ethyl acetate extracts of SSF-fermented wheat using *Asp. oryzae* var. *effuses*, *Asp. oryzae* and *Asp. niger* (Cai et al., 2012).

During milk fermentation in the presence of *Leu. mesenteroides* ssp. *cremoris*, *Lac. jensenii* and *Lac. acidophilus* strains, antioxidative peptides released (4–20 kDa) were responsible for RSA and inhibition of lipid peroxidation (Virtanen et al., 2007). Low molecular weight bioactive peptides and FAA (His, Tyr, Thr and Lys) in commercial yoghurt provided antioxidant activities by inhibiting oxidation in a liposome model, in addition to possession of strong DPPH radical scavenging and high  $\text{Fe}^{2+}$  chelation (Farvin et al., 2010). An antioxidative undecapeptide (Ala-Arg-His-Pro-His-Pro-His-Leu-Ser-Phe-Met) isolated from milk fermented with *Lac. delbrueckii* subsp. *bulgaricus* strain demonstrated scavenging activity against DPPH radical (Kudoh et al., 2001). Soy whey fermented using *Lac. plantarum* B1-6 when compared to unfermented, possessed more TPC and isoflavone aglycone, higher ABTS, hydroxyl and superoxide RSA, ferric reducing antioxidant power, and greater protection against oxidative DNA damage (Xiao et al., 2015). Milk-kefir and soymilk-kefir fermented using kefir grains LAB and yeasts strains showed significant anti-mutagenic property against different mutagens, as a result of scavenging activity against DPPH radicals, inhibition of linoleic acid peroxidation and ferrous ion chelation (Liu et al., 2005).

Fermentation of soybeans to produce *tempe*, *natto*, *kinema* and *douchi* by mould or bacterial strains has also led to antioxidant effects in the methanolic extract or water-soluble fractions of these food products. Enhancement in TPC, DPPH scavenging activity,  $\text{Fe}^{3+}$  reducing power,  $\text{Fe}^{2+}$  chelation, inhibition of lipid peroxidation and oxidation of LDL correlated with increased FAA, peptide content, free isoflavones and phenolic acids, protease and  $\beta$ -glucosidase activities, in most of the investigations, suggesting their potential to mitigate oxidative stresses (Sanjukta and Rai, 2016). Earlier, free soluble phenol in fermented underutilized legume seeds significantly enhanced reducing power, DPPH scavenging ability and inhibition of lipid peroxidation more than the bound phenols (Obboh et al., 2009). The ethanolic extract of *Bac. subtilis* or *Asp. oryzae* fermented red beans decreased MDA as well as increased GSH and SOD in the liver tissue of Sprague–Dawley rats, while only *Bac. subtilis* extract increased the levels of ascorbic acid and  $\alpha$ -tocopherol in the liver tissue; *Asp. oryzae* also increased ascorbic acid in the brain tissue better than the control (Chou et al., 2008). In an attempt to demonstrate the beneficial functions of antioxidant compounds in the management of diabetes mellitus (DM), Lim et al. (2012) assessed the *in vivo* anti-diabetic potential of fermented soybean extract with a *Bac. subtilis* strain, previously isolated from *chungkookjang*. Intra-peritoneal administration of the extract caused significant reduction in the plasma glucose level in addition to significant increases in plasma insulin level and activities of SOD, GSHPx, CAT and malondialdehyde (MDA) in streptozotocin (STZ)-induced diabetic rats, suggesting hyperglycemia inhibition (i.e. hypoglycemic action), due to the protection of pancreatic  $\beta$ -cells from free radical-mediated oxidative stress.

### Anti-diabetic

Both *in vitro* and *in vivo* anti-diabetic effects of *meju* and *chungkookjang* fermented soybean products, rich in isoflavonoid aglycones and small peptides have been investigated. While peptide fractions in *chungkookjang* slightly enhanced glucose-stimulated insulin secretion, daidzein extract in *meju* and *chungkookjang* better improved insulin-stimulated glucose uptake by activating peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) in 3T3-L1 adipocytes than unfermented soybeans. Furthermore, mouse insulinoma (Min6) cells treated with genistein and peptides had greater glucose-stimulating insulin secretion capacity, as genistein and daidzein stimulated glucagon-like peptide-1 (GLP-1) secretion in enteroendocrine NCI-H716 cells, generating insulinotropic actions (Kwon et al., 2006, 2011). Experiments with type-2 diabetic male Sprague-Dawley (SD) rats fed *meju* and *chungkookjang*, prepared with microbial starter strains, significantly improved glucose homeostasis and tolerance, by way of glucose-stimulating insulin secretion and increased pancreatic  $\beta$ -cell mass, than the unfermented products (Yang et al., 2013; Yang et al., 2012). Fermented soybean diets, enriched with phenolic compounds, in STZ-induced diabetic rats reduced blood glucose, thiobarbituric acid reactive species (TBARS) contents, pancreatic MDA,  $\alpha$ -amylase, intestinal  $\beta$ -glucosidase and acetylcholinesterase activities, with corresponding increase in pancreatic glutathione peroxidase (GPx) and GSH (Ademiluyi et al., 2014, 2015).

### Anti-cancer

Apart from the lunasin anti-cancer peptide present in soybean, only very few reports are available on the anti-cancer compounds produced during food fermentation. Hydrophobic peptides in Korean traditional soy sauce displayed anti-tumour activity by their cytotoxic effects on different *in vitro* cell lines, including human colon cancer cells (Kim et al., 1998). *Bac. subtilis* natto T-2 and *Bac. natto* TK-1 in *natto* produced cyclic lipopeptide and lipopeptide bio-surfactant respectively, which induced apoptosis in human leukemia cells and inhibited the proliferation of human breast cancer cells (Cao et al., 2009b; Wang et al., 2007). Also, a surfactin-like compound from *Bac. subtilis* CSY 191-fermented *cheonggukjang* resulted in growth suppression of human breast cancer (MCF-7) cells (Lee et al., 2012). In camel milk fermented with *Lac. lactis* and *Lac. acidophilus* strains, the water-soluble extract ( $\leq 3$  kDa) significantly inhibited proliferation of Caco2, MCF-7 and HELA carcinoma cell lines (Ayyash et al., 2018). Fractionated peptides released by *Lac. helveticus* in fermented milk suppressed the growth of fibrosarcoma tumours induced by methylcholanthrene crystals, and increased the number of immunoglobulin A (IgA)-secreting cells in BALB/c mice (LeBlanc et al., 2002).

### Fibrinolytic Enzymes

Developments in the study of fibrinolytic enzyme activities and its potential thrombolytic property started when Sumi et al. (1987) observed that one of the *natto* beans developed a clear zone on a fibrin plate, indicating that insoluble fibrin (i.e. blood clot) around the bean was digested by an unknown enzyme produced by *Bac. subtilis* (*natto*), which was thereafter characterized and named

nattokinase (NK). Subsequently was the report of oral administration of NK in *natto* to healthy adults, which increased fibrinolytic activity two-folds in plasma, together with fibrin/fibrinogen degradation products in serum, and tissue plasminogen activator (tPA) (Sumi et al., 1990). In furtherance to this, Fujita et al. (1995) described the passage of NK through intestinal cells of rats, showing degradation of fibrinogen and appearance of NK in the plasma. Fibrinolytic enzymes from other protein-based fermented foods apart from *natto* include: choggokkinase from *chonggakjang*, myulchikinase from *myul-chi-jeot-gal*, katsuwo kinase from *shiokara*, subtilisin DFE from *douhchi*, subtilisin DJ-4 from *doenjang*, metalloprotease from fish *jeotgal* and TPase from *tempe*. They are specific in their actions toward fibrin clots. Fibrin, the key protein constituent of blood clot, is formed following fibrinogen degradation by thrombin (Wolberg, 2007). Its presence is checked by fibrinolysis, to maintain a balance in homeostasis, by endogenous plasmin, which is activated from the non-active plasminogen by tPA. However, an imbalance situation arises in the human physiology, when there is challenge in hydrolysing fibrin. This results in its excessive accumulation in the blood vessels, which interfere with blood flow, to cause thrombosis, leading to myocardial infarction, ischemic heart disease, CVD, high BP and stroke (Mine et al., 2005).

For treatment purpose, different thrombolytic agents [e.g. urokinase, streptokinase, staphylokinase and tissue-type plasminogen activator (t-PA)] are available for clinical use, and they follow same mechanism for plasmin activation as previously described. They are however expensive, and suffer some drawbacks, such as short half-life in their specificity towards fibrin, gastrointestinal bleeding, allergic reactions, etc (Blann et al., 2002). Fibrinolytic enzymes produced by food-grade, edible microorganisms in traditional fermented foods that have the ability to degrade fibrin and inhibit thrombin, are cheap, highly specific toward fibrin, with safe records of consumption and no side effects. They have been isolated and characterized, and recommended as alternative therapy for the prevention and management of thrombosis (Kim et al., 1997; Montriwong et al., 2012; Singh et al., 2014; Stephani et al., 2017). However, much more than enzyme purification and identification, is the need for sufficient evidence-based and empirical scientific investigations to demonstrate the therapeutic efficacy of these enzymes in animal models and human subjects, involving clinical trials. Unfortunately, only very few studies exist on this aspect of therapeutic effectiveness. For example, subcutaneous administration of NK from *Bac. natto*, preceding intravenous kappa carrageenan to the tail of rats, produced infarcted regions that were significantly shorter in mean length in rats administered NK than those in control rats, signifying the anti-thrombosis prophylactic effects of NK (Kamiya et al., 2010). The fibrinolytic enzyme from *Stenotrophomonas* sp. in Indonesian soybean fermented food dissolved thrombin and reduced blood clot induced by  $\kappa$ -carrageenan injection in the tail of Wistar rats (Nailufar et al., 2016). In human subjects with cardiovascular risk factors, oral intake of NK for 2 months significantly decreased plasma levels of the CVD-associated coagulation factors of fibrinogen, factor VII and factor VIII (Hsia et al., 2009). Bacilopeptidase F preparations, a serine protease secreted by *Bac. subtilis* (*natto*) that was orally administered to human volunteers showed fibrinolytic and amidolytic activities by shortening euglobulin lysis time and positive changes in local blood flow (Omura et al., 2004).

### Production of Exopolysaccharides (EPSs)

Complex polysaccharide metabolites are generally synthesized by wild-type microorganisms, autochthonous or starter cultures that are involved in the fermentation of different food substrates. Though these polysaccharides are secreted outside the microbial cells as extracellular metabolites, they are either adherent, remaining tightly bound to the cell wall surface appendages (e.g. capsule), referred to as capsular polysaccharides (CPSs) or permanently unattached to the cell surface as EPSs. EPSs may have two forms; those loosely attached to the bacterial surface and the ones freely released to the cell's external environment, which forms mucus, ropiness and slimy materials (Badel et al., 2011). Structurally, EPSs are long-chain, high molecular weight carbohydrate polymers, consisting of branched, repeating sugar units (mainly glucose, galactose and rhamnose), substituted sugars or sugar derivatives, including substituents such as phosphate and acetyl group (De Vuyst and Degeest, 1999; Du et al., 2017). They could be homopolysaccharides (HoPSs), composed of only one repeating monosaccharide moiety (D-glucose or D-fructose of two major groups: glucans and fructans), and examples are cellulose, dextran, mutan, alternan, pullulan, levan and curdlan, or heteropolysaccharides (HePSs), comprising different sugar molecules e.g. glucose, galactose, rhamnose, mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine and glucuronic acid, to form gellan and xanthan (Fabera et al., 1998; Laws et al., 2001). Because the biosynthesis of EPSs is a complex one, and the fact that the mechanism of polymerization of the repeating unit is unclear, its discussion remains out of the scope of this review.

Other than the technological properties (i.e. viscosity, texture, rheology and firmness) of EPSs in fermented foods, they have been reportedly found to impart a number of physiological and health beneficial functions on the consumers, which include adhesion and colonization of probiotic microorganisms for competitive exclusion of food-borne pathogens, prebiotic activity, acting as a physical barrier to many pathogenic bacteria. Other health benefits include serum and blood cholesterol reduction, immunomodulation and immunostimulatory effects, antimicrobial, antioxidant, antihypertensive, anti-diabetic, anti-cancer, anti-tumour, anti-proliferative, anti-allergic, anti-ulcer, anti-viral, anti-biofilm formation of pathogens, generation of short chain fatty acids (SCFAs) upon degradation in the gut by the colon microbiota, and protection against the harsh gut environment (Caggianiello et al., 2016; Dilna et al., 2015).

Purified EPS (EPS\_DN1) produced by *Lac. kefirifaciens* in *kefir* completely inhibited *Lis. monocytogenes* and *Sal. Enteritidis* at 1% least concentration, exerting bactericidal effects against them *in vitro* (Jeong et al., 2017). EPSs of *Leu. citreum*, *Leu. mesenteroides*, *Leu. pseudomesenteroides* and *Ped. pentosaceus* obtained from Tunisian fermented foods showed pre- and post anti-biofilm activities at 1 mg/mL against *Esc. coli*, *Ent. faecalis* and *Sta. aureus*, with minimum adhesion inhibition of 86.9% and 53.4% for *in vitro* pre- and post-treatments, respectively (Abid et al., 2018).



*Lac. plantarum* LRCC5310 isolated from *kimchi* produced an EPS that had anti-rotavirus effect against human rotavirus (HRV) Wa strain. At 1.95 mg/mL, it reduced the viral RNA copy numbers significantly, when compared to the control; it also caused cytopathic effects and interference towards the viral cells, due to strong adherence to MA104 cell lines. In the *in vivo* study with the same EPS but rotavirus EDIM (RV-EDIM) strain, neonate mice pre-treated with EPS for 2 d, followed by administration of RV-EDIM together with EPS at 1 mg/mouse for 5 d, significantly lowered the number (50%) that developed RV-EDIM-induced diarrhoea than in control (Kim et al., 2018). For mice with acute diarrhoea and severe dehydration, the mean diarrhoea score and rotavirus shedding with this EPS also decreased significantly at 8 d post-infection in comparison with those in control (Kim et al., 2018). The EPS of *Lac. delbrueckii* ssp. *bulgaricus* 1073R-1 from traditional Bulgarian yoghurt at 20 µg/d orally administrated to BALB/c mice for 21 d, prior to intranasal infection, significantly decreased influenza virus (H1N1) titre, and increased its antibodies (IgA, IgG<sub>1</sub>) at 4 d post-infection, when compared to the control (Nagai et al., 2011). However, the acidic EPS (APS) prolonged the survival rate of influenza virus-infected mice, and not the neutral EPS (NPS).

EPS prepared from *Lac. plantarum* YW11 used to ferment Tibetan *kefir* showed *in vitro* AOA against hydroxyl radicals at 75% of 1.22 mg/mL, superoxide anion at 62.71% of 1.54 mg/mL, DPPH at 35.11% of 0.63 mg/mL, and 41.09% ferrous ion chelation at 1.07 mg/mL concentration. In the oxidant-induced stress experiment by subcutaneous injection of 500 mg/kg per day of 5% D-galactose in an ageing mouse, followed by 2.5 mg/mL EPS of *Lac. plantarum* YW11, there was a significant reduction in serum MDA, which reflects lipid oxidation inhibition as well as increased GSHPx, SOD, CAT and total antioxidant capacity (TAOC) activities (Zhang et al., 2017). Pyrosequencing data analysis of the gut microbiota of the ageing mouse revealed gut modulation and improvement, where *Lac. plantarum* YW11 EPS recovered the microbiome and phylotypes initially decreased or eliminated by D-galactose, with further increase in SCFAs content (Zhang et al., 2017). *Wei. confusa* OF126 strain isolated from *ogi*, having EPS of  $1.1 \times 10^6$  Da exhibited hydroxyl radical and DPPH activities of 86.5% and 67.4%, respectively at 4 mg/mL (Adesulu-Dahunsi et al., 2018). *Ent. faecium* BDU7 cultured from *ngari* was assayed for EPS; its purified form (8 mg/mL) showed significant scavenging of DPPH (63.5%), superoxide anion (77.3%) and hydroxyl (38.4%) radicals (Abdhuil et al., 2015). The antioxidant activity of a purified EPS ( $6.9 \times 10^5$  Da) from *Lac. lactis* subsp. *lactis* in Chinese pickled cabbage, revealed significant decrease in MDA and increased SOD and CAT in mice serum in a concentration-dependent manner (Pan and Mei, 2010). An EPS (LPC-1) extracted from *Lac. plantarum* C88 found in Chinese dairy *tofu*, demonstrated strong RSA of 85.21% hydroxyl radical and 52.23% DPPH at 4 mg/mL. LPC-1 also significantly inhibited the formation of MDA and exerted AOA against H<sub>2</sub>O<sub>2</sub>-induced injury in Caco-2 cells (Zhang et al., 2013).

Wang et al. (2018) characterized a neutral EPS (EPS0142) produced by *Lac. plantarum* JLK0142 from *tofu*. EPS0142 significantly induced macrophage-derived nitric oxide (NO) production in RAW 264.7 cell lines, in a dose-dependent manner, without any cytotoxic effect, as well as improved phagocytic activity. High dose of EPS0142 also administered to previously cyclophosphamide-induced immunosuppressed female BALB/c mice, significantly increased the spleen index and splenic lymphocyte proliferation, including the intestinal immunoglobulin A (sIgA) content and the levels of IL-2 and TNF- $\alpha$  cytokines. EPS extracted from milk fermented with *Lac. lactis* subsp. *cremoris* FC, and orally administered to male BALB/c mice before skin exposure to 2,4,6-trinitro-1-chlorobenzene (TNCB), significantly suppressed skin thickening induced by TNCB and penetration of mast cells in skin lesions (Gotoh et al., 2017). There was also the regulation of IL-4, IFN- $\gamma$ , IL-6 and TNF- $\alpha$  over-expression, as a result of TNCB exposure, and stimulation of bone marrow cell proliferation in dose-dependent EPS-treated Payer's patch cell of C3H/HeJ mice. EPS derived from *Lac. delbrueckii* ssp. *bulgaricus* 1073R-1 as previously described, and the respective yoghurt product, caused immunostimulation of IFN- $\gamma$  and augmentation of NK cells production in female BALB/c mice spleen cells, but not other yoghurts that also contain lactobacilli cultures (Makino et al., 2006, 2016). EPS fraction (B-EPS) from *Bac. subtilis* J92 isolated from *kimchi* increased NO, TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , and their proteins and mRNA expressions in IFN- $\gamma$ -primed RAW 264.7 macrophages cell lines, including cytokine (IL-2 and IFN- $\gamma$ ) production by CD3/CD28-stimulated splenocytes (Jung et al., 2015). In addition, post-orally administered B-EPS significantly lowered the immunosuppression effects of cyclophosphamide in mice thymus and spleen, in a concentration-dependent manner.

EPS-producing *Str. thermophilus* strains in fermented milk, as well as their purified EPS in sterile milk, prevented the development of gastritis ulcer, when previously fed to BALB/c mice for 7 d before acetyl-salicylic acid (ASA)-induced gastritis, based on histological parameters and immune responses (Rodríguez et al., 2009). Two novel homogeneous EPSs synthesized by *Lac. casei* SB27 that was previously isolated from fermented *yak* milk, significantly inhibited the proliferation of HT-29 colorectal cancer cells, as an anti-tumour agent; induced apoptosis by the activation of *caspase-3* and *-8* genes in addition to up-regulation of pro-apoptotic genes *Bad* and *Bax* (Di et al., 2017). EPS of probiotic *Ent. faecium* K1, an isolate from a traditional fermented milk product, *kalarei*, showed significant cholesterol reduction potential, lowering the concentration from 100% to 48.81% *in vitro* (Bhat and Bajaj, 2018). *Lac. delbrueckii* subsp. *bulgaricus* strains from homemade yoghurt that produced high amount of EPS, removed more cholesterol from the medium, compared to those strains with low EPS production (Tok and Aslim, 2010).

### Probiotic Properties of Fermented Foods

Probiotics are defined as preparation of live microorganisms, which when consumed in adequate amounts ( $10^7$ – $10^9$  cfu/g or mL), induce health beneficial effects by qualitatively or quantitatively influencing gut microbiota, modifying immune status and contributing to general well being of the host, beyond basic nutrition (FAO/WHO, 2002; Phippen et al., 2009). Most fermented foods and beverages contain high population of viable microorganisms, and they serve as vehicles for the delivery of probiotics. For a probiotic microorganism to exert health benefits and other positive desirable effects on the host when administered, it is expected to be

resistant to gastric acidity of the stomach and tolerant to bile salts of the small intestine, produce antimicrobial compounds against pathogenic microorganisms, adhere to GIT mucosal and epithelial cell linings, as well as *in vivo* persistence (colonization) for competitive exclusion of pathogens, in addition to a long history of safety and non-pathogenicity (Ouwehand et al., 2002).

Even though the precise mechanisms by which probiotics perform their functions in the host have not been fully elucidated, some manner of probiotic functions has been proposed. These include up-regulation of immune responses (e.g. IgA) towards pathogens or vaccines, down regulation of inflammatory responses, production of bacteriocins and SCFAs, improving gut mucosal barrier function, enhanced stability and recovery of commensal microbiota when disturbed, as well as modulation of host gene expression and delivery of functional proteins (e.g. lactase) (Sanders, 2009). A probiotic *Lac. gasseri* SBT2055 used to prepare fermented milk significantly reduced abdominal visceral and subcutaneous fat, weight and body mass index (BMI) in adults with obese tendencies, in a randomized controlled trial (Kadooka et al., 2010). Fermented milk curd containing probiotic *Lac. acidophilus*, *Lac. casei* and *Lac. lactis* biovar *diacetylactis* had anti-tumour effect in rats, inhibiting 1,2-dimethylhydrazine (DMH) colon genotoxic compound, as a result of significant reduction in DNA damage, in comparison to the control (Kumar et al., 2010). The anti-diabetic effect of *dahi* fermented with probiotic *Lac. acidophilus* and *Lac. casei* was evaluated in high fructose-induced type-2 diabetic male albino Wistar rats. *Dahi*-supplemented diet significantly reduced blood glucose, glycosylated hemoglobin, glucose intolerance, plasma insulin, liver glycogen, plasma total cholesterol, triacylglycerol, low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (vLDL-C), and blood free fatty acids that were initially increased after high fructose feeding (Yadav et al., 2007). High cholesterol diet supplemented with cereal-mix fermented food containing probiotic *Pic. kudriavzevii* OG32, significantly lowered serum total cholesterol, triacylglycerol and LDL-C in rats, when compared to the control high cholesterol feed without probiotic supplementation (Ogunremi et al., 2015). Total serum and liver cholesterol, including the atherogenic index of rats fed high cholesterol chow, supplemented with milk fermented by probiotic *Lac. plantarum* HLX37, significantly decreased by 23.33%, 32.37% and 40.23% respectively, when compared to the hyperlipidemia diet (Guan et al., 2017). Probiotic bacteria in fermented milk were able to maintain consistent microbial community shift in the human GIT, where *Bacterioidetes* species increased during the intervention programme (Unno et al., 2015). Chung et al. (2014) investigated the effects of probiotic *Lac. helveticus*-fermented milk on cognitive functions in healthy older adults, in a double-blind, randomized control experiment. Their results showed an improvement in cognitive functioning, in relation to neuropsychological and cognitive fatigue.

## Conclusion

Fermented foods and beverages constitute a significant component of human nutrition, dietary supply and calories intake in different parts of the world. Fermentation of diverse plant and animal substrates by microorganisms and their enzymes provides desirable features, such as post-harvest preservation of perishable food materials, nutritional enrichment, bio-preservative effects and specific health-promoting benefits. Nowadays, fermented foods and beverages are consumed not only for nutritional values, wholesomeness or palatability, but importantly for their health beneficial functions. Live microorganisms and/or their metabolites in fermented foods are responsible for various health-promoting properties. An example is lactic acid, the primary metabolite in non-alcoholic fermented cereal foods (pH < 4.2) that demonstrates potential health benefits, by inhibiting pathogens causing food-borne diseases and human illnesses. Bioactive peptides, free amino acids and polyphenols, flavonoids, isoflavones and enzymes, which are naturally enriched in fermented foods possess antimicrobial, antihypertensive, antioxidant, anti-diabetic, anti-cancer, anti-tumour, anti-mutagenic, anti-proliferative and anti-thrombosis health benefitting properties. In addition, exopolysaccharides (EPSs) consumed in fermented foods can serve as prebiotics; they are also metabolized by colon microbiota to produce short chain fatty acids (SCFAs), which induce apoptosis of cancer cells and stimulate immune responses in the host. Furthermore, fermented foods contain viable probiotic microorganisms that confer health benefits on the host. However, to justify their development as functional foods and nutraceuticals, there is need for further and detailed scientific investigations on the in-depth characterization of the bioactive compounds and mechanism of actions in animal models and human intervention programmes, involving clinical trials.

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# Hypoallergenic Foods: Development and Relevance in the Management of Food Allergy

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## Glossary

**Antigen-presenting cells (APCs)** specialized cells that process antigens and display their peptide fragments on the cell surface.

**CD4<sup>+</sup> antigen-specific T helper cells** type of T cell which usually reacts with antigenic peptides associated with class II MHC.

**Cupin** protein family that contains 11S and 7S seed storage proteins, and germins.

**Dendritic cells (DCs)** professional antigen-presenting cells that link the innate and adaptive immune systems by capturing and then presenting antigens to T cells.

**Epitope** binding site for antibodies.

**FcεRI receptor** a receptor on the surface of mast cells and basophils that binds free IgE. When antigen binds this IgE and cross-links FcεRI, it causes mast cell activation.

**Gene silencing** prevention of the expression of a specific gene.

**Lipid Transfer Proteins** class of food allergens with low molecular weight found in plant derived foods and highly resistant to pepsin digestion.

**Major Histocompatibility Complex Class II** molecules made by antigen-presenting cells (macrophages, dendritic cells, and B cells) which help trigger an acquired immune response.

**Prolamin** type of gluten protein found in certain grains. These proteins are insoluble in water but soluble in alcohol/water mixtures.

## Nomenclature

APC antigen-presenting cell

BAT basophil activation test

CD4 cluster difference 4

DBPCFC double blind, placebo controlled food challenge

DC dendritic cell

ELISA enzyme-linked immunosorbent assay

FcεRI high-affinity IgE receptor

GMO genetically modified organism

HPP high pressure processing

IgE immunoglobulin E

IL13 interleukin-13

IL4 interleukin-4

IUIS International Union of Immunological Societies

MHC-II Major Histocompatibility Complex Class II

nsLTP non-specific lipid transfer protein

SPT skin prick test

## Introduction

Food allergy has emerged as a prominent global public health and food safety issue. It is defined as an “adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food” (NIAID, 2010). This definition comprises immunological reactions, which may be mediated either by immunoglobulin-E (IgE) antibodies or other immunological pathways (non IgE-mediated). IgE-mediated food allergy also known as immediate type hypersensitivity (type I reaction) represents the main form of food allergy, which causes the most severe reactions (Johnston et al., 2014; Kimber and Dearman, 2002). In this paper, food allergy is understood to refer to IgE-mediated reactions of the human immune system to food.

The true prevalence of food allergy is difficult to establish because factors such as allergy definitions, study populations, methodologies, geographic variation, age, dietary exposure, and other factors influence the estimates. Taken together, current available data substantiate food allergy prevalence nearing 5% in adults and approaching 8% in children (Longo et al., 2013; Sicherer and Sampson, 2014; De Silva et al., 2014). Although there are methodological limitations, there is a large consensus that prevalence of food allergy has been increasing in recent decades (Sicherer and Sampson, 2014; Rona et al., 2007; Tang and Mullins, 2017). To date, elimination of causative foods remains the principal treatment for food allergies. This can lead to nutritional deficiencies unless a proper nutritional support is given. Given the negative impact of food allergy, on health, psychosocial (Sicherer et al., 2001; De Blok et al., 2007), and socio-economics of food allergic sufferers and their families (Cornelisse-Vermaat et al., 2008; Fernández-Rivas and Miles, 2003; Fox et al., 2009), the prevention and treatment of allergic reactions to foods is a major challenge that must be addressed.

The development of hypoallergenic foods and ingredients is sought as one approach that could potentially contribute to the treatment and prevention of food allergies by avoiding elicitation of allergic reactions and reducing new sensitizations and cross-reactions (Mahler and Goodman, 2017). This is particularly relevant as recommendations about the prevention of food allergy and atopic disease through diet have changed radically in recent years (Du Toit et al., 2016). The paradigm has, indeed, shifted from recommending avoidance of common food allergens to considering early consumption to prevent allergy development. The availability of hypoallergenic foods, would theoretically improve the life quality of food allergy sufferers through increasing dietary variation and reducing restrictions on product selection (van Putten et al., 2010).

This article discusses the current knowledge and approaches being taken to develop hypoallergenic foods, and highlights their usefulness and challenges in the implementation of efficient food allergy management strategies.

## **Food IgE Allergy Mechanism and Definition of Terms**

To facilitate understanding an overview of IgE-mediated food allergy mechanism and term definitions is first provided hereafter.

### **Food IgE Allergy Mechanism**

The initial phase in the development of IgE-mediated allergy is termed sensitization, which occurs when a food allergen comes into contact with the immune system, generally through the epithelial barrier of the gut. Food allergen are internalized by antigen-presenting cells (APCs) such as dendritic cells (DCs), processed and presented to T cells, with Major Histocompatibility Complex Class II (MHC class II) molecules, on the surface of APCs. Upon stimulation by APCs, the naïve CD4<sup>+</sup> antigen-specific T helper (Th0) cells will either develop into Th1, Th2 or Th17 effector cells or regulatory T-cells (Treg). In individuals where signals from the APCs will cause differentiation of Th2 cells, production of IL-4 and IL-13 from these cells will drive B-cell class switch to IgE production and secretion of allergen specific IgE. The secreted allergen-specific IgE will subsequently bind to tissue mast-cells and blood basophils via the high-affinity receptor FcεRI. Upon second encounter with the same allergen, cross-linking of FcεRI-bound IgE occurs leading to mast-cells activation and release of potent inflammatory mediators, such as histamine, proteases and leukotriene (Rindsjö and Scheynius, 2010). Mast-cells and basophils are activated within a few minutes of IgE cross-linking, therefore this process is called an immediate allergic reaction; symptoms occur shortly after allergen contact. The immediate allergic reaction leads to intense inflammation that can become life threatening (Valenta et al., 2015; Berin, 2015).

### **Food Allergens**

Food allergens are defined as those specific components of food or ingredients within food (typically proteins) that are recognized by allergen-specific immune cells and elicit specific immunologic reactions, resulting in characteristic symptoms (NIAID, 2010). There is a global diversity in the foods that cause the majority of allergic reactions and the current regional regulatory lists of allergenic foods requiring labelling reflects to some degree regional patterns and prevalence of food allergies worldwide (Chan et al., 2011). Most of the research is concentrated on those few foods that are responsible for the majority of observed allergic reactions, namely, milk, egg, peanuts, tree-nuts, soy, wheat, fish, shellfish, fruits of the latex group (e.g. kiwi, banana), fruits of the *Rosaceae* family (e.g. apples, pears, prunes), and vegetables of the *Apiaceae* family (e.g. carrot, celery), thereby guiding food hypoallergenization efforts worldwide.

Each allergenic food contains a multitude of different proteins that can cause allergy. Owing to the development of proteomics, spectroscopic methods and gene cloning, numerous allergens have been isolated, purified and well characterised. By convention in the systematic International Union of Immunological Societies (IUIS) nomenclature (King et al., 1994), allergens are designated by the first three letters of the genus, the first letter of the species name according to the Linnaean taxonomic system and an Arabic number reflecting the chronological order in which the allergen was identified and characterised (e.g. Ara h 1 stands for allergen 1 from *Arachis hypogea* (peanut)) (EFSA Panel on Dietetic Products, 2014). Up to date structural information, biochemical characteristics and clinical relevance for each known allergen are compiled in a number of open-access databases. The most important ones are: the IUIS official website; the Allergome database (Mari et al., 2006); the Protein family (Pfam) database, which assigns sequences of clinically proven food allergens to protein families (Jenkins et al., 2005); AllFam,

which merges the Allergome allergens database with data on the Pfam database (Radauer and Breiteneder, 2007); the Structural Database of Allergenic Proteins (SDAP) provides detailed structural data on allergens in the IUIS Nomenclature, including sequence information, Protein Databank files (PDB-files) and computational tools to analyse IgE epitopes (EFSA Panel on Dietetic Products, 2014; Lorenz et al., 2015).

Currently, known food allergens are clustered according to their common structural, biochemical and functional features into a relatively small number of protein families, indicating that conserved structures and biological activities play a role in determining or promoting allergenic properties of proteins (Breiteneder et al., 2007; Breiteneder and Mills, 2009; Scheurer et al., 2015). Thus, around 65% of those from plants belong to just four protein families, the prolamin, cupin, Bet v 1-like, and profilin families (Breiteneder and Mills, 2009; Radauer et al., 2008), with the most comprehensively characterized plant allergenic proteins being profilins, seed storage proteins (2S albumins, 7S/11S globulins), and pathogenesis related proteins such as non-specific lipid transfer proteins (nsLTPs) (Scheurer et al., 2015). Similarly, animal food allergens are grouped into three main families, the tropomyosins, parvalbumin, and caseins (Breiteneder and Mills, 2009; Kuehn and Hilger, 2015). Food allergens are further classified either as class I or class II food allergens. Class I allergens act as the primary sensitizing agents via the gut and elicit an allergic reaction upon ingestion (e.g., storage proteins, such as peanut Ara h 1 and Ara h 2, milk  $\beta$ -lactoglobulin Bos d 5, nsLTPs). They are generally very stable to digestive enzymes and are often associated with severe and anaphylactic reactions. Class II food allergens, instead, are allergens, which IgE-reactivity results from primary sensitization to homologous allergens from different source, particularly inhalant allergens (e.g., Bet v 1 family, profilins, such as hazelnut Cor a 2). They are frequently associated with mild allergic reactions (Lorenz et al., 2015).

The allergenicity of food proteins depends on the reactivity to specific T-cell and B-cell epitopes (Lorenz et al., 2015). T-cell epitopes and B-cell epitopes differ in the way they are recognised by the immune system. T-cell epitopes refer to the regions on the allergen that are recognized after degradation, when presented to T-cells as peptides in (MHC-II) molecules (Bohle, 2006). These epitopes are generally short linear segments, located anywhere along the amino acid sequence and may be buried in its three-dimensional fold (Dall'Antonia et al., 2014). B-cell epitopes also called IgE-binding epitopes refer to the regions on the allergen, which are recognized in their native conformation by free IgE or membrane-bound B-cell receptors (Dall'Antonia et al., 2014; Van Regenmortel, 2009). IgE-binding epitopes can be linear/sequential epitopes comprising continuous amino acids sequences or conformational epitopes, which are formed by spatially adjacent amino acids segments that are distantly separated in the sequence and brought into physical proximity by protein folding. Allergens are typically defined based on their ability to bind specific IgE antibodies and induce IgE-mediated immediate reactions, while the allergen's potential to trigger T-cell reactivity is in many cases not very well studied and is not taken into account when categorizing a protein as an allergen (Schulten et al., 2014). Even though most allergens can be grouped into a small number of structural classes, many allergens do not exhibit any known physicochemical, functional or structural properties that would account for their allergenicity (Scheurer et al., 2015). Only few structural features are currently known to be common for certain allergens. As an example, important plant food allergens of the prolamin superfamily (such as nsLTPs and 2S albumins) contain the conserved pattern of cysteine skeleton that form three or four intramolecular disulfide bonds that gives stability to heating and digestion to these proteins (Breiteneder and Mills, 2005; Radauer and Breiteneder, 2009). However, the available information on allergen structures indicates that allergens are heterogeneous, and IgE-epitope repertoire too large to make specific IgE epitopes a realistic target for allergenicity prediction (Scheurer et al., 2015; Aalberse and Crameri, 2011; Dall'Antonia et al., 2014; Matsuo et al., 2015). Moreover, there is a general consensus that the molecular basis of allergenicity is likely not defined solely by the epitope structure, but is determined by additional factors, including the amount and duration of exposure to the immune system and environmental conditions (Scheurer et al., 2015). Nonetheless, molecular characteristics of allergens, in particular the identification of T-cell and B-cell epitopes, constitute essential information for the design of effective hypoallergenization approaches.

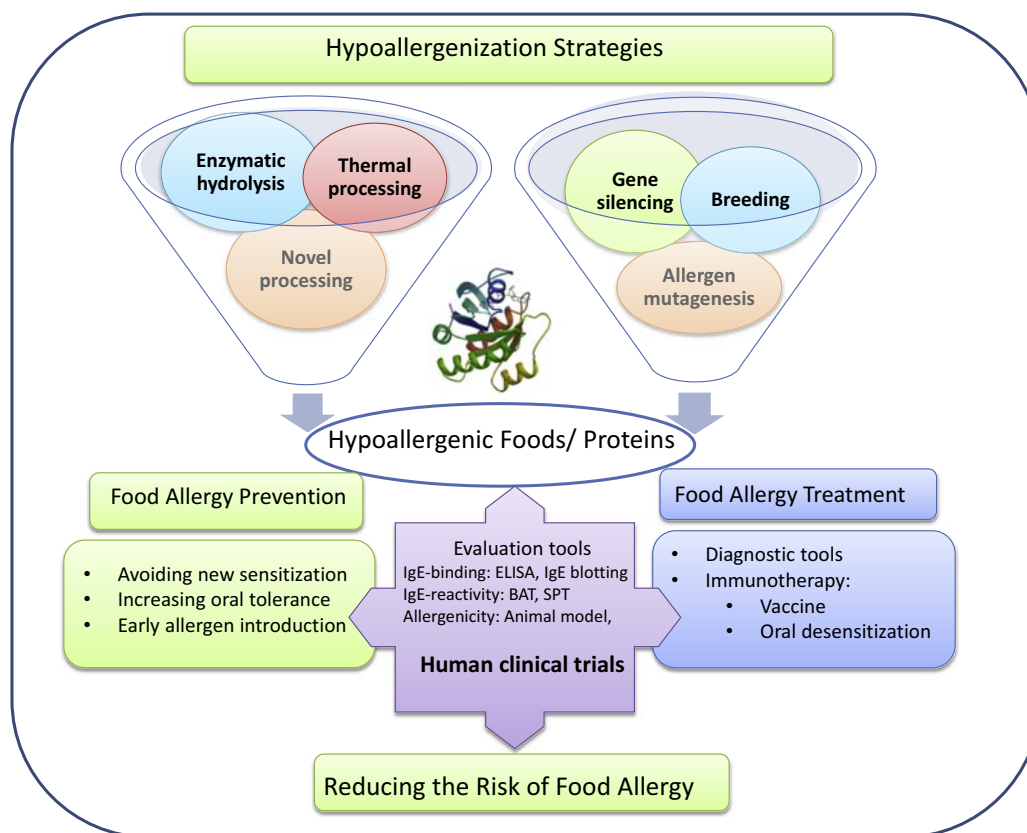
### Hypoallergenic Foods

There is actually no legal definition or standards that govern the utilisation of the term "hypoallergenic food", although it is commonly understood to refer to foods that are having little like hood of causing allergic reactions in humans. Hypoallergenic foods are also defined as being foods that have significantly reduced in vivo capacity to elicit an allergic reaction compared with naturally occurring foods (Mahler and Goodman, 2017; Muraro et al., 2004). Foods that are precisely defined as hypoallergenic to date are primarily restricted to cow's milk formulations treated using food processing techniques (hydrolysis, heat treatment, and/or ultrafiltration), with the aim of destroying or inactivating IgE-binding and T-cell epitopes (Beyer, 2007). For a formula to be considered hypoallergenic, it should be well tolerated in double-blind, placebo-controlled food challenge (DBPCFC) by at least 90% (with 95% confidence) of individuals who are allergic to the parent protein from which that formula has been derived (Muraro et al., 2004; Beyer, 2007; Chung and Reed, 2014).

### Food Hypoallergenization Strategies

The principle of hypoallergenization consists in blocking, reducing or eliminating the capacity of allergens to induce new sensitization or to trigger allergic reactions in already-sensitized individuals. Elimination or reduction of food allergenicity has been attempted for years by various investigators, essentially through the use of a wide range of conventional and novel thermal and





**Figure 1** Strategies behind hypoallergenic foods.

non-thermal food processing techniques (e.g. wet and dry heat, enzyme hydrolysis, glycation, lactic fermentation, irradiation, high pressure processing, etc.) (Verhoeckx et al., 2015; Vanga et al., 2017) or genetic engineering tools (Herman et al., 2003). Current state of the science with regard to food hypoallergization strategies (Fig. 1.) is discussed below. Their potential benefits, limitations, and perspectives are highlighted.

### Food Processing Approaches

Food processing induces several physical and biochemical changes to proteins such as unfolding, aggregation and degradation resulting in different reversible and irreversible changes on the levels of quaternary, tertiary and secondary structures, and cleavage or formation of intra and/or inter-molecular covalent and non-covalent interactions. Interactions with other constituents of the food matrix during processing such as sugars, lipids, and polyphenols can lead to additional modifications (Mills et al., 2009). In this way IgE-epitopes on an allergen can either be destroyed, modified, or masked thereby interfering with its ability to be recognized by IgE (Soler-Rivas and Wichers, 2001). Food processing approaches are undoubtedly the most attempted strategies for the reduction of food allergenicity (EFSA Panel on Dietetic Products, 2014; Verhoeckx et al., 2015; Vanga et al., 2017; Jiménez-Saiz et al., 2015; Rahaman et al., 2016). A majority of published works on the reduction of food allergenicity by processing were essentially based on the evaluation of IgE-binding capacity using in vitro immunological assays (e.g. ELISA, immunoblotting). However, IgE binding of food proteins does not always translate to clinical symptoms (Shi et al., 2013; Panda et al., 2015). Thus, the assessment of cross-linking and degranulation capacity is essential (Panda et al., 2015; Vissers et al., 2011). Clinical reactivity by oral food challenge remains the ultimate tool to be performed before concluding the effect of any processing method on the allergenicity of any food (Høst and Halken, 2004). In the context of this report, we would like to highlight those processing techniques that demonstrated reduction in food and/or protein allergenicity, and which were minimally supported by additional bio potency tests, such as release of allergic mediator (histamine and cytokines) test, animal allergic models, skin prick test (SPT) or human oral challenges.

### Thermal Processing

Heating is the most common technological treatment applied to food preservation and quality improvement. It includes blanching, pasteurization, sterilization, boiling, cooking, baking, roasting, frying, steaming, etc. It is now widely proven that thermal treatments can lead to significant alterations in allergen structure through denaturation, aggregation, fragmentation and Maillard reaction



(Mills et al., 2009; Davis et al., 2001). The nature and extent of such changes are however, dependent on the type (e.g., wet vs dry heat), the temperature and duration of heating treatment as well as on the intrinsic characteristics of the protein and the physico-chemical conditions of its environment, resulting either in an increased, decreased or unaltered IgE binding activity of food allergens (Verhoeckx et al., 2015; Jiménez-Saiz et al., 2015; Rahaman et al., 2016; Wal, 2003; Blanc et al., 2011; Verma et al., 2012).

For example, numerous studies have shown that extended boiling (100 °C, 1–16 h) reduced peanut IgE-binding through a combination of leaching of allergens into cooking water, fragmentation and denaturation of allergens (Blanc et al., 2011; Beyer et al., 2001; Turner et al., 2014). The reduction in allergenicity of 2 to 4 h boiled peanut extracts was demonstrated in vivo on 20 peanut-allergic subjects by a reduction of 55% of the mean SPT diameter when compared to raw extract (Tao et al., 2016). However, boiled peanut extracts maintained their ability to stimulate T-cell activation and proliferation. In two clinical studies, roasting of hazelnut resulted in reduced allergenicity in patients with birch pollinosis allergy by DBPCFC (Hansen et al., 2003; Worm et al., 2009). Still, 5/17 and 17/20 of patients, respectively, developed oral symptoms showing that the allergenicity was not completely abolished. The decrease in allergenicity of hazelnut was attributed to the decreased IgE-binding of the birch pollen related allergens, Cor a 1 and Cor a 2 (Pastorello et al., 2002; Schocker et al., 2000). In contrast, seed storage proteins and nsLTPs in hazelnut, almond, and cashew nuts were reported to be very stable regardless of the heat treatment and conditions (Pastorello et al., 2002; Venkatachalam et al., 2002; Venkatachalam, 2008; Masthoff et al., 2013).

The effect of extensive heating such as baking on the allergenicity of bovine milk and egg proteins has been investigated by few clinical studies. These studies have shown that a majority, 68% and 50%–85%, of milk and egg allergic children, respectively, tolerated baked (180 °C, 20–30 min) milk and egg products in oral challenges (Nowak-Węgrzyn et al., 2008) (Des Roches et al., 2006; Lemon-Mulé et al., 2008; Turner et al., 2013). Moreover, through these studies, it was also observed that incorporation of baked forms of milk and egg foods into the diets of children who are allergic to milk and eggs has generated immunological changes that could lead to the development of oral tolerance to the unheated forms of milk and eggs (Lemon-Mulé et al., 2008; Kim et al., 2011), indicating the potential role of hypoallergenic foods in the induction of oral tolerance. Larger and well-designed randomized clinical studies are, however, still needed to assess the accuracy of this hypothesis (Lambert et al., 2017). In contrast, moderate forms of heat treatments (temperatures <80 °C) still retained to a great extent, the allergenic properties of egg (Des Roches et al., 2006; Lemon-Mulé et al., 2008; Turner et al., 2013; Bartnikas and Phipatanakul, 2013; Cortot et al., 2012), and milk proteins (Bu et al., 2009; Loss et al., 2011; Waser et al., 2007). Partial reduction of allergenicity is achieved, presumably by destroying conformational epitopes (Thomas et al., 2007), while reactivity to linear epitopes is retained (Bu et al., 2013). It is important to appreciate here, that apart from the allergen intrinsic properties and processing conditions (type, temperature and duration), the food matrix composition and structure may also affect the digestibility and release of allergens, thereby modifying the way they are presented to the gut immune system, and subsequently modulating the overall food allergenicity (Wickham et al., 2009; Mackie et al., 2012).

### Enzymatic Hydrolysis

The principle behind enzymatic hydrolysis (proteolysis) as means for reducing food protein allergenicity is to hydrolyse protein peptide bonds into smaller peptides and even amino acids, thereby disrupting the structure of both linear and conformational epitopes present in food allergens. The extent of hydrolysis is highly dependent on the amino acid sequence, secondary structure, protein modification, and enzyme specificity (Thomas et al., 2007). Trypsin, pepsin and chymotrypsin are frequently used for hydrolysis, but other enzymes of plant and microbial origins have been also investigated (e.g., papain, alcalase, pronase, etc.). Currently, the only commercially available food preparations that are considered hypoallergenic are extensively hydrolysed milk formulas or free amino acids based formulas (Bahna, 2008; Baker et al., 2000; Niggemann et al., 2008; Oldæus et al., 1991; Terheggen-Lagro et al., 2002). Their hypoallergenicity for children with confirmed cow milk allergy was demonstrated with oral food challenges in a number of clinical trials (Berni Canani et al., 2013). However, it was reported recently that extensively hydrolysed formulas may still elicit reactions in some children with cow milk allergy (Chauveau et al., 2016). Enzymatic hydrolysis has been also attempted for reducing the allergenicity of other food allergens. For example, hypoallergenic wheat flour was developed using the combined hydrolytic action of cellulase and actinase (Watanabe et al., 2000). In vitro IgE-reactivity to treated wheat was abolished, and only 2/15 of wheat-allergic patients developed an immediate reaction after oral challenge with the hypoallergenic wheat-based products (Tanabe, 2008). Cashew protein also showed a decreased allergenicity in a mice allergic model when hydrolysed to very low molecular weight peptide (Kulis et al., 2012). Similarly, enzymatic hydrolysis of soluble protein extracts of three legumes – kidney bean, black gram, and peanut resulted in an important reduction in the bio potency of hydrolysates by SPT. Only 1/10 kidney bean-sensitive individuals, 2/6 black gram sensitive individuals and 1/7 peanut-sensitive individuals were found positive to their respective hydrolysates (Kasera et al., 2015). In another study, combining the application of heat treatment and enzymatic hydrolysis in egg resulted in low allergenicity and induction of oral tolerance in mouse allergic model (Hacini-Rachinel et al., 2014). From these studies, it appears, that enzymatic hydrolysis has the potential to reduce allergenicity of some food proteins by destroying specific IgE-epitopes, but care should be taken to not uncovering previously masked epitopes or simply leaving protein fragments with retained IgE-binding (Bu et al., 2013). Human oral challenges should be performed for more accurate evaluation of the allergenicity reduction. Moreover, hypoallergenic protein hydrolysates and peptides that have diminished capacity to cross-link IgE on mast-cells and basophils, but maintained capacity to target CD4<sup>+</sup> T-cells, are postulated as useful candidates for immunotherapy (Tao et al., 2016; Kulis et al., 2012).

### **Combined Conventional and Novel Food Processing Approaches**

In a search of more effective food hypoallergenization strategies, researchers have showed keen interest to evaluate the potential of emerging novel food processing techniques in reducing protein allergenicity (e.g., high hydrostatic pressure (HPP) (Lee et al., 2016), microwave (Garino et al., 2012),  $\gamma$ -irradiation (Oh et al., 2009), high-intensity ultrasound (Li et al., 2013), pulsed ultraviolet light (Chung et al., 2008), and so on (Vanga et al., 2017).

As for the conventional techniques, these novel processes can modify protein structure and consequently lead to a partial or complete reduction, or increase in protein immunoreactivity/allergenicity. To date, it remains difficult to draw conclusions because most of these reports are mainly focused on in vitro assays, and none of them produced results of clinical importance (Vanga et al., 2017). Nonetheless, combinations of novel processes with conventional (thermal or enzymatic hydrolysis) that may act synergistically appear to be more effective in achieving reduction in protein allergenicity. In a study combining the effect of high pressure and enzymatic hydrolysis, it was shown that milk  $\beta$ -lactoglobulin's peptic and chymotryptic hydrolysates treated at 400 MPa, had an abrogated allergenicity in a milk allergy mouse model (López-Expósito et al., 2012). Such high pressure induced protein unfolding facilitated enzymatic digestion and resulted in shorter peptide fragments (7–10 residues long, MW < 1.5 KDa) that had lost their ability to cross-link 2 human IgE antibodies to induce mast-cell degranulation. Others studies demonstrated the efficiency of combining high pressure and thermal treatment in the reduction of allergenicity of walnut (Yang et al., 2017), peanut (Long et al., 2016) and shrimp (Long et al., 2015) proteins in mouse allergic models. In another study, the synergistic effect of high pressure (autoclaving at 2.56 atm/30 min) and roasting treatment was also found to be efficient in reducing IgE binding properties of peanut protein extracts and abolishing skin reactivity in 8 peanut allergic patients (Cabanillas et al., 2015) by SPT. These results are promising, but need substantiation by relevant human oral challenges, to further confirm the usefulness of this approach for producing hypoallergenic foods.

### **Genetic Engineering Approaches**

#### **Breeding and Genetic Modification**

Breeding and genetic modification approaches for eliminating allergenic proteins from crop plants has long been explored for the development of hypoallergenic foods and ingredients based on (a) screening of low-allergenic cultivars from the existing biodiversity of a given crop, (b) breeding using characterized genotypes and genetic markers for low allergenicity, and (c) genetic modification to silence native genes encoding allergenic proteins (Gilissen et al., 2006; Riascos et al., 2010; Scheurer and Sonnewald, 2009).

In soybean for example, the three major allergens designated as Gly m Bd 60K, Gly m Bd 30K, and Gly m Bd 28K were eliminated by the development of a mutant line, Tohoku 124. Oral challenge test conducted with soybean-sensitive patients reported that about 80% of the soybean-allergic patients could ingest hypoallergenic products prepared from this mutant line without any adverse reactions (Ogawa et al., 2000). In another example, peanut cultivars missing either an Ara h 2 or Ara h 3 isoform were cross-bred and produced a variety lacking both isoforms (Perkins et al., 2006). Gene silencing by means of RNA interference (RNAi) offers the possibility to inhibit expression of entire gene families (Small, 2007) and it was successfully used for the silencing of Ara h 2; Ara h 1/Ara h 3 (Ananga et al., 2008) and Ara h 6 (Chu et al., 2008) in peanuts, and of PR-10 isoforms in carrots (Peters et al., 2011).

Although genetic engineering technologies have shown great promise to produce crops with a reduced or “null” content of allergenic proteins, these technologies are found to be limited by several big drawbacks (Riascos et al., 2010): (a) the development of allergen-free crop is unrealistic given the multitude of allergenic proteins; (b) hypoallergenization through conventional breeding is time consuming (Schenk et al., 2011); (b) the potential risk of sensitization to newly overexpressed proteins and questions regarding the long-term stability of transgenic suppression of allergenic proteins; (c) the consumer acceptance of transgenic food (GMOs); (d) the requirement for tight control on labeling. All these concerns require further attention from the perspective of food allergy management.

#### **Allergen Site-Directed Mutagenesis**

Site-directed mutagenesis is a molecular biology method that is used to make specific and intentional changes to the DNA sequence of a gene to produce hypoallergenic mutants of specific food allergen that can be used in immunotherapy with few adverse reactions (Mahler and Goodman, 2017; Patel et al., 2017). The approach consists in mutating the sequence of IgE binding epitopes or by targeting the secondary structure of the protein, such as overlapping (offset of one or two amino acid positions), substitution, or disrupting cysteine–cysteine disulphide bridges in protein domain. The produced hypoallergenic variant of the protein is then expressed in *Escherichia coli* (*E. coli*) and tested for reduced IgE immunoreactivity. T-cell reactivity is generally unaffected. Successful results were observed with egg allergens, in particular Gal d 1 (Dhanapala et al., 2017), as well as other major allergens such as shrimp allergen Pen a 1 (Reese et al., 2005), and peanut recombinant of Ara h 1, Ara h 2, and Ara h 3 (EMP-123) (Wood et al., 2013). However, these results remain to be translated into well-designed clinical studies. Thus, while the developed mutated peanut recombinant EMP-123 product did not bind IgE and showed reduced allergic symptoms in mouse allergy models, acute allergic reactions were still found in human clinical trial (Wood, 2016).

## Conclusion and Perspectives

A variety of food processing and genetic engineering technologies have been investigated in a proof-of-concept trials to reduce the allergenicity of food either by reducing their IgE-binding capacity or eliminating certain major allergens (Fig. 1). Based on the findings of these initial trials, producing hypoallergenic foods is technically feasible, but it is clear, that at present, there are a number of challenges and limitations that make absolute certainty regarding the absence of potential for allergic reactions not possible for most hypoallergenic foods. Examples of these challenges are (a) the multiplicity of the allergenic proteins contained in a whole food, (b) the variability of the impact of food processing and the food matrix on the allergenic properties of proteins, (c) the intra- and inter-individually variability of sensitization and thresholds of reactions against allergens, and (d) the presence of unknown allergenic proteins in the food that have not been identified yet. Moreover, in order to ensure the safety of food-allergic individuals, a thorough safety assessment and substantiation by human clinical studies using scientifically appropriate standards are necessary to confirm their presumed hypoallergenicity. Regulatory requirements that define and control the utilisation of the term “hypoallergenic food” will also need to be developed and reinforced throughout the entire food chain. In this regard, in vitro testing of IgE-reactivity remains a valuable tool for food quality control and assurance as well as for labelling.

With the progress being made in the understanding of factors that contribute to the development of sensitization and tolerance to foods, and with the substantial progress being made in clinical and molecular allergy diagnostics, hypoallergenic foods and proteins will certainly find their utility in food allergy treatment and prevention in the future through (a) the setup of efficient well-targeted and personalized management strategies, (b) the development of specific hypoallergenic protein molecules to be used for improved immunotherapeutic strategies tailored to the sensitization pattern of each patient, and (c) by facilitating and rendering more effective the new global approach to allergy prevention, which has recently shifted from recommending avoidance of common food allergens to consideration of early consumption strategy to prevent allergy development.

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## Relevant Websites

IIUS official website: <http://www.allergen.org>.  
 Allergome database: <http://www.allergome.org>.  
 Protein family (Pfam) database: <http://pfam.sanger.ac.uk>.  
 Pfam database: <http://www.meduniwien.ac.at/allergens/allfam/>.  
 Protein Databank files (PDB-files) and computational tools to analyse IgE epitopes: <http://fermi.utmb.edu>.

## Insects as a Novel Food

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### Glossary

**Entomophagy** The practice of eating insects

**Larva** Immature form of insects that undergo complete metamorphosis

**Nymph** Immature form of insects that undergo incomplete metamorphosis

**Pupa** Immature form of insects between larva and adult stages

### Overview

Insects are a group of invertebrate animals within the class Insecta, phylum Arthropoda. They are the largest group of animals on the planet with an estimated diversity of 1.4 million to over 5 million species (Capinera, 2008). Incorporation of insects into human diet is not entirely novel. Historically, these six-legged creatures composed an important food source for our ancestors. Evidence of insect consumption by early American Indians dates back to 7000 BC (Van Itterbeeck and Van Huis, 2012). This is not surprising considering that insects are virtually eaten by all primates (McGrew, 2014). Insectivory may even play a role in human evolution as some of the first bone tools (1.7 million year old) were developed for the extraction of termites (Lesnik, 2011) that served as a nutrient-dense dietary supplement for large-brained hominins (Lesnik, 2014). Today, as many as 2 billion people of 3071 ethnic groups in 130 countries practice insect-eating regularly (Costa-Neto and Dunkel, 2016). For unintentional entomophagy (insect-eating), this number is expanded to the entire human population as food infestation by pests are unavoidable. That is why the United States Food and Drug Administration established the Food Defect Action Levels (levels of natural or unavoidable defects in foods that present no health hazards for humans) instead of a zero tolerance for insect contaminants in food. For example, the acceptable maximum of insects in canned citrus fruit juices are five *Drosophila* eggs or one maggot per 250 mL (FDA, 2016). Despite the fact that insects are already in our food, the bugs on the menu concept is still relatively new and somewhat repulsive for the general public in developed countries.

### Entomophagy Around the World

Insects are considered as ordinary food items in certain regions. Fig. 1 illustrates a food cart selling edible insects along with other arthropods in China. In his pioneer works on entomophagy, Professor Gene R. DeFoliart documented a large number of insect species consumed worldwide: 148 in Mexico, 12 in Central America and Caribbean Islands, 65 in South America, 83 in Southern Africa, 163 in Central and Eastern Africa, 25 in North and West Africa, 16 in Southwest Asia, 52 in South-Central Asia, 151 in Southeastern Asia, 66 in Eastern Asia, and 84 in Oceania (Defoliart, 2002). An updated inventory comprises 1544 edible insect species and this number is still just a tiny fraction of the total insect species (Dossey et al., 2016). Although widely practiced around the world, entomophagy is generally absent in Canada, United States, and Europe but this was not always the case. Ancient Greeks and Romans enjoyed insects, particularly grasshoppers and cicadas, as food. Aristotle (3rd century BC) himself described cicada nymphs and egg-bearing females as delicacies (Defoliart, 2002). Up until the famine of 1688, cockchafer grubs were still eaten by peasants and mountain inhabitants in Ireland as an important protein source (Bodenheimer, 1951).

The prejudice against insect-eating was likely developed alongside the agricultural advancement. When food became readily available, insects faded away from our diet because insect-gathering was no longer economical (Bodenheimer, 1951). Over time, these once considered delicacies turned into something alien and disgusting, and entomophagy became a taboo. This prejudice was later brought to North America. In the 1600s, the European colonists classified the New England lobster as an insect and refused to eat it (Kiple and Ornelas, 2000). It is interesting how lobster has been widely accepted as a premium food in the Western world today while insect-eating is still considered repulsive, unusual, or newsworthy at best. Recently, using insects as a “novel” food has become a trending topic in the food science community. So what has changed to make us re-embrace this once abandoned food?

### Sustainability of Insect Farming

The fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC) underscores that climate change has had detectable negative impacts on crop yields (IPCC, 2014). This comes in the background of growing global population. It is projected





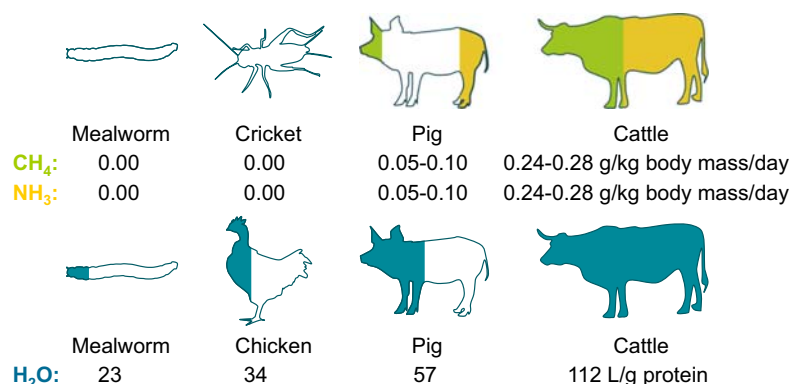
**Figure 1** A food cart selling (left to right, front to back) fried shrimps, crabs, cicadas, cockroaches, grasshoppers, bamboo weevils, silkworm pupae, and scorpions in Sichuan, China.

that by 2050 the global population will reach 9 billion. To feed the planet, current food production has to be doubled in the context of water and farmland shortages (Van Huis et al., 2013). Agriculture consumes approximately 70% of freshwater and livestock production is particularly water intensive. It is estimated that 22,000 L of water are required to produce 1 kg of beef (Van Huis, 2013). In addition, agriculture (e.g., enteric fermentation in livestock, manure management, and rice cultivation) remains a main source of anthropogenic methane emissions worldwide (Kirschke et al., 2013). Increasing agricultural activities may worsen climate change and complete the vicious cycle. Therefore, improving sustainability of food production is imperative to meet the growing demand for food and to address the pressing environmental challenges.

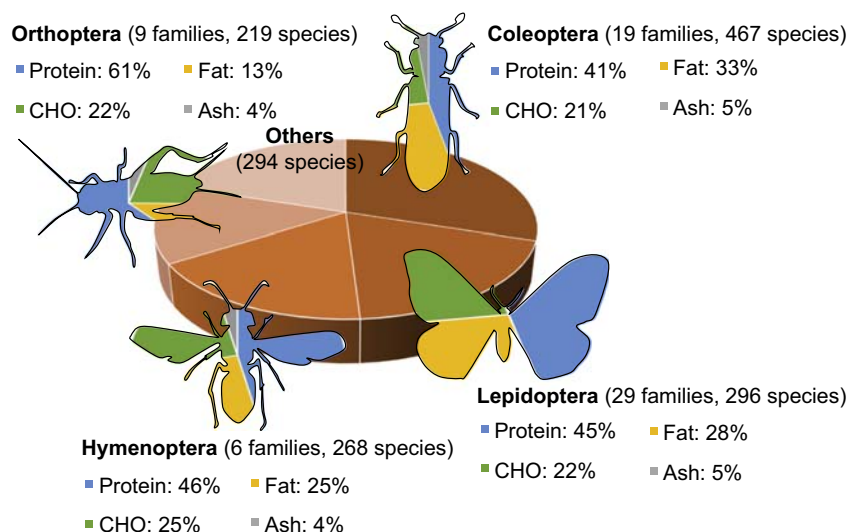
The Food and Agriculture Organization (FAO) of the United Nations has suggested using insects as an alternative food source. Insects have higher feed conversion rate as compared to the conventional livestock. For example, the feed-to-food conversion rate of cricket (*Acheta domesticus*) is twice that of chicken, four times that of pig, and 12 times that of cattle (Van Huis et al., 2013). Moreover, insects have low environmental footprints such as water use and emission of greenhouse gas and ammonia (Fig. 2) (Van Huis, 2013). Species such as yellow mealworms (*Tenebrio molitor*) and buffalo worms (*Alphitobius diaperinus*) are drought resistant and can live on organic side streams (Ramos-Elorduy et al., 2002) while the black soldier fly (*Hermetia illucens*) larvae have great potential to recycle organic waste and to be utilized as nutritious animal feed (Nguyen et al., 2015).

### Nutrient Composition of Insects

The most commonly eaten insect species belong to the orders of Coleoptera (30%), Lepidoptera (19%), Hymenoptera (17%), and Orthoptera (14%) (Dossey et al., 2016). As the most diverse group of animals, insects exhibit considerable variation in nutritional composition among species (Rumpold and Schlüter, 2013) (Fig. 3). Nutrient compositions of 471 edible insects are available in the FAO/INFOODS Food Composition Database for Biodiversity (Charrondière et al., 2013).



**Figure 2** Environmental footprints of the production of select animals. Data from Oonincx et al. (2010) and Miglietta et al. (2015).

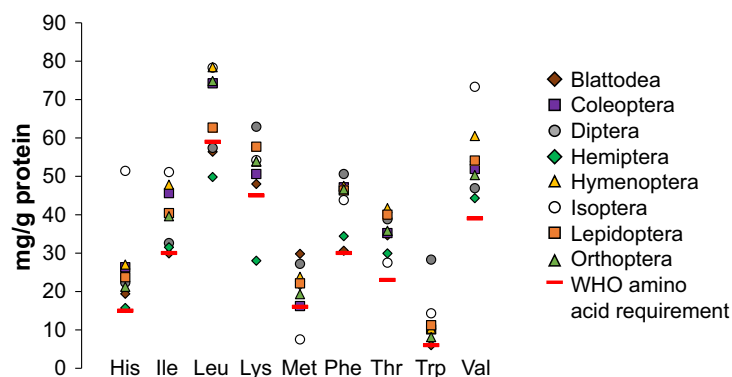


**Figure 3** Proximate composition (dry weight basis) of commonly consumed insect orders. Data from Rumpold and Schlüter (2013) and Dossey et al. (2016).

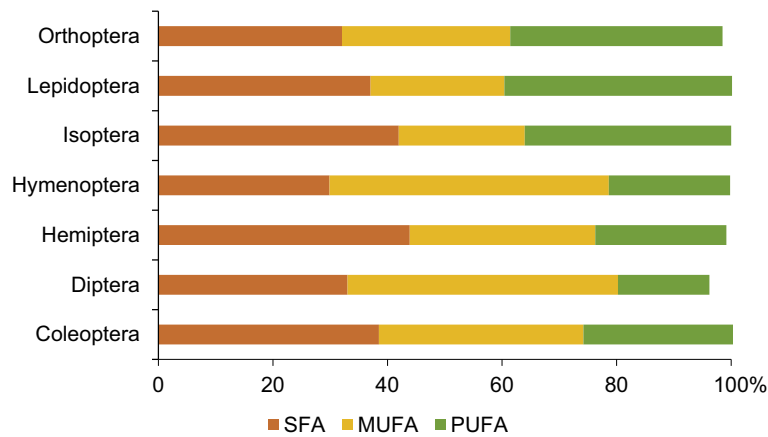
Insects are a good source of proteins with well-balanced amino acid composition. Protein content of 203 edible insects in the FAO/INFOODS Database ranged from 1.8% to 72.7% with an average of 23.3%. Based on the definition of FAO/WHO, the majority of insects (89%) are regarded as “high in protein” (over 10 g proteins/100 g edible portion on fresh weight basis) (FAO/WHO, 2007). It is worth noting that most reported protein content of insects are calculated from the total nitrogen content using a nitrogen-to-protein conversion factor of 6.25, which overestimates the protein content due to the presence of non-protein nitrogen in insects. A conversion factor of 4.76 was obtained for *T. molitor*, *A. diaperinus*, and *H. illucens* larvae based on amino acid analysis (Janssen et al., 2017). With regards to protein digestibility, insect proteins are comparable to other animal proteins and are superior to many plant proteins (Rumpold and Schlüter, 2013). Essential amino acid scores of insect proteins ranged from 46% to 96%, which are above the FAO/WHO recommended score of 40% (Ramos-Elorduy et al., 1997). Essential amino acid composition of different insect orders is illustrated in Fig. 4.

The fat content of edible insects vary considerably from 0.66% to 77.13% (Rumpold and Schlüter, 2013). The fatty acid makeup of insect fat is generally preferable with high levels of polyunsaturated fatty acids (PUFA), particularly in isopteran (36%), orthopteran (37%), and lepidopteran (40%) insects (Fig. 5). Moreover, many insects exhibit a low ratio of omega-6/omega-3 fatty acids, which is desirable in reducing the risk of chronic diseases (Simopoulos, 2002). For example, toasted silkworm pupa (*Bombyx mori*) has a good omega-6/omega-3 ratio of 0.3 (Pereira et al., 2003).

The exoskeletons of insects are mainly composed of the polysaccharide chitin, which can serve as a dietary fiber. Fiber content of edible insects ranged from 0.12% to 29.13%. In addition to dietary fibers, insects are rich in a variety of micronutrients such as the vitamins riboflavin, pantothenic acid, biotin, and in some cases folic acid, and the minerals copper, iron, magnesium, manganese, phosphorous, selenium, and zinc (Rumpold and Schlüter, 2013). The minerals are not only abundant but also readily available. For example, bioavailability of iron from buffalo worm (*A. diaperinus*) is superior to that of sirloin beef (Latunde-Dada et al., 2016). Due to their high iron and zinc content, many edible insects play an important role in alleviating deficiencies in pregnant women’s diet in developing world (Belluco et al., 2013). Apart from the nutritional values, numerous bioactive components in insects with



**Figure 4** Essential amino acids in edible insect proteins. Data from Rumpold and Schlüter (2013).



**Figure 5** Fatty acids composition of edible insect fat (SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids). Data from [Rumpold and Schlüter \(2013\)](#).

immunological, analgesic, antibacterial, diuretic, anesthetic, and antirheumatic properties have been used for medicinal purposes ([Costa-Neto, 2005](#)).

Although a substantial amount of nutrient composition data are available for edible insects, they are subject to a large variation. Small sample size, species-specific metabolism, varying accuracy of sampling and/or analytical techniques, and contamination may contribute to the variation ([Ooninx and Dierenfeld, 2012](#)). Meanwhile, most insects analyzed are collected from the wild with highly variable growing environment and food, which may also affect the results. On top of that, many species undergo complete metamorphosis and their nutrient composition may change in different life cycle stages. Thorough nutrient analyses and life cycle assessments of different species reared in controlled conditions are therefore, warranted.

### Insect Processing Technology

Some insects are eaten alive such as certain isopteran and hemipteran species consumed in Africa ([Van Huis, 2003](#)) and the cheese skippers (larvae of *Piophilidae casei*) in the maggoty cheese *casu marzu* ([Overstreet, 2003](#)). However, edible insects are more often subjected to processing methods including appendix removal, degutting, baking, boiling, drying, frying, grilling, pickling, roasting, smoking, steaming, and toasting before consumption ([Rumpold et al., 2014](#)). Such processing methods are important for enhancing flavor, shelf-life, and safety of the products. A wealth of traditional processing methods and recipes around the world are documented in The Food Insects Newsletter ([Defoliart et al., 1988–2000](#)). The historic and cultural accumulation of entomophagy practices lays a foundation for developing edible insect-based products while modern food processing technologies offer innovative approaches. For example, cold plasma treatment and high hydrostatic pressure treatment have been applied as effective decontamination techniques for mealworms (*T. molitor*) ([Rumpold et al., 2014; Bußler et al., 2016a](#)).

Edible insects are also ground or milled into granular or paste form and used as food ingredients. For instance, many insects are prepared as chutneys in India ([Rumpold et al., 2014](#)) and ground giant waterbugs (*Lethocerus indicus*) are an essential ingredient for certain chili pastes in Thailand and the Lao People's Democratic Republic ([Van Huis et al., 2013](#)). In contrast to eating whole insects, using ground insects as ingredients in conventional food products is also preferred by Western consumers ([Gmuer et al., 2016; Tan et al., 2017](#)). Many edible insects, in bits and pieces, have made their way into baked goods, bars, burgers, chips, meat balls, nuggets, pasta, rice, schnitzels, and sausages ([Van Huis, 2016; Kim et al., 2016; Tan et al., 2017; Gmuer et al., 2016](#)). Likewise, extracting and isolating proteins, fats, and carbohydrates from insects as food ingredients is a good strategy for utilizing insects in regions where people cannot readily accept the intact form of insects in their food. Proteins of insects are often isolated by alkaline extraction ([Azagoh et al., 2016](#)) or alkaline extraction followed by isoelectric precipitation ([Bußler et al., 2016b](#)) although protein extraction in water have also been reported ([Yi et al., 2013](#)). [Mariod and Fadul \(2015\)](#) described extraction of gelatin from melon bugs (*Coridius viduatus*) and sorghum bugs (*Agonoscelis versicoloratus*) using distilled water, mild acid, and hot water, with an ascending extraction efficiency in that order. Edible insect oils are isolated by either solvent extraction or supercritical CO<sub>2</sub> extraction with the latter technology demonstrating a high yield of solvent-free oil at low extraction temperature ([Purschke et al., 2017; Mariod et al., 2010](#)). Chitins are prepared by removing minerals, proteins, and pigments from the exoskeleton of insects ([Kaya et al., 2016](#)). The isolated components can be further processed into value-added products such as bioactive peptides. Bioactive peptides with antioxidant ([Zielińska et al., 2017](#)), antihypertensive ([Wu et al., 2015](#)), and antidiabetic ([Zhang et al., 2016](#)) activities have been produced by enzymatic hydrolysis of insect proteins using either mammalian or microbial enzymes ([Nongonierma and FitzGerald, 2017](#)). Chitosan is a polycationic derivative of chitin that can form flexible and durable film with the help of plasticizing agent. Chitosan film is edible and biodegradable. It also possesses antimicrobial activity and good thermal stability, making it an excellent food packaging material ([Martínez-Camacho et al., 2010](#)).

Different processing techniques may affect the nutritional value and functional properties of edible insects in different ways. It is reported that solar drying and toasting resulted in a 7% decrease in protein digestibility and a 11%–68% reduction in vitamin content of grasshoppers (*Ruspolia differens*) (Kinyuru et al., 2010) whereas smoke drying effectively reduced the cholesterol content of palm larvae *Rhynchophorus phoenicis* and *Oryctes monoceros* by 41% and 80%, respectively (Edijala et al., 2009). For the *R. phoenicis* larvae, roasting and grilling respectively resulted in a 35% and 29% reduction in water holding capacity while smoking led to a seven-fold increase in protein solubility and a 50% decrease in oil holding capacity (Womeni et al., 2012). Therefore, it is necessary to optimize the processing methods for each insect species based on the desired attributes of the final products.

## Safety Concerns of Eating Insects

Despite the environmental and nutritional benefits of edible insects, the potential safety issues associated with insect-based foods need to be identified and addressed. Insects may contain physical hazards, toxins, antinutrients, allergens, pathogens, and parasites. Food safety is particularly worrisome for edible insects collected from the wild due to possible exposure to harmful substances. Entomophagy-induced pesticide and lead poisonings have been reported (Defoliart, 1999; Handley et al., 2007). Bioaccumulation of arsenic and cadmium by insects is also known (Van Der Fels-Klerx et al., 2016). Consequently, the United States Food and Drug Administration requires edible insects to be raised specifically for human food in good manufacturing practice facilities and prohibits wildcrafted insects to be sold as food (Ramaswamy, 2015).

Many insects are armed with hairs, spines, and stingers that can cause irritation or choking. One example is spiny elm caterpillar (*Nymphalis antiopa*), an edible insect in Mexico (Ramos-Elorduy et al., 2011), that stings with its setae (Hossler, 2010) (Fig. 6). Another example is the grasshoppers with heavily spined tibiae, which can cause internal hemorrhage, intestinal occlusion, or even death in animals and humans (Defoliart, 2002). Therefore, the heads, legs, and wings of the grasshoppers and the hairs and spines of the caterpillars are often removed before cooking (Defoliart, 2002).

Other insects defend themselves with venoms, toxins, or bad smell. We are well alarmed of the painful stings or bites by ants, bees, wasps, caterpillars, and assassin bugs and the obnoxious smell of certain true bugs, beetles, and caterpillars. A myriad of poisonous substances are present in a variety of insect species including benzoquinone in tenebrionid beetles, cantharidin in meloid beetles, cyanogenic substances in coleopteran and lepidopteran insects, leptinotarsin and Bushman arrow poisons in chrysomelid beetles, metabolic steroids in dytiscid beetles, pederin in staphylinid beetles, pyrrolizidine alkaloids in cinnabar moths, toluene in longhorn beetles, and heart-toxic cardenolides in monarch butterflies and laygaed bugs (Belluco et al., 2013; Schmidt, 2008). Some of the chemical hazards can be removed or inactivated through cooking or digestion while some cannot (Belluco et al., 2013). Careful assessment of the toxicity of different insect species is warranted for developing safe products for human consumption. Although not recommended, consumption of toxic insects are practiced. For example, cantharidin- and pederin-containing beetles are used as anti-fungal agents in folk medicine. These toxic terpenoids can cause severe blistering of the skin (Mitsuhashi, 2008). Cantharidin is also the active component of the well-known ‘aphrodisiac’ Spanish fly (*Lytta vesicatoria*), which is banned due to its lethal toxicity (Karras et al., 1996).

Antinutrients are another entomophagy-related health concern. Consumption of African silkworm (*Anaphe* spp.) pupae is responsible for an acute seasonal ataxic syndrome in Nigeria for over 40 years due to the presence of a highly active and heat-resistant thiaminase (Nishimune et al., 2000). Antinutrients of plant origin such as oxalates, phytates, saponins, and tannins, which are likely accumulated from feed, are also found in edible insects (Omotoso, 2006; Musundire et al., 2014; Chakravorty et al., 2016). In order to utilize insects as a nutritious food source, such antinutritional factors need to be mitigated, eliminated, or inactivated.

For sensitive individuals, insects may cause adverse immune responses. Non-food-mediated allergic reactions such as Hymenoptera venom allergy are excluded from discussion. Food allergies to bee larva and pupa, cicada pupa, *Clanis bilineata*, cochineal insect (*Dactylopius coccus*), field cricket (*Gryllus bimaculatus*), grasshopper, lentil weevil (*Bruchus lentis*), locust, mopane caterpillar (*Imbrasia belina*), and silkworm pupa (*B. mori*) have been documented (Ji et al., 2009; Dicello et al., 1999; Srinroch et al., 2015; Armentia et al., 2006; Kung et al., 2011; Ji et al., 2008). The allergens identified include arginine kinase (*B. mori*, *G. bimaculatus*), hexamerin 1B (*G. bimaculatus*), phospholipase (*D. coccus*), and tropomyosin (*T. molitor*) (Broekman et al., 2015; Srinroch et al., 2015; Liu et al., 2009; Ohgiya et al., 2009). Allergens of insect origin can act either as a primary allergen (i.e., the original sensitizing molecule) or as a cross-reactive allergen with the latter being more common in Western countries. Arginine kinase and tropomyosin are well-known invertebrate pan-allergens responsible for insect-shellfish cross-reactivity (Reese et al., 1999; Binder et al., 2001). Therefore,



**Figure 6** Spiny elm caterpillar (*Nymphalis antiopa*).



individuals with shellfish allergy are recommended to avoid entomophagy. Moreover, patients allergic to inhalant arthropod allergens (e.g., cockroach and house dust mite) may experience oral allergy syndrome when eating insects.

Some insect species are notorious for spreading diseases because they are vectors of pathogenic microorganisms such as bacteria, virus, fungi, and protozoa (Van Huis et al., 2013). Unlike conventional livestock, many insects are consumed with their gut microflora. Practices such as fasting and evisceration may affect the microbial composition of the insect food product (Van Huis et al., 2013). In general, raw insects are high in microbial load. The microbial counts were as high as 8.6 log cfu/g in fresh mealworm larvae (*T. molitor*) and grasshoppers (*Locusta migratoria*) sold for human consumption (Stoops et al., 2016). Microbial contamination is also common in improperly processed or stored insects. Pathogenic bacteria including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus cereus* were found in rhinoceros beetle grubs (*Oryctes monocerus*) (Banjo et al., 2006) and mycotoxin producing fungi (e.g., *Aspergillus*, *Penicillium*, and *Fusarium*) were isolated from stored mopane caterpillars (*I. belina*) (Mpuchane et al., 2000). Proper processing and storage are therefore important. For example, boiling for 5 min is effective in eliminating Enterobacteriaceae but not sporeforming bacteria in mealworm larvae (*T. molitor*) and house crickets (*A. domesticus*). Consequently, storage at refrigeration temperature is recommended for these processed insects (Klunder et al., 2012).

Insects may also carry parasites or act as parasites themselves. Consumption of aquatic insects such as dragonflies and damselflies in Southeast Asia is associated with foodborne intestinal flukes (Chai et al., 2009). A case of *Gongylonema* infection due to accidental ingestion of an uncooked insect has been reported in the United States (Wilson et al., 2001). The cheese skippers (*P. casei*) in *casu marzu* are sometimes consumed alive in Italy and are repeatedly reported for causing intestinal myiasis (Peckenschneider et al., 1952). Although thoroughly cooked insects pose no risk of parasite infection, it is recommended to avoid consumption of insects that are known vectors of parasites.

## Consumer Acceptance

Besides the safety concerns, consumer acceptance is another challenge to the utilization of insects as human food. Due to unfamiliarity in Western countries, insects are considered disgusting and often evoke negative emotions in consumers (Lensvelt and Steenbekkers, 2014; Looy et al., 2014; Gmuer et al., 2016). Among the Western consumers who are willing to taste insect-containing foods, insects eating is mostly out of curiosity (Yen, 2009; Verkerk et al., 2007). Consumer acceptance studies have been performed in Netherlands, Belgium, Italy, Hungary, Germany, and China (De Boer et al., 2013; Vanhonacker et al., 2013; Verbeke, 2015; Megido et al., 2016; Cicatiello et al., 2016; Sogari, 2015; Laureati et al., 2016; Gere et al., 2017; Hartmann et al., 2015). According to these investigations, the major barriers of consumer acceptance towards insect-based foods are unfamiliarity/culture prejudice, safety concerns, nutritional value, and flavor (Lensvelt and Steenbekkers, 2014; Tan et al., 2015; Cicatiello et al., 2016). Several studies have shown that if the insect ingredients are incorporated in familiar food products instead of having the insects visible, consumers in Western countries are more likely to accept the product (Gmuer et al., 2016; Schösler et al., 2012; Hartmann et al., 2015; Megido et al., 2016). In fact, studies have shown that the taste of insect-based foods was well accepted by consumers with a preference to known flavors and textures (Caparros Megido et al., 2014; Elzerman et al., 2013).

The fact that most people in Western cultures associate insects with negative emotions and disgusting feelings is probably because they are only familiar with insects that are pests or hazards while not having much exposure to the edible species. Insect tasting helps consumers get acquainted with entomophagy, and therefore, is considered important to decrease food neophobia (Megido et al., 2016). In order to increase the consumer acceptance, activities that expose more consumers to insect-based food products and educate consumers about the sustainability, nutritional values, and safety of edible insects should be promoted. Recently, many insect-based food companies have emerged, introducing new products and recipes to the market (Dossey et al., 2016). Information on edible insects are also advocated through dedicated insect food festivals (e.g., the Annual Montana State University Bug Buffet), conferences (e.g., the Insects to Feed the World International Conference), and organizations such as the North American Coalition for Insect Agriculture.

Apart from consumers' willingness to try insect-based foods, a higher level of consumer acceptance is their willingness to pay for the products. A gap between the intention to eat insects and the actual purchase of insect-based products is likely to exist, even among the consumers who have declared an interest towards entomophagy (Cicatiello et al., 2016). Tan, Verbaan and Stieger (2017) reported that even with high interest and good products, willing consumers still hesitate to consume insect-based foods regularly due to other practical and socio-cultural factors.

## Conclusions

Entomophagy is an old idea that dates back to prehistory and it is regularly practiced in many regions until now. Although eating insect is still adventurous for Western consumers, people are getting acquainted with this novel food. Thanks to their nutritional values and environmental benefits, these little creatures may not only provide a solution for the food security challenge on this planet, but also serve as an animal protein source for astronauts during deep space exploration in the future (Tong et al., 2011). Despite the great potential of edible insects, more studies are warranted for nutrition and safety assessment, mass production and processing, product development, and consumer testing. Collaborative efforts from researchers, insect farmers, food manufacturers, regulatory agencies, and consumers are needed to incorporate edible insects in our daily diet.

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## Low-Glycemic Foods: Pulses

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### Nomenclature

AUC area under the curve

BG blood glucose

GI glycemic index

GIP gastric inhibitory polypeptide

GLP-1 glucagon like peptide 1

Min minutes

PYY peptide tyrosine tyrosine

VAS visual analog scale questionnaire

### Overview

Pulses and pulse ingredients (fractions and flours) have many positive nutritional attributes. Regular consumption of pulses (1/2 cup/day) is related to higher quality diets, including higher intakes of fiber, protein, folate, zinc, iron, and magnesium as well as lower intakes of saturated fat and total fat compared to diets low in pulses (Mitchell et al., 2009). Substantial scientific evidence supports the notion that low glycemic index (GI) foods can positively influence many physiological processes, which are relevant to health such as postprandial glycaemia. Based on their inherent nutritional composition, pulses are a very suitable food for glycemic control. Pulses contain high amounts of protein, which make them unique in comparison to other plant foods. In fact, the protein content of pulses ranges from 17%–30% of dry weight which is typically twice the amount found in cereals (Boye et al., 2010). Pulses are limited in sulphur-containing amino acid content, but are high in lysine (McCrory et al., 2010). Higher intakes of protein in a meal have been shown to decrease the glycemic response (Smith et al., 2012). Pulses also contain high amounts of complex carbohydrates including both soluble and insoluble fiber (Tosh and Yada, 2010), as well as resistant and slowly digestible starch (Tosh and Yada, 2010; Hoover et al., 2010). Pulses contain about 15%–32% total dietary fiber; 33%–75% is insoluble fiber with the remainder being soluble fiber (McCrory et al., 2010). A variety of health benefits are associated with the intake of dietary fiber, including improved glycemia and insulin sensitivity in non-diabetic and diabetic individuals (Anderson et al., 2009). Similar to dietary fiber, demonstrated health effects of resistant and slowly digestible starches are improved glycemic and insulin responses (Nugent, 2005; Anderson et al., 2010; Bodinham et al., 2010).

### The Glycemic Index

The GI is defined as the incremental area under a two-hour blood glucose (BG) response curve (AUC) following a 12h fast and ingestion of a food (Sc et al., 1981; Wolever et al., 1991). For glycemic studies, the test food has a certain quantity of available carbohydrate, usually 50 g, and is compared relative to a control food containing the same amount of available carbohydrate. A lower GI suggests slower rates of digestion and absorption of the carbohydrate in the test food, and usually equates to a lower insulin demand. In vivo testing is considered standard.

Substantial scientific evidence supports the notion that low GI foods can influence many physiological processes which are relevant to health. The ingestion of high GI foods where there is rapid absorption can pose a challenge to beta-cells. It has been suggested that hyperglycemia as a result of consuming a high GI diet may contribute to developing insulin resistance. This can lead to diminished beta cell function as a result of the increased insulin demand (Schofield and Sutherland, 2012), which may increase risk of developing type 2 diabetes (Grill and Bjor, 2000).

### Effects of Whole Pulse Consumption on Post-prandial Glycemia

The effects of whole pulses on short term glycemic control have been assessed in over 80 published human studies including GI and postprandial glycemic response studies. A variety of whole pulses have been studied (Jenkins et al., 1980; Potter et al., 1981; Jenkins et al., 1982; Traianedes and O'Dea, 1986; Dilawari et al., 1981; Mollard et al., 2011; Thompson et al., 2012; Mollard et al., 2012).

The following paragraphs discuss relevant whole pulse research findings. A summary of these findings along with other relevant findings are summarized in [Table 1](#).

The father of the GI, Dr David Jenkins, examined the BG response of 35 carbohydrate rich foods in his early glycemic research. The foods tested included varieties of pulses, potatoes, grains, breads, pasta, cereals, and biscuits. The pulse treatments included butter beans, haricot beans, kidney beans, soya beans, black eye peas, chickpeas, marrowfat peas, and lentils. Blood glucose samples were obtained by fingerpick at baseline and were followed over 2 h following the consumption of the treatment ([Jenkins et al., 1980](#)). Results showed that the mean BG for each pulse treatment was significantly lower than the mean curves of all other foods tested for at least two measurement time points each. The AUC and mean peak in BG were significantly lower for all dried legumes than the other treatments ([Jenkins et al., 1980](#)). This study established that pulses are low glycemic compared to other common carbohydrate foods.

[Potter et al. \(1981\)](#) examined the effect of varying dietary fiber content of foods, including pinto beans, on plasma insulin and BG response. The treatments were a glucose formula control, brown rice, pinto beans, and all bran cereal. The bran and pinto bean treatments resulted in lower BG compared to control at 30, 60, and 180 min. For insulin, the response to bran was lower at 30 and 60 min compared to the control. Consumption of the pinto treatment also had lower insulin concentrations at 30 min compared to control. Bran and pinto bean both resulted in lower insulin concentrations compared to rice at 30 min. The authors concluded that dietary fiber was most likely responsible for the differences in insulin and glucose concentrations. Fiber content was analyzed for each treatment, and the higher the BG and insulin, the lower the non-digestible fibers including lignin, hemicellulose, and cellulose. Pinto beans and all bran were very similar in non-digestible fiber content which corresponds to their similar BG and insulin responses ([Potter et al., 1981](#)). This study demonstrates that the fiber content of pinto beans is comparable to that of bran and possesses acute insulin and BG lowering effects.

[Jenkins et al. \(1982\)](#) also examined the effects of processing on lentils. The treatments were a white bread control, boiled 20 min lentils, boiled 20 min blended lentils, 1 h boiled lentils, and 1 h boiled 12 h oven dried lentils. The BG response to the bread and 12 h dried lentil treatment were very similar. The BG responses to the bread and 12h dried lentil treatment were higher than the 20 min boiled lentil treatment at 15, 30, and 45 min following treatment consumption. There were no differences in BG response between 20 min boiled lentils, 1h boiled lentils, and 20 min blended lentils. Through in vitro digestion testing it was found that carbohydrates were more rapidly digested from the lentils that were dried for 12 h compared to the lentils boiled for 20 min, which explains the higher BG response ([Jenkins et al., 1982](#)). This research indicates that processing can significantly affect the BG effects observed. Lentils that were dried for 12 h compared to boiled for 20 min resulted in an increased BG response due to carbohydrate being more rapidly digested. Blending does not appear to increase BG but prolonged exposure to heat does ([Jenkins et al., 1982](#)). This study established that 12 h of dry heat cooking raises the BG response to lentils. The effects of processing are supported by other research studies that have shown that the digestion of starch is also affected by the duration of pressure cooking, and temperature ([Traianedes and O'Dea, 1986](#)).

Studies examining the glycemic response of pulses when served in a mixed meal have shown that the glycemic effects are retained ([Mollard et al., 2011](#); [Dilawari et al., 1981](#); [Mollard et al., 2012](#); [Thompson et al., 2012](#)). [Mollard et al. \(2012\)](#) investigated the acute effects of BG following a meal with pulses and after a pizza meal 4 h later. The treatments included chickpeas, lentils, navy beans, and yellow peas served with pasta and homemade tomato sauce. The control was the pasta and sauce alone. All treatments resulted in a significantly lower BG compared to the control over the testing period, navy beans also resulted significantly lower compared to chickpeas. Following the pizza meal, BG was not affected by treatment. In response to all pulse treatments, BG before the pizza meal was significantly lower compared to control ([Mollard et al., 2012](#)).

Complementing the previous study, [Thompson et al. \(2012\)](#) examined the effects of three pulse varieties on BG using mixed meal formats. Treatments were long grain rice (control), pinto beans, black beans, and red kidney beans. The bean treatments were served with rice and were matched for 50 g of available carbohydrate. Results demonstrated that the BG response was lower compared to the control for all bean treatments at 90, 120, and 150 min and for pinto beans at 180 min. The black and pinto bean treatments led to a lower overall BG response compared to the red kidney beans though black beans had lower total fiber. The authors expected the bean treatments to result in a more uniform BG response because they were matched for carbohydrate content, however, the difference in fiber content among the beans may have been responsible for the effects observed ([Thompson et al., 2012](#)). Similar to the research by [Mollard et al. \(2012\)](#), some pulse treatments performed more favourably than others. The study concluded that bean and rice meals elicit an attenuated BG response compared to rice served alone, and that the difference in BG between bean types warrants further investigation ([Thompson et al., 2012](#)).

In summary, research of whole pulses consumed alone has demonstrated that compared to common carbohydrate foods that they are low glycemic ([Jenkins et al., 1980](#); [Traianedes and O'Dea, 1986](#)) and possesses acute insulin ([Traianedes and O'Dea, 1986](#); [Potter et al., 1981](#)) and BG lowering effects ([Potter et al., 1981](#); [Jenkins et al., 1982](#); [Traianedes and O'Dea, 1986](#); [Wong et al., 2009](#)). Additionally, the benefits of whole pulses when consumed alone are retained when consumed within a mixed meal ([Mollard et al., 2012](#); [Thompson et al., 2012](#)). While whole pulses have shown to be low glycemic, it appears that some pulses reduce glycemic response to a greater degree than others.

**Table 1** Acute glycemic response to whole pulses

Study	Participants	Treatment	Results
Jenkins et al. (1980)	Groups of 5–10 healthy volunteers of 110% ideal body weight. Age was not specified.	50 g carbohydrate portions of eight dried and boiled legumes (butter beans, haricot beans, kidney beans, soya beans, blackeye peas, chick peas, marrowfat peas, lentils), and 35 other high carbohydrate foods.	- Mean BG for each dried legume was significantly lower than the mean curves of all other foods at least two time points. The AUC and mean spike in BG was significantly lower for dried legumes than the other treatments.
Potter et al. (1981)	Eight healthy sedentary men aged 22–45 y.	1. Glucose formula (control, 102 g) 2. Brown rice (97 g) 3. Pinto beans (118 g) 4. All bran (106 g) Meals were blended and had water added so they were of equal volume. Matched for fat, protein, and carbohydrates.	Blood Glucose: - Bran and pinto bean was significantly lower than the control at 30, 60, and 180. Insulin: - Bran was significantly lower at 30, 60, and 120 compared to the control, and beans was significantly lower at 30. Bran and beans were also significantly lower compared to rice at 30 min. - The insulin response peak of the control was nearly twice that of pinto beans and bran. - There were no significant differences in BG between 20 min lentils compared to blended lentils or 1 h boiled lentils. - 12 hour dried lentils had a significantly higher BG response than 20 min lentils at some time points.
Jenkins et al. (1982)	Eight healthy participants (two men, six women) aged 21–37 y.	1. White bread control 2. Boiled 20 min lentils 3. Boiled 20 min blended lentils 4. One hour boiled lentils 5. One hour boiled, 12h oven dried lentils Meals were served with tomato, 500 mL of water, as well as tea or coffee.	- 12 hour dried lentils had a significantly higher BG response than 20 min lentils at some time points.
Traianedes et al. (1986)	Six normal weight participants (four men, two women) aged 25–40 y.	1. D-glucose (control) 2. Home cooked baked haricot beans 3. Heinz vegetarian baked haricot beans The home cooked beans were served with a sauce made of molasses, mustard and tomato.	- The home cooked beans BG AUC was significantly lower than canned beans.
Wong et al. (2009)	Healthy normal weight men aged 18–35 y. Study I: n = 14 Study II: n = 14 Study III: n = 15	I: Canned navy beans manufactured in Canada, canned navy beans manufactured in the UK, homemade navy beans made following a recipe, glucose drink (control). II: Canned navy beans in tomato sauce, canned navy beans maple style, canned navy beans pork and molasses, homemade navy beans pork and molasses, white bread (control). III: chickpeas, lentils, navy beans, yellow peas, white bread (control), water (control).	I: All beans resulted in a reduced BG with the exception of at 90 and 120 min compared to control. II: Homemade pork and molasses navy beans and canned navy beans in tomato sauce led to a reduced BG net AUC compared to control. III: BG net AUC was significantly reduced compared to white bread control for all beans except navy.

(Continued)

**Table 1** Acute glycemic response to whole pulses—cont'd

<i>Study</i>	<i>Participants</i>	<i>Treatment</i>	<i>Results</i>
Mollard et al. (2011)	25 men aged 20–30 y with a BMI between 20–24.9 kg/m <sup>2</sup> .	<ol style="list-style-type: none"> <li>1.Chickpea</li> <li>2.Lentil</li> <li>3.Yellow pea</li> <li>4.Macaroni &amp; cheese (control)</li> </ol> Pulse treatments were canned and served with macaroni pasta and served with homemade tomato sauce. All pulse treatments contained 40 g available carbohydrate. Overall available carbohydrate was matched between treatments at about 100 g, meals were isocaloric.	<ul style="list-style-type: none"> <li>- No differences in pre-meal pizza AUC.</li> <li>- Chickpea and lentil significantly lowered post-pizza meal BG AUC, yellow pea did not.</li> </ul>
Mollard et al. (2012)	24 healthy males aged 20–30 y of normal weight.	<ol style="list-style-type: none"> <li>1.Chickpeas</li> <li>2.Lentils</li> <li>3.Navy beans</li> <li>4.Yellow peas</li> <li>5.Pasta and tomato sauce (control)</li> </ol> Treatments were served with pasta and homemade tomato sauce. 44% of energy from the meals was from pulses. The meals were isocaloric.	<ul style="list-style-type: none"> <li>- BG effect of treatment was significantly lower compared to control for all treatments to 260 min.</li> <li>- AUC was significantly lower compared to the control for all treatments.</li> </ul>
Mollard et al. (2014)	15 healthy males aged 18–35 y of normal weight.	<ol style="list-style-type: none"> <li>1.White bread (control)</li> <li>2.Chickpeas</li> <li>3.Lentils</li> <li>4.Navy beans</li> <li>5.Yellow peas</li> </ol> The meals were isocaloric. A pizza meal was served at 135 min following treatment consumption	<ul style="list-style-type: none"> <li>- All pulses led to a mean decreased BG response compared to control. Chickpea and lentil were also lower compared to yellow pea</li> <li>- Pre-pizza BG AUC was lower for chickpea, navy bean, and lentil compared to control.</li> <li>- Post-pizza BG AUC was lower for lentil compared to control</li> </ul>



**Table 2** Acute glycemic response to pulse flours

Study	Participants	Treatment	Results
Hall et al. (2005)	11 participants (nine men, two women) aged 25–45 y with a BMI of 20.9–28.6 kg/m <sup>2</sup> .	1. White bread (control) 2. White bread with 7.7 g of Australian sweet lupin flour (ASLF) added Consumed with margarine, apricot jam and decaffeinated tea.	- BG response of the ASLF bread was significantly lower than the control. - Insulin response was significantly higher for the ASLF bread than the control.
Johnson et al. (2005)	11 healthy participants (nine men, two women) aged 25–45 y.	1. White bread (control) 2. Chickpea flour 3. Extruded chickpea flour	- BG of the chickpea bread was significantly lower than the control at 90 min, and the extruded chickpea bread BG was significantly lower than the control at 120 min.
Marinangeli et al. (2009)	20 healthy participants (seven men, 13 women) aged 22–67 y, with a BMI between 21–42 kg/m <sup>2</sup> .	1. Boiled whole yellow pea (control) 2. White bread (control) 3. Banana bread with WYPF 4. Biscotti with WYPF 5. Pasta with WYPF	- Decreased iAUC BG response for WHPF biscotti and banana bread compared to white bread.
Anderson et al. (2014)	Healthy young men Ex. I (n = 17) Ex. II (n = 12) Ex. III (n = 12)	Ex. I/II/III 1. Whole navy bean/lentil/chickpea 2. Pureed navy bean/lentil/chickpea 3. Powdered navy bean/lentil/chickpea	- Overall no significant differences seen between the treatments due to processing. - For lentil and chickpea treatments mean BG was lower over 120 min compared to control, for navy bean only peak BG was lowered compared to control.

### Effects of Pulse Flour Consumption on Post-prandial Glycemia

Limited pulse flour research exists, however, research has demonstrated that despite milling, varying benefits of whole pulses are retained compared to control (Anderson et al., 2014; Marinangeli et al., 2009; Johnson et al., 2005; Hall et al., 2005). The following paragraphs discuss relevant pulse flour research findings. A summary of these findings along with other relevant findings are provided in Table 2. Hall et al. (2005) examined the effects of Australian sweet lupin flour (ASLF) bread on glycemic response. Treatments were a white bread control and ASLF containing bread. The BG peak was seen at 30 min for both treatments and the BG response of the ASLF bread was lower than the control. The peak insulin response was seen at 30 min for both treatments and the insulin response was significantly higher for the ASLF bread than the control (Hall et al., 2005). This study demonstrates that the acute beneficial BG effects of lupins are still observed when milled to a powder and made into bread.

Another aspect of processing was examined by Johnson et al. (2005) by incorporating chickpea flour, and extruded chickpea flour into bread and comparing it to a white bread as control. The treatments all had 50 g of available carbohydrate and were very similar in energy, protein, and fat. The control bread contained 3 g total fiber, chickpea flour bread contained 5 g total fiber, and extruded chickpea flour bread contained 6 g total fiber. The chickpea bread replaced 24.3% of the white bread flour in the chickpea bread treatments, this amount was chosen because it kept the bread palatable. For all treatments, BG peaked at 30 min and returned to and fell beneath baseline between 60–90 min. There were no differences in BG iAUC for any treatments. Consuming chickpea bread resulted in a lower BG compared to the control at 60 min, and at 120 min the extruded chickpea bread resulted in lower BG compared to control. At 60 min both treatment breads had lower insulin compared to the control. The insulin iAUC was also significantly higher for chickpea bread than the control bread. The authors suggested that perhaps the incorporation of chickpea flour was too low or the particle size too small to observe the glycemic and insulin benefits seen by whole chickpeas. They believed that further studies with varying particle size and greater incorporation of chickpea flour are needed (Johnson et al., 2005). It was surprising to see a hyperinsulinemia effect from the chickpea flour, which also warrants further investigation.

Marinangeli et al. (2009) explored the glycemic response of whole yellow pea flours (WYPF) in food products. Treatments were WYPF incorporated into banana bread, biscotti, and pasta. Controls were boiled whole yellow peas, white bread, and whole wheat flour (WWF) (Marinangeli et al., 2009). WYPF biscotti and WYPF banana bread resulted in a significantly lower (61.9% and 55.1%) iAUC BG response compared to WB. The WYPF biscotti resulted in a 29.2% BG reduction compared to the WWF biscotti. However, WYPF pasta resulted in significantly higher BG iAUC than boiled yellow peas. The WYPF pasta did not result in a lower BG response compared to WWF. This difference in BG response suggests that the manner in which the treatments are cooked affects the glycemic effect exhibited. It was not indicated whether WYPF possesses the same beneficial effects as whole yellow peas, but the cooking method utilized appeared to be responsible for the beneficial effects of WYPF. This research supports the use of whole pea flour in novel foods products intended to be low glycemic (Marinangeli et al., 2009).

In another study examining pulse flours, Anderson et al. (2014) found that the beneficial effects of whole pulses are not diminished when ground to a powder. Three experiments were conducted, each with a control of whole-wheat flour. In experiment I, the treatments were whole navy bean, pureed navy bean, and navy bean powder. In experiment II, whole lentil, pureed lentil, and lentil powder were provided. In experiment III, whole chickpea, pureed chickpea, and chickpea powder were provided. There were 38.8 g of available carbohydrate in each treatment and water was added so treatments were of equal weight (Anderson et al., 2014).

Participants consumed a fixed-size pizza meal at 120 min. In experiment I, prior to the pizza meal there was a significant time by treatment interaction. At 15 min the bean powder treatment did not differ from the control in BG response. At 30 min the three bean treatments resulted in a significantly lower BG compared to control. At 45 min the navy bean powder resulted in lower BG compared to the pureed treatment. At 60 min whole wheat resulted in lower BG compared to the whole navy bean treatment. The difference in pre pizza meal BG net AUC was significant for the navy bean powder compared to the control, and was intermediate for the other two treatments. In experiment II there was a significant time by treatment interaction for BG; mean BG was also significantly lower compared to the control for all lentil treatments at all time points preceding the pizza meal. Compared to the control, the BG net AUC was lower for whole and powdered lentils. In experiment III, time points preceding the pizza meal had a time by treatment interaction and BG was significantly lower for powdered, pureed, and whole chickpeas compared to the control. The authors concluded that pureeing and commercial processing does not negate the acute glucose regulation benefits of consuming pulses as there were overall no significant differences seen between the treatments due to processing (Anderson et al., 2014).

The four published studies discussed above are encouraging in that it is evident that the BG lowering effects of consuming whole pulses are retained when pulses are processed to flours (Anderson et al., 2014; Marinangeli et al., 2009; Johnson et al., 2005; Hall et al., 2005). More studies exploring pulse flour incorporation are needed to determine the relative efficacy of specific pulse types across different food matrices, the most effective doses of incorporation, and the effectiveness following different processing methods.

### Pulse Fraction Extraction

Two primary fractionation processes are utilized on pulses; dry and wet fractionation. The dry fractionation process involves several steps: dehulling, pin milling, and air classifying fractions (Doney and Jolene, n.d.). During dehulling, the outer kernel is removed. This process is easier with lentils and peas and more difficult with beans and chickpeas due to differences in characteristics. Dehulling can be done by impact or by stone dehulling processes. Pin milling utilizes rapid pulverization to achieve product that is very fine. Air classification of fractions is then conducted utilizing a fluidized tower system. Particles are separated in a vacuum based upon particle size. Protein fractions range from 2–20  $\mu\text{m}$ , starch fractions range from 20–40  $\mu\text{m}$  (Tulbek, 2010).

The dry process allows for increased concentrations of target pulse components. In the dry fractionation process protein fractions have a 53% protein content compared to 22% in whole peas. The fiber fractions have 87% fiber content, which is the same achieved with the wet process. The carbohydrate fraction reaches 75% of starch/sugar content compared to 48% in whole pulses (Doney and Jolene, n.d.).

With wet fractionation the process has different steps: dehulling, milling, steeping, precipitation, decanting, dewatering, and drying (Doney and Jolene, n.d.). The dehulling process is the same as dry fractionation. Milling is performed using the same process or by hammer mill. The fine product is then solubilized in an NaOH solution for 1 h following which the protein and starch can be removed by ultrafiltration or by decanter. Protein may also be precipitated. Drying of protein and starch is then initiated by fluidized bed drying or by spray drying (Tulbek, 2010). An 86% protein content is achieved in the protein fraction, and the carbohydrate fraction achieves a 99% starch/sugar content (Doney and Jolene, n.d.).

### Effects of Pulse Fractions on Post-prandial Glycemia

The investigation of the effectiveness of the different pulse fractions is important in understanding the compositional aspects of pulses that are responsible for any shift in acute postprandial glycemia. It is also important to determine whether the benefits are retained when whole pulses are fractionated and incorporated into different food matrices. Limited pulse fraction studies exist thus far; however, studies that have been conducted are summarized in the following paragraphs and summarized in Table 3.

Smith et al. (2012) studied the effects of yellow pea protein and fiber on post-prandial glycemia. Two experiments were conducted. In experiment I, participants received an ad libitum pizza meal at 30 min, and in experiment II at 120 min (Smith et al., 2012). Treatments were tomato soup used as a control, and tomato soup with 10 g or 20 g of fiber or protein. In the treatments there were 9.5–10.5 g of available carbohydrate (Smith et al., 2012). In experiment I, BG was significantly lower over the whole treatment period for both protein treatments compared to the control. BG was overall significantly lower with protein 20 g treatment compared to control and fiber 10 g treatment. Both protein treatments had a significantly lower BG immediately following the pizza meal compared to the control and fiber 10 g treatment. The glycemic responses to yellow pea protein pre-meal were independent of dose; however, response to the post-pizza meal was dose dependent. In experiment II, there were no differences in BG response across treatments. The authors attributed the null results in experiment II to be due to pea protein's rapid digestion. The authors suggested that the null results from the fiber treatments, though surprising, may have been because the fiber was taken from the hull only and not from the entire pea. Pea hull contains 45.8% more insoluble fiber and 21% more soluble fiber than dehulled peas that contain fiber derived from the cotyledon of the pulse. There may be different benefits derived from consuming hull fiber vs consuming cotyledon and hull fiber in combination (Smith et al., 2012).

Mollard et al. (2014) conducted a study investigating the effects of pea fractions on glycemia when served with tomato sauce and noodles. Five treatments included yellow peas, pea hull fiber and pea protein, pea hull fiber, pea protein, and the control. An ad libitum pizza meal was given 135 min following treatment consumption (Mollard et al., 2014). The combined pea protein and

**Table 3** Acute glycemic response to pulse fractions

Study	Participants	Treatment	Results
Smith et al. (2012)	Experiment I (pizza meal at 30 min) n = 19 healthy men. Experiment II (pizza meal at 120 min) n = 20 healthy men.	Experiment I and II had the same five treatments: 1. Tomato soup (TS) 2. TS + 10 g yellow pea fiber 3. TS + 20 g yellow pea fiber 4. TS + 10 g yellow pea protein 5. TS + 20 g yellow pea protein	Ex I: BG was overall significantly lower with protein 20 g treatment compared to control and fiber 10 g treatment. Ex II: No BG differences.
Mollard et al. (2014)	15 healthy young men.	1. Tomato sauce and noodles (control) 2. Yellow peas 3. Pea hull fiber and pea protein 4. Pea protein 6. Fiber alone	- Combined pea protein and fiber treatment lowered the glycemic response compared to the control, this was not seen by the fiber or protein treatment alone.

fiber treatments lowered the BG response compared to the control similar to yellow peas. This was, however, not seen by the fiber or protein treatment alone (Mollard et al., 2014). The authors suggested that the dose of protein and fiber alone may have been too low to observe a difference in BG, however, the fiber treatment was observed to have resulted in the highest BG responses. This study establishes that fractions of pea fiber and protein combined retain the effects of whole peas, but suggests that fractions alone do not.

Results of the pulse fraction studies suggest that pea protein and fiber combined are more effective at reducing BG than protein or fiber alone. Smith et al. (2012) found that protein at a dose of 10 g as well as 20 g reduced BG following the consumption of a second meal at 30 min. However, when the second meal was served at 120 min there were no differences (Smith et al., 2012). Mollard et al. (2014) served a pea protein treatment of similar protein content (18 g) with tomato sauce and noodles and found that the protein treatment alone did not reduce BG significantly, but that the combined protein + fiber treatment lowered BG compared to control. A second meal was supplied at 135 min and did not produce significant differences in BG post-meal (Mollard et al., 2014). These results suggest that effects of pea protein alone are transient and that pea protein and fiber combined may have synergistic effects that allow BG lowering effects to last longer than protein alone. So far it remains unclear as to whether a specific fraction is responsible for the BG reduction effects. Further research is needed to investigate whether protein alone lowers BG, and to investigate whether there are synergistic effects when protein and fiber are consumed together. Research with varying amounts of protein and fiber are also needed to determine if there is a specific dose needed to elucidate effects.

### Potential Mechanisms of Action of Pulses

A low glycemic response ensues when pulses are consumed whole. When pulses are milled as served as flours the low glycemic response is mainly retained (Marinangeli et al., 2009; Anderson et al., 2014; Hall et al., 2005; Johnson et al., 2005). It is when pulses are processed into fractions that individual aspects of pulse composition can be investigated for their role in glycemic response.

As previously discussed, research indicates that the protein content of pulses appears to be a stronger modulator of BG response than pea fiber (Smith et al., 2012), however, in other research findings, fiber appears to be a strong contributing component when served in combination with protein (Mollard et al., 2014). One explanation on the effectiveness of pulse protein may be increased secretion of insulin. If pulse protein is insulinotropic, that could explain the beneficial effects seen on post-prandial blood glucose. The viscous properties of pulse fiber may decrease the speed of protein and carbohydrate absorption (Mollard et al., 2014). When served alone pulse protein appears to digest more quickly similar to whey protein (Smith et al., 2012). Pulse protein combined with fiber may also decrease gastric emptying (Mollard et al., 2014). The mechanisms behind the low glycemic response of pulses are still unclear. Further research is needed involving fractions, varying processing conditions, and hormonal response.

The effects of pulses on intestinal satiety should also be considered as a modulator of BG response. Close interaction between nutrients and the intestinal wall causes the release of satiety signal hormones. Increasing the viscosity of digested food as it travels through the small intestine increases its residence time, and time that nutrients are being absorbed. Prolonged residence of digested food in the small intestine also impacts gastric emptying through the release of satiety hormones (Paradis et al., 2011). GLP-1 and GIP are secreted during and after the consumption of a meal containing carbohydrates. They slow gastric emptying and stimulate insulin release (De Silva and Bloom, 2012). PYY increases following a meal, remains elevated for up to 6 h and slows gastric emptying (De Silva and Bloom, 2012). These and other metabolic factors involved with glucose metabolism should be explored to help elucidate the low glycemic effect of pulses.

Apart from differences in composition there are some effects of processing that should be considered. When processing pulses, starch granule damage may occur. Damaged starch granules are digested more quickly than non-damaged starch granules (Sandstedt and Mattern, 1960) and may explain differences in BG response among typically low GI pulses that have been processed. Pulse research has shown that the digestion of starch has been affected by the duration of time of pressure cooking, and temperature (Traianedes and O'Dea, 1986). Lentils that were dried for 12 h compared to boiled for 20 min produced carbohydrates that resulted

in increased BG response due to being more rapidly digested. Blending does not appear to increase BG but prolonged exposure to heat does (Jenkins et al., 1982).

Another aspect to consider is the effects of processing on protein. For example, extrusion improves pulse protein digestibility by causing protein to unfold and denature. Extrusion also increases protein digestion by inhibiting protease inhibitors that hinder protein digestion (Hood-Niefer and Tyler, 2010; Day and Swanson, 2013).

Along with inhibiting protease inhibitors that prevent protein digestion, extrusion also decreases other anti-nutrients. Some anti-nutrients, however, have desirable effects such as  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibition (Sievenpiper et al., 2009).  $\alpha$ -glucosidase and  $\alpha$ -amylase are important glucosidases needed by humans to digest starch (Yilmazer-Musa et al., 2012). These glucosidases are crucial to carbohydrate digestion as all carbohydrates other than monosaccharides require being broken down by enzyme activity prior to absorption. Indeed,  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibition impedes carbohydrate digestion leading to lower postprandial BG concentrations (Kim, 2013; Heacock et al., 2005; Paradis et al., 2011). Heating used during extrusion can decrease or inactivate compounds that contain anti-nutrition factors such as the  $\alpha$ -amylase inhibitor, which affects digestibility (Martín-Cabrejas et al., 1999). The temperature used for pulse cooking impacts the degree at which anti-nutrition factors are inactivated. These anti-nutrients could be more resistant during certain cooking processes in some pulses than in others leading to differences in glycemic response.

## Conclusion

In summary, pulses are well established as low glycemic foods that are high in protein, fiber, and other nutrients. Despite the merits of consuming pulses, they are not consumed as readily as they could be, this could be a result of unfamiliarity of cooking with pulses and lengthier preparation time.

Incorporating pulse fractions into commercial products could not only increase the nutritional content of the products but would also increase the number of individuals consuming pulses by having them available in a ready to consume form that individuals are familiar with, and many consume often. Greater awareness of how to cook with pulses and pulse fractions could also aid in increasing consumption. While it is well established that pulses are low glycemic, the exact mechanisms warrant further investigation.

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## Relevant Websites

- Pulse Canada and Pulse USA <http://pulses.org/nap/>.
- Pulses in Canada <http://www.statcan.gc.ca/pub/96-325-x/2014001/article/14041-eng.htm>.
- Pulses in the USA <https://www.ers.usda.gov/data-products/vegetables-and-pulses-data/>.



# Microencapsulated Food Ingredients

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## Introduction

Microencapsulation technology is used in a wide range of pharmaceutical, nutraceutical, and food and beverage industries for the delivery and/or protection of sensitive ingredients. The technology isolates or embeds bioactive ingredients (e.g., flavors, probiotics, omega fatty acids, vitamins, minerals, and enzymes) within a physical barrier of one or more macromolecules (proteins, polysaccharides and lipids), to produce capsules of varying morphologies [microcapsules (well-defined core-shell morphology) and microspheres (bioactive ingredients are disbursed within the matrix)] (Thies, 1996). As such, the capsules consist of two parts: the encapsulated substances, also known as core, internal, or payload phase, and the protective materials, also known as wall, external, or coating phase. Generally, microencapsulation offers protection to sensitive core materials from moisture, oxygen, light, pH, and temperature to prolong shelf-life; converts liquid materials into an easily handled and dispersed solid powder; prevents nutrient loss of the encapsulant; provides controlled release of core ingredients; and masks undesirable flavor, taste and odor (Desai and Park, 2005; Lakkis, 2008; Nedovic et al., 2011).

This chapter provides a brief review of the microencapsulation of food ingredients (flavors, probiotics, omega fatty acids, micronutrients, and enzymes), addressing issues surrounding: the coating materials and techniques for encapsulation, the physicochemical and release properties of the microcapsules themselves, and processing challenges. Table 1 presents examples of the microencapsulation of different core materials using different processing techniques.

## Microencapsulation of Flavors

Flavors determine the overall acceptability and preference of food and beverage products among different consumers. The perception of flavor is triggered by various components, including aromatic compounds (perceived by olfactory epithelium, e.g., burnt, musty, and fresh), gustatory components (perceived by taste receptor cells on the tongue, e.g., sweet, sour, salty, bitter, and umami), chemical heat principals (perceived by filiform taste buds, e.g., spicy and numb), and physical properties of foods (perceived by cells on the tongue and palate, e.g., crispy, hard, and soft) (Delwiche, 2004). However, many flavor compounds are susceptible to environmental changes (e.g., light, pH, humidity, and temperature), which results in ingredient–ingredient interactions altering the flavor profile and in turn decreases consumer acceptance. Therefore, microencapsulation is introduced to maintain a consistent flavor in food products over a period of storage and provide controlled release during processing and consumption.

Depending on the affinity of the flavor components for certain encapsulation operations or materials, different techniques can be used (Table 1). For example, spray drying and chilling are appropriate for both nonpolar and polar flavor systems, whereas co-extrusion is typically used for nonpolar systems. The specific wall material used can determine the controlled release, flavor masking abilities, and level of protection. It is required that wall materials should have generally recognized as safe (GRAS) status, film-forming capacity, good dispersability in the solvents, neutral taste, and chemical inertness to the flavor compounds during processing and storage. Food grade biopolymers such as proteins, lipids, gums, and cellulose, are common encapsulation matrix materials (Shahidi and Han, 1993; Zuidam and Heinrich, 2010). Charve and Reineccius (2009) selected proteins (sodium caseinate, whey and soy protein isolates) and hydrocolloids (gum acacia and modified starch) to encapsulate limonene and aldehydes [(E)-2-hexenal, (E)-cinnamaldehyde, and citral] using spray drying. They found hydrocolloids (especially gum acacia) greatly prevented the flavor loss, whereas proteins effectively limited limonene oxidation during 28 d of storage at 40 °C, with a 1:4 ratio of flavors to wall materials providing the best retention (Charve and Reineccius, 2009; Zuidam and Heinrich, 2010). Therefore, both type and concentration of encapsulation matrix materials are identified as important factors influencing flavor encapsulation.

Spray drying has been the most widely used encapsulation technique in the flavor industry, but many novel techniques (spray chilling, melt extrusion, molecular inclusion complexation, and coacervation) were recently developed to satisfy product development and manufacturing requirements (production efficiency, particle size, storage stability, cost, and production yield) (Gouin, 2004; Sobel et al., 2014). Due to simple operation procedures and low temperature, spray chilling (also known as spray cooling) is an attractive method to entrap flavor compounds and form solids via crystallization. Therefore, it is critical for the selected wall materials (stearin, waxes, polyols, and mono- or diacylglycerols) to undergo solidification at low temperature (Sillick and Gregson, 2012). Oriani et al. (2016) used three different lipids (palmitic acid, oleic acid and palm fat) to convert ginger oleoresin into a powder that remained solid at room temperature using spray chilling; they reported that the best retention of pungent (>95%) and volatile (>80%) compounds occurred when using palmitic acid alone.

Molecular inclusion complexation is another novel technique to utilize the porous structure of materials (cyclodextrin and hydrophobic modified starches) to entrap flavor molecules, and is typically used to maintain flavor stability and mask undesirable taste (Yu and Huang, 2010; Sobel et al., 2014). Saldanha do Carmo et al. (2017) formed cyclodextrin inclusion complexes with limonene for enhanced flavor retention in non-alcoholic beverages. Additionally complex coacervation has been utilized as an



**Table 1** Microencapsulation of flavors, probiotics, omega fatty acids, and enzymes using different techniques

Core material	Wall material	Technique	Main results of encapsulation	References
<b>Flavors</b>				
Limonene, (E)-2-hexenal, (E)-cinnamaldehyde, and citral	Sodium caseinate, WPI, SPI, gum acacia, and modified starch	Spray drying	Improved flavor retention, prevent oxidation and non-enzymatic browning reaction	Charve and Reineccius, 2009
Ginger oleoresin	Palmitic acid, oleic acid and palm fat	Spray chilling	Efficient pungent and volatile compound retention and conversion of viscous oil into powder	Oriani et al., 2016
Curcumin	Hydrophobically modified starch	Molecular inclusion complexation	Increased bioaccessibility and stability, and enhanced in vitro anti-cancer activity of curcumin	Yu and Huang, 2010
<b>Probiotics</b>				
<i>Lactobacillus</i> spp.	Sodium alginate (double coating)	Freeze drying	Increased survivability under simulated digestive conditions and increased heat tolerance	Rather et al., 2017
<i>Lactobacillus acidophilus</i> and <i>Bifidobacterium</i> spp.	Hydrogenated vegetable oil, ethylcellulose E100, maltodextrin, sodium alginate, microcrystalline cellulose, and calcium alginate	Fluidized bed drying	Improved heat resistance, and ability to withstand relative high humidity and shear force	Penhasi et al., 2010
<i>Lactobacillus</i> spp.	Sorbitol and trehalose	Vacuum drying	High stability of bacteria at non-refrigerated temperatures for three months	Foerst et al., 2012
<b>Omega fatty acids</b>				
Canola oil	Lentil protein isolate, maltodextrin, sodium alginate, and lecithin	Spray drying	Better oxidative stability, and higher entrapment efficiency	Chang et al., 2016
Olive oil	Gelatin and sodium alginate	Complex coacervation	Improved thermal stability, controlled release properties, no interaction between core and wall materials, and free flowing spherical structure	Devi et al., 2012
Fish oil	Soybean soluble polysaccharide, maltodextrin, and octenyl succinic anhydride starch	Fluidized-bed coating	Very high entrapment efficiency (96%–99%), great protection against oxidation	Anwar and Kunz, 2011
<b>Enzymes</b>				
Lactase	Shellac, gum acacia, and hydroxypropylmethyl cellulose	Spray coating	Protected lactase activity under acidic conditions, and effectively reduced lactose in the dairy products	Soloman, 2013
Lipase	Sodium chloride and hydroxypropylmethyl cellulose	Fluidized-bed coating	Decreased oil rancidity, maintained lipase activity and extended the shelf-life	Plijter and Meesters, 2000
Proteinase (Flavourzyme)	Lecithin and cholesterol	Liposome	Entrapment efficiency of 25%, accelerated ripening of Iranian white cheese	Jahadi et al., 2016

encapsulation method, for example, [Yang et al. \(2014\)](#) encapsulated vanilla oil with hydrocolloid materials (chitosan, gum Arabic, and genipin) using complex coacervation to improve the thermostability and flavor retention of the vanilla oil.

### Microencapsulation of Probiotics

Probiotic microorganisms have garnered much attention for their health benefits and disease prevention effects. They are defined as non-pathogenic and non-toxicogenic living microorganisms (e.g. *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*) that provide nutritional and therapeutic benefits to the host such as modulating immune response, increasing resistance to infectious intestinal diseases, and preventing colon cancer ([Schrezenmeir and de Vrese, 2001](#); [Kotzampassi and Giamarellos-Bourboulis, 2012](#)). However, considering the fragility of probiotic microorganisms to a variety of adverse environmental conditions and manufacturing processes, encapsulation can be used to extend shelf-life, protect the probiotics, and control their release in the intestinal tract ([Table 1](#)).

Polymeric materials (e.g., high amylose starch, pectins, and carrageenans) are most frequently used for probiotic encapsulation to immobilize the bacteria to prevent cell death in the stomach and release them across the intestinal tract. For example, high amylose starch is able to form a strong gel matrix through the hydration of starch granules in boiling water under stirring, which is non-digestible in the stomach but readily degradable by gut microflora in the intestine ([Cook et al., 2012](#)). Due to the simple preparation and crosslinking conditions, alginate has been extensively studied for its use in microencapsulation. However, those polymeric matrices tend to form a porous structure that decreases microcapsule stability; an extra coating of biopolymers is often a means to help circumvent this issue. [Rather et al. \(2017\)](#) double coated probiotics (composed of *Lactobacillus* spp.) in alginate to produce freeze dried microcapsules that survived treatment under simulated digestive conditions and had increased heat tolerance as compared to free cells. Co-encapsulation has also been utilized to protect probiotics during digestion and increase their viability upon release. [Eratte et al. \(2015\)](#) co-encapsulated a probiotic bacteria with tuna oil in complex coacervates consisting of whey protein isolate and gum Arabic; the oil increased bacteria survival through *in-vitro* digestion as well as the cell surface hydrophobicity, which is used as a measure of the ability of the bacteria to adhere to the lining of the intestine.

Comparing with other methods (spray drying and fluidized bed drying), freeze drying and vacuum dehydration are most favorable for use in probiotic microencapsulation, due to low temperature, better encapsulation efficiency, and less contamination concerns ([Roser, 1991](#)). During the freeze drying, lyophilization under vacuum is applied after freezing the liquid containing the probiotics, therefore, the formation of ice crystals may result in loss and damage of living microorganisms. [Harel and Tang \(2014\)](#) stated that appropriate amounts (typically >50%) of food grade cryoprotectants (glycols, sugars, and salts) can be added to the liquid as antifreeze agents, to minimize the generation of ice crystals through depressing the freezing point of the probiotic liquid. In addition, controlled low-temperature vacuum dehydration, which involves the dehydration of non-frozen liquids containing probiotics at low temperature under vacuum, was also developed to avoid the damage from ice crystals associated with freeze drying. This is a faster drying technique due to the increased surface area and the vacuum condition, which can be easily controlled by the adjustment of the applied vacuum ([Harel and Tang, 2014](#)). [Foerst et al. \(2012\)](#) used sorbitol and trehalose for encapsulation of *Lactobacillus paracasei* F19 by vacuum drying and reported that while trehalose encapsulation produced the highest survival rate after drying, sorbitol provided the best bacteria protection during long-term storage of the probiotics at 20 and 37 °C. In addition the authors reported that vacuum drying produced cells with much higher stabilities than freeze dried cells. The applications of probiotics in nutraceuticals, functional foods, and supplements have been rapidly expanding through various delivery forms (e.g., tablets, soft gel capsules, and dry powders) and numerous probiotic products are commercially available in the market place.

### Microencapsulation of Oils Rich in Omega Fatty Acids

Omega fatty acids (omega-3, -6, and -9) play an essential role in the prevention and treatment of cardiovascular diseases and immune response disorders, development of the central nervous systems for infant growth, and maintenance of mental health ([Shibasaki et al., 1999](#)). However, because of their unsaturated structure, they are susceptible to oxidative deterioration and readily produce free radicals, which are deemed to negatively affect the shelf-life, sensory properties, and overall acceptability of food products ([Velasco et al., 2003](#)). As such, microencapsulation offers a means to protect the sensitive omega fatty acids, decrease nutrient loss, increase shelf-life, mask unpleasant taste, and transform oils into an easily handled and dispersed solid powder in the marketplace ([Desai and Park, 2005](#)) ([Table 1](#)). The number of commercial food products (e.g., dairy products, baked goods, snacks, and beverages) fortified with encapsulated omega fatty acids (especially omega-3) is dramatically increasing as advances in storage stability and desirable sensory properties of the products are made.

The use of just one wall material can create challenges in providing high entrapment efficiencies and protection to the core material; however, this can be overcome by using a combination of polysaccharides and proteins. Typically the wall material involves the use of proteins (e.g., whey proteins, gelatin, and soy protein isolate) because of their emulsifying and film-forming properties, and polysaccharides (e.g., maltodextrins, starches, and chitosan) because of their matrix forming properties, and specifically the use of maltodextrin as a drying agent ([Young et al., 1993](#); [Gharsallaoui et al., 2010](#); [Nesterenko et al., 2013](#)). [Chang et al. \(2016\)](#) investigated the microencapsulation of canola oil (rich in oleic acid, linoleic acid, and  $\alpha$ -linolenic acid) using different wall materials [lentil protein isolate (LPI), maltodextrin, sodium alginate and lecithin], and found that the combination of LPI,

maltodextrin, and sodium alginate produced microcapsules with better entrapment efficiency (~88%) and protection against oxidation in comparison with the mixture of LPI and maltodextrin.

Among the various encapsulation techniques (spray drying, freeze drying, coacervation, extrusion, and fluidized-bed coating), spray drying is the most commonly applied, due to its low cost and wide availability of equipment (Desai and Park, 2005). For example, Sanguansri et al. (2013) and Can Karaca et al. (2013) encapsulated omega fatty acid-rich oils, within a polysaccharide (glucose and maltodextrin, respectively) and protein (sodium caseinate and chickpea protein isolate, respectively) mixture using spray drying to generate capsules with relatively high entrapment efficiencies (>85%). However, using extremely high temperatures (160–180 °C) for spray drying may result in damage of the microcapsule wall structure (pores, shrinkages, and invaginations), adversely affecting the oxidative stability of the omega fatty acids (Bakry et al., 2016). Limited oil payload (5%–50%) is also a drawback for spray drying encapsulation applications in the food industry (Desai and Park, 2005). Therefore, research activities surrounding encapsulation by complex coacervation have increased as coacervation provides a number of potential advantages over spray drying, such as higher oil payload (up to 99%), lower surface oil, better storage stability and controlled release properties (Xiao et al., 2011). Olive oil was encapsulated within a gelatin and sodium alginate matrix using complex coacervation at pH 3.5–3.8, in which the entrapment efficiency and release characteristics of oil from the microcapsules were greatly dependent on the polymer concentration and oil concentration (Devi et al., 2012). Yan and Jin (2011) invented single- and multi-core (fish oil) capsules with multiple layers using a mixture of polysaccharides (pectin, gum Aracia, and carboxymethyl guar gum) and proteins (gelatin and whey protein) by complex coacervation, which delivered 140–180 mg of omega-3 fatty acids (per g powder) and greatly increased the shelf-life of the oil to 18 months when stored at 4 °C.

## Microencapsulation of Enzymes

Enzymes (e.g., amylase, cellulase, isomerase, lactase, lipase, and pectinase) extracted from plants, animals, or microbial fermentations have been widely used in food applications (e.g., baking, brewing, sweeteners, dairy, oil, and juice productions) (Schwimmer, 1981; Dale, 2014) as they can catalyze specific biochemical reactions to alter or strengthen the texture, flavor, and storage stability of food products (Schwimmer, 1981). However, these enzymes are susceptible to degradation in response to changes in temperature, moisture, pH, shear stress, and air, through oxidation, deamidation, and glycation, which adversely affects their performance in various applications (Dale, 2014). Encapsulation is an effective way to mitigate enzyme exposure to detrimental environmental conditions, but is dependent on the specific application and associated challenges (Table 1).

As mentioned previously, spray drying has been universally used to produce microcapsules, and this includes entrapping enzymes for use in food applications. Horn (2003) described how  $\alpha$ -amylase was substantially coated with an oil using spray drying and then incorporated into dough before baking, in which the enzyme was released at an acceptable rate to retard staling of the baked product. Many other approaches (e.g., wet granulation, fluidized-bed coating, and liposome) have been studied to efficiently encapsulate enzymes. Plijter and Meesters (2000) utilized fluidized-bed coating to entrap lipase onto sodium chloride particles, which was then further covered by a hydroxypropylmethyl cellulose layer and utilized in baking applications to decrease oil rancidity during storage. Moreover, using liposomes to entrap enzymes is a technique used for accelerating ripening in cheese production. Jahadi et al. (2016) entrapped proteinase within liposomes using a heating method, which does not need organic solvents and allows for scale-up of the liposome production, for use in Iranian white brined cheese.

Enzyme encapsulation has provided unique benefits in food applications, such as improving production processes, optimizing product quality, and delivering the enzyme at the correct time and place (Panesar et al., 2010; Dale, 2014). It can be homogeneously distributed in the baking products to booster the dough characteristics by amylases, improve dough quality by proteases, and extend the shelf-life by lipases. It can also be used in dairy products to modify the texture, promote flavor formation, and reduce the aging process (Dale, 2014). The production of syrups and sweeteners is another area that uses enzyme encapsulation, in which glucose isomerase is immobilized to have better stability under high temperature for promoting the conversion of glucose to fructose, so, the reaction is continuously run and enzymes can be reused to decrease production cost (Dale, 2014).

## Conclusion

Microencapsulation of flavors, probiotics, omega fatty acids, and enzymes, in addition to other core materials, has been extensively investigated. Selection of the appropriate wall materials and encapsulation techniques are crucial as they primarily determine the success of the encapsulation through measures of entrapment efficiency, stability of capsules and protection of the core material. Effective targeted release properties and efficient delivery of the health benefits of the core materials are becoming drivers of the utilization of microencapsulation and as such new innovations in encapsulation as well as the use of microencapsulated ingredients in the food industry continues to grow.

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# Multifunctional Foods

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## Overview

Foods contain biologically active substances that exert various activities, including plural functions (Yamada, 2017). Thus, foods are basically multifunctional in nature. However, contents and composition of these functional factors are fairly different with foods. Therefore, we have to take various foods to maintain our health. On the other hand, unbalanced diet may lead to the occurrence of various food style dependent diseases. To avoid such diseases, it is important to produce and afford nutritionally balanced foods.

In addition, excess intake of energy and some food components induce such diseases. Among food components, excess intake of lipids, especially unsaturated fatty acids, induces various diseases. To avoid induction of such diseases, it is necessary to produce and afford multi-functional foods which prevent various diseases simultaneously. Fortunately, we can utilize multifunctional factors to produce multifunctional foods. Dietary fiber (DF) is a typical multifunctional factor present in various plant foods and used for production of healthy foods in various countries. Though oxidized products of unsaturated fatty acids induce various diseases, they can also inhibit the occurrence of other diseases. Thus, the addition of antioxidants is important to utilize the abilities of unsaturated fatty acids.

Since antioxidants suppress the oxidation of unsaturated fatty acids, they can suppress the expression of their many inconvenient effects. In addition, antioxidants exert multifunctional effects independent with their anti-oxidative activity. Effective use of these multifunctional factors may greatly enhance the ability of multifunctional foods.

In designing multifunctional foods, maintenance of safety is very important. Highly effective functional factors often induce side effects when they are taken excessively. To enhance the safety of multifunctional foods, simultaneous uses of biologically active factors may be effective. Some multifunctional factors are reported to have synergic effects. For example, simultaneous use of  $\alpha$ -tocopherol (Toc) and sesamin exert their biological effects at lower doses. Such decreases in dosage are attributes that contribute to the maintenance of safety of multifunctional foods, as well as reduction of production cost.

## Biologically Active Food Components

As summarized in Table 1, foods contain various biologically active substances. Digestive polysaccharides and sugars are important energy sources for animals. However, persistent elevation of serum glucose level leads to the occurrence of diabetes. On the other hand, hardly digestible polysaccharides suppress the degradation of digestible polysaccharides and incorporation of glucose. This leads to the prevention of diabetes. In addition, hardly digestible polysaccharides can bind lipid-soluble components such as carcinogenic components and lipids. This leads to the expression of anti-cancer and lipid-metabolism improving activities. Thus, hardly digestible polysaccharides are called DF and are widely used for the production of health-oriented foods. Some oligosaccharides are also hardly digestible. In addition, such oligosaccharides enhance the growth of *Lactobacillus* and *Bifidus* bacteria which improve the circumstance of gut. Thus, these saccharides are widely used as low-calorie biologically active components.

Proteins are polymers composed of about 20 amino acids. Since humans cannot synthesis some amino acids in sufficient quantities, they are called essential amino acids (EAA), which should be taken from various foods. Balanced intake of EAA is important to keep health, because they are necessary for *in vivo* synthesis of various regulatory proteins. Food proteins are digested to peptides or amino acids in the digestive track and incorporated into our body. Some peptides have been reported to exert biological effects such as enhancement of Ca absorption and inhibition of angiotensin-inducing enzyme activity. The former is useful for the prevention of osteoporosis and the latter for the inhibition of blood pressure elevation.

Lipids are hydrophobic, water-insoluble components present in foods. Major component is neutral lipid (triacylglyceride) in which glycerol is bound to three fatty acids. The function of neutral lipid is highly dependent on fatty acid composition. Animal fats are rich in saturated fatty acids and oleic acid (OA, C18:1n-9) while most plant oils are rich in polyunsaturated fatty acids (PUFA) that belong to the n-6 series. On the other hand, fish oils are rich in PUFAs that belong to the n-3 series. PUFAs with 20 carbons are oxidized by lipoxygenase or cyclooxygenase to give various eicosanoids, which have diverse biological effects. Leucotrienes (LT) produced from arachidonic acid (AA, C20:4n-6) are one of food allergy inducers, but the activity of LT produced from eicosapentaenoic acid (EPA, C20:5n-3) is very low. In addition, EPA-derived LT suppresses the expression of allergic effect of AA-derived LT, through a competition with AA-derived LT. Thus, the composition of PUFAs in diets is highly important in the prevention of food allergy.

PUFAs are easily oxidized *in vivo* and the oxidized products induce various diseases. To prevent the *in vivo* oxidation of PUFAs, intake of antioxidants is important. Vitamins are low molecular regulatory factors present in various foods, and balanced intake of vitamins is important to maintain healthy condition. Among them, vitamin E is a lipid-soluble antioxidant which efficiently suppresses the oxidation of unsaturated fatty acids in lipophilic environments such as in cell membranes. On the other hand, vitamin C is a water-soluble antioxidant, which is effective in the prevention of oxidation in foods or in body fluids. In addition, various types of antioxidants are present in foods. These antioxidants prevent the occurrence of food style-dependent diseases via



**Table 1** Biologically active food components

<i>Food components</i>		<i>Functions</i>
Saccharides	Monosaccharides	Glucose is a major energy source of body, especially of brain. Fructose and galactose are also available for energy source. Sugar alcohols are hardly absorbable, and used as low-calorie sweetener.
	Oligosaccharides	Digestible ones such as sucrose are utilized as an energy source. Hardly digestible ones are used as low-calorie sweetener. Some of them enhance the growth of lactic acid bacteria.
	Polysaccharides	Digestible ones such as starch are utilized for energy source. Hardly digestible ones called dietary fibers exert various biological effects.
Proteins	Amino acids	Essential amino acids should be taken from foods. Nonessential amino acids can be synthesized via TCA cycle. These amino acids can be converted to various biologically substances such as nucleic acid bases via TCA cycle
	Peptides	Degraded products of proteins. Some of them exert biological activities such as enhancement of mineral absorption, inhibition of blood pressure elevation, anti-oxidative activity etc.
	Proteins	Digestible ones afford essential and nonessential amino acids. Some that are hardly digestive can act as dietary fibers. Most functional proteins lose their activities through digestion. Those that exert their activities in the digestive track such as digestive enzymes are useful for our health.
Lipids	Fatty acids	They are composed of saturated and unsaturated fatty acids. The former is divided into 3 groups, such as short-chain, medium-chain, and long-chain fatty acids. Short-chain fatty acids enhance excretion of feces and middle-chain fatty acids are effective energy source of digestive tract disease patients. Long chain fatty acids are classified into n-3, n-6, and n-9 groups depending on the position of the first double bond counted from methyl end. They exert various biological effects, varying with groups.
	Neutral lipids	The glycerol molecule is bound to 3 fatty acids. Their functions differ based on fatty acid composition.
	Phospholipids	The glycerol molecule is bound to 2 fatty acids and one basic component. Main component of cell membrane. Their functions are different with fatty acid composition.
	Lipid soluble components	Cholesterol is important as a component of cell membrane and a starting material of bile acid synthesis. Increase of serum cholesterol level leads to the occurrence of circulatory disease.
Vitamins	Water-soluble	Vitamin Bs and C. The former is important for the regulation of metabolism. The latter is essential for collagen synthesis and exerts anti-oxidative activity.
	Lipid-soluble	Vitamins A, E, D and K. Vitamin E effectively suppress oxidation of unsaturated fatty acids especially in lipophilic circumstances such as within the cell membrane
Others	Minerals	Ca is important for maintenance of bones and teeth. Fe is essential for the transport of oxygen. K is useful for the decrease of blood pressure. Some of minerals regulate the metabolism as co-factors of various enzymes.
	Antioxidants	The components with anti-oxidative activity.

suppression of unsaturated fatty acids oxidation. In addition, some antioxidants induce various biological effects independent of their anti-oxidative activity. This means that antioxidants are important multi-functional factors just like DF and PUFAs.

### Multifunctional Activity of Dietary Fats

**Table 2** shows typical multifunctional factors in foodstuffs. Among them, most popular multifunctional factor is dietary fibre (DF). DF is a group of polysaccharides which are not digestible or hardly digestible by human digestive enzymes. Some of them are water-insoluble and the others are water-soluble. Typical water-insoluble DF is cellulose. Because of its hydrophobic surface, it adsorbs lipophilic food components, such as dietary fats or carcinogenic compounds. Since DF cannot be absorbed in our body, it inhibits the absorption of these lipophilic components. This leads to the expression of its lipid metabolism regulating and anti-cancer activities.

On the other hand, water-soluble DF (WSDF) are partially digested by intestinal bacteria to give short-chain fatty acids (SCFA), such as acetic, propionic and butyric acids. Since acetic acid is absorbed at intestinal wall and used as an energy source, WSDF afford some amounts of energy. On the contrary other SCFA exert some physiological activities. For example, butyric acid arrests the cell cycle of diploid mammalian cells at both G1 and G2 phases, and the cells arrested at the G2 phase are rapidly converted to tetraploid G1 cells (Yamada and Kimura, 1985). This leads to the production of polyploid cells with a high efficiency (Yamada et al., 1985). On the other hand, the cells transformed with oncogenes are hardly arrested to the G2 phase by butyric acid and the cells that passed the G2 phase lose viability quickly. This leads to the expression of its anti-cancer effects (Yamada et al., 1992).

In addition, WSDF can express lipid metabolism regulating and anti-cancer activities simultaneously, since they have lipophilic part on their surface as well as water-insoluble DF. A previous work tried to clarify the difference in physiological activities between two types of DF and found that WSDF exert much stronger immuno-regulatory activity than cellulose (Lim et al., 1997). Among WSDF, the rats fed glucomannan (GM) or pectin (P) gave higher serum IgA and IgG levels and a lower serum IgE level than those fed cellulose.

In the feeding experiments, lipid metabolism regulating activity of food components can be assayed simultaneously with immuno-regulatory activity. The rats fed WSDF such as guar gum (GG), GM or P gave lower serum cholesterol and triglyceride levels than those fed cellulose, when aged eight-month-old Sprague-Dawley (SD) rats were fed the diets containing the above DFs for



**Table 2** Typical multifunctional factors in foodstuffs

Food components	Characteristics
Dietary fibers	Water-insoluble ones are non-digestible. Cellulose, hemicellulose and lignin are plant cell wall components. Chitin is a polymer of N-acetyl glucosamine isolated from shells of crustacean. Deacetylated chitin (chitosan) is acid-soluble. Intestinal regulation, anti-cancer, anti-lipidemia, and anti-diabetes. Water-soluble ones are partially digestible and give smaller calories than digestible polysaccharides. Glucomannan and fructomannan are present in vegetables, and pectin in fruits. Gums isolated from various plants (Arabia gum, karaya gum, tragacanth gum, locust bean gum, guar gum), seaweed polysaccharides (agarose, arginic acid, carageenan) and bacterial products (xanthan gum and pullulan) are also used in food industries. In addition to the effects of water-insoluble DF, they exert strong immune-regulatory effects.
Fatty acids	Oleic acid (OA, C18:1 <i>n</i> -9) is a representative monoenoic fatty acid, which enhances growth of mammalian cells. Since it can be produced from a saturated fatty acid rich in animal tissue, it is not essential. Animal cells cannot introduce second double bond in monoenoic fatty acids. Thus, polyunsaturated fatty acids (PUFAs) should be taken from plant foods. Linoleic acid (LA, C18:2 <i>n</i> -6) is a starting material of n-6 series PUFAs synthesis and metabolized to $\gamma$ -linolenic (GLA, C18:3 <i>n</i> -6), dihomo- $\gamma$ -linolenic (DGLA, C20:3 <i>n</i> -6), arachidonic (AA, C20:4 <i>n</i> -6) acids. DGLA and AA are further metabolized to eicosanoids which exert various biological effects. PUFAs that belong to n-3 series such as eicosapentaenoic (EPA, C20:5 <i>n</i> -3) and docosahexaenoic (DHA, C22:6 <i>n</i> -3) are metabolized from $\alpha$ -linolenic acid (ALA, C18:3 <i>n</i> -3). EPA is further metabolized to eicosanoids which exert various biological effects.
Antioxidants	It is composed of various compounds with anti-oxidative activity. Some polyphenols with more than two OH groups in benzene ring exert strong anti-oxidative activities. Catechins are antioxidants rich in green tea and exert diverse biological effects such as anti-cancer, anti-allergic and anti-obesity effects. Flavonoids (flavone, flavonol, anthocyanin) are antioxidants present in vegetables and fruits. In addition, various foods contain various non-polyphenolic antioxidants.

3 weeks (Yamada et al., 1999a). On the other hand, these results were not obtained in 8-month old aged SD rats (Yamada et al., 2003). The results suggest that the dietary effects of WSDF are age-dependent. Similarly, the young SD rats fed GG, GM or P gave significantly higher serum IgA levels than those fed cellulose, but the effect was not observed in aged rats.

In the feeding experiments, lymphocytes were isolated from spleen and mesenteric lymph node (MLN), and cultured for 24 hr in the absence of DF to determine the effect on Ig productivity of these lymphocytes. In this experiment, lymphocytes isolated from MLN of young SD rats fed WSDF gave higher IgA, IgG, and IgM productivities than those isolated from the rats fed cellulose.

In the case of splenocytes, the stimulating effects were weaker than those observed in MLN. In aged rats, the stimulating effect was observed only in MLN lymphocytes, but not in splenocytes. These results suggest that the feeding effect of WSDF is dependent on immune tissue, as well as age of rats.

As shown above, feeding experiments using experimental animals afford an excellent system to study multifunctional activity of food components. When some biological effects were observed in animal experiments, cultured cells are often used to clarify the regulatory mechanism of food components. However, the *in vitro* system was not effective in WSDF. When established cells or lymphocytes with Ig-producing activity were cultured in the presence of WSDF or SCFA, such changes in Ig productivity were not induced. This suggests that the effect of WSDF was not induced through the direct interaction of WSDF with immune cells.

### Multi-functional Activity of Polyunsaturated Fatty Acids

Biological activities of unsaturated fatty acids are dependent on their structures. Linoleic acid (LA, C18:2*n*-6) has a double bond at the sixth position from its methyl terminal and metabolized to dihomo- $\gamma$ -linolenic acid (C20:3*n*-6) and finally to arachidonic acid (AA, C20:4*n*-6) via  $\gamma$ -linolenic acid (GLA, C18:3*n*-6) and dihomo- $\gamma$ -linoleic acid (DGLA, C20:3*n*-6). The AA in membrane phospholipids (PL) is cut out by phospholipase and then oxidized with lipoxygenase to 4-series LT, which induce type I allergy. On the other hand,  $\alpha$ -linolenic acid (ALA, C18:3*n*-3) has a double bond at the third position from its methyl terminal and metabolized to EPA (C20:5*n*-3) and docosahexaenoic acid (DHA, C22:6*n*-3). EPA is also oxidized with lipoxygenase to 5-series LT, which suppress type I allergy. Since n-3 PUFAs can be substituted with AA in membrane PL, they can suppress allergic reaction through the reduction of membrane AA level.

In addition, dietary n-3 PUFAs decreased serum triglyceride and cholesterol levels (Huglund et al., 1991). The lipid metabolism-regulating activity can be observed in the rats fed fish oils rich in EPA or DHA, as well as the above immuno-regulatory effects (Hung et al., 1999). The fish oils with different EPA and DHA contents can be prepared by mixing some fish oils, but it is impossible to prepare the fish oil containing solely EPA or DHA, because all fish oils contain both components. To clarify the difference of biological activity between EPA and DHA, purified EPA and DHA esters were used in the feeding experiment and it was found that EPA ester exerted stronger anti-allergic effect than DHA esters, as expected (Hung et al., 2000).

In addition, conjugated linoleic acid (CLA) exerts multifunctional effects, such as anti-cancer (Yamasaki et al., 2002), lipid metabolism-regulating (Yamasaki et al., 2003), and immune-regulatory activities (Yamasaki et al., 2004). CLA is a generic term for the positional and geometric isomer of LA. LA is converted to CLA by intestinal bacteria and small amount of CLA is detected in dairy products. The naturally produced CLA has 9-cis, 11-trans (9c, 11t) conformation. On the other hand, 10-trans, 12-cis (10t, 12c) CLA is produced at the similar level with 9c, 11t CLA, when LA is heated in alkaline solution. Among them, 10t, 12c CLA exerts much stronger biological effects than 9c, 11t CLA. This means that the artificially produced CLA is more excellent effector than naturally produced CLA. To use the artificial product for human health improvement, confirmation of safety is essential.

## Multi-functional Activity of Antioxidants

There are various types of natural and synthesized antioxidants which are used for the inhibition of oxidation of food component *in vitro* or *in vivo*. They exert various physiological activities in addition to the anti-oxidative activity. Among them, biological effects of polyphenolic compounds were widely studied. Diphenol compounds with two OH groups on a benzene ring at ortho or para position exert strong anti-oxidative activity, as well as anti-allergic effects (Yamada et al., 1999b, 1999c). These polyphenols may exert the activities through the binding to cell surface or through the interaction with intracellular components after their incorporation into cells. In general, triphenol compounds exert stronger activity than diphenol compounds on cell surface (Yamada et al., 1999b, 1999c), probably due to stronger interaction with cell surface (Yano et al., 2007).

Among tea polyphenols, epigallocatechin gallate (EGCG) with 2 triphenol groups exerts strong biological activities. For example, it exerts much stronger toxicity against rat 3Y1 diploid fibroblasts transformed by E1A than other tea polyphenols, but their toxicities against normal 3Y1 cells are unchanged (Yamada et al., 1993). This means that EGCG is more effective anti-cancer compound with low toxicity against normal cells than other catechins. Since it strongly suppresses both histamine and LTB<sub>4</sub> release from rat peritoneal exudates cells stimulated with a calcium ionophore A23187 (Yamada et al., 1999c), EGCG may also be useful for prevention of type I allergy.

In the case of vitamin E derivatives, clarification of their tissue distribution is important (Okabe et al., 2002). Tocopherol (Toc) derivatives are distributed to various tissues, but the presence of tocotrienol (T3) derivatives is limited in several tissues. When a mixture of Toc and T3 is administered into the stomach of SD rats and their tissue contents are determined after around 16-hr fasting, T3 can be detected only in small number of tissues. On the other hand, T3 derivatives are detected in various tissues, when their tissue levels are determined without fasting. Though  $\alpha$ -Toc is detected in various tissues and the levels are fairly stable, the levels of  $\alpha$ -T3 and  $\gamma$ -T3 are usually much lower than that of  $\alpha$ -Toc. In the adipose tissues, small amounts of  $\alpha$ - and  $\gamma$ -T3 are detected at 0 hr and the levels are increased with the elongation of ingestion time.

Detection of T3 derivatives without T3 administration and the increase of T3 levels after T3 administration suggest that T3 derivatives are accumulative in the adipose tissues. In other tissues, T3 derivatives are not detectable at 0 hr and accumulated with the elongation of ingestion time in some tissues. In the liver and MLN, the levels of T3 derivatives were highest at 8 hr after ingestion and decreased at 24 hr. In these tissues, T3 may be incorporated quickly and then released or metabolized thereafter. The quickness of disappearance in some tissues may be the reason why T3s are detectable only in limited tissues. T3 derivatives often exert stronger biological effects than Toc derivative in cell culture assays (Sakai et al., 2006). However, such biological effects cannot be expressed in the tissues where T3 are not present. Thus, clarification of tissue distribution is essential for the determination of target tissue of biologically active substances.

## Design of Multi-Functional Foods

Some biologically active substances interact with other substances. For example, two anti-oxidative components, such as  $\alpha$ -Toc and sesamin, exert a synergic effect (Gu et al., 1994). When rats were fed these compounds simultaneously, these components exert anti-allergic effects at the doses where each component exerts no activity. In addition, tea polyphenol administration enhances the anti-allergic effect of n-3 PUFA. Though LTB<sub>4</sub> productivity of peritoneal exudates cells isolated from the rats fed perilla oil rich in n-3 PUFA was significantly lower than that from the rats fed safflower oil rich in n-6 PUFA, administration of tea polyphenols further decreased LTB<sub>4</sub> of the cells (Matsuo et al., 2000). In the case of EGCG, coexistence of phosphodiesterase inhibitor strongly enhanced the expression of its biological activity (Kumazoe et al., 2013). Such combinational use of biologically active components allows us to decrease the dose of these components. This leads to the improvement of safety and reductions in production costs. Thus, the studies on the interaction of biologically active substances are also important to produce multifunctional foods with a high safety.

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# Nutritional, Functional and Bioactive Protein Hydrolysates

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## Glossary

**Amphiphilic** A compound possessing both hydrophilic (water soluble) and lipophilic (fat soluble) properties

**Anticoagulant** The effect of hindering or preventing the process of blood forming a clot (a solid mass)

**Antidiabetic** Tending to alleviate or prevent diabetes by control of glucose (sugar) in the blood

**Antigenic** Substance that stimulates antibody production when introduced to the immune system. Future interactions with the antibody and similar substances can induce allergy symptoms

**Antihypertensive** The ability to lower high blood pressure

**Antimicrobial** Killing or preventing the growth of microorganisms, particularly pathogenic microorganisms

**Endoprotease** Proteolytic enzymes that catalyzes the cleavage peptide bonds within the molecule (nonterminal amino acids)

**Exoprotease** Proteolytic enzymes that catalyzes the cleavage of peptides bonds from the end of a polypeptide chain (terminal amino acids)

**Hydrophobic interaction chromatography** A technique to separate molecules based on hydrophobic properties

**Ionizable** Molecules capable of dissociating into electrically charged atoms or radicals

**Isoelectric precipitation** Precipitation of materials at its isoelectric point (pI), at which the net charge is zero and is least soluble

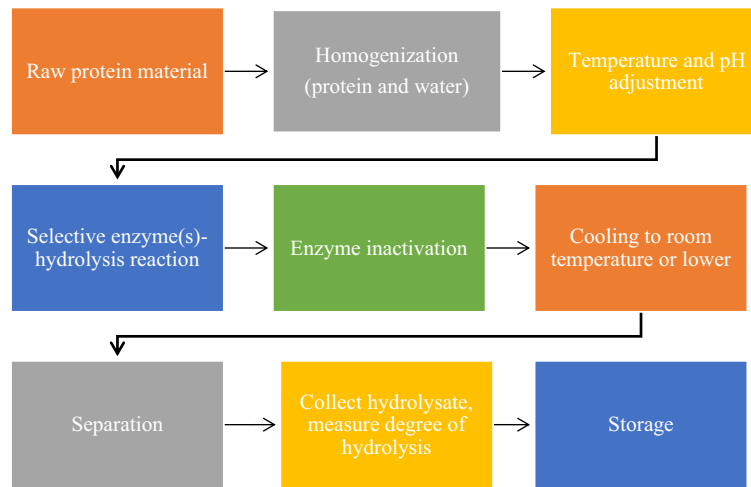
**Physicochemical** Relating to both physical and chemical properties.

## Protein Hydrolysates Definition

Protein hydrolysates are defined as the products derived from hydrolysis of the protein's peptide bonds, producing peptides with different sizes and free amino acids. This type of protein structure modification has an effect on its physicochemical and functional properties (Severin and Xia, 2006). The most notable modifications that affect functionality include a decrease in molecular weight of the peptide chain, an increase in polar groups ( $-\text{NH}_4^+$ ,  $-\text{CO}_2^-$ ) which increase hydrophilicity, and a change in molecular configuration. Acids, alkalis or enzymes can be used to cleave the peptide bonds. While acid/alkali hydrolysis are less expensive procedures, they are difficult to control and yield products with lower nutritional quality and functionality (Lahl and Windstaff, 1989; Sinha et al., 2007). Nevertheless, acid hydrolysis is used in the production of flavor enhancers (Pasupuleti and Braun, 2010). Although more expensive than chemical hydrolysis, enzymatic hydrolysis using proteases derived from plants and microorganisms that cleave peptide bonds at specific sites is the preferred method (Haard, 2001). Research on the degree of hydrolysis and enzyme specificity has allowed for decrease in cost associated with enzymatic hydrolysis (Pacheco-Aguilar et al., 2008).

## Procedure for Enzymatic Hydrolysis

The process for enzymatic hydrolysis varies in literature. A general overview of the process is given in Fig. 1. The process begins with homogenization of the starting material as either the whole food (i.e. whole milk or meat slurry) or protein that has been extracted, isolated, or concentrated prior to hydrolysis. An appropriate protein to water ratio (e.g. 1:2, w/v) is required to ensure sufficient water for enzyme mobility. Pasteurization of the solution may follow depending on the protein's autolytic activity. Temperature (between 40–60 °C) and pH (6–8) are adjusted to create optimal conditions for enzyme activity. Commercial protease(s) is added to the slurry in various concentrations and the enzymatic reaction varies from minutes to hours depending on the extent of hydrolysis required (Kristinsson and Rasco, 2000). During the enzymatic reaction an initial rapid phase occurs where large peptide bonds are hydrolyzed at specific sites; later the rate of enzymatic hydrolysis decreases as it reaches a stationary phase (Shahidi et al., 1995; Haard, 2001). The choice of enzyme specificity is an important factor influencing amino acid sequence of peptides or amino acid residues that lead to different nutritional, functional and bioactive properties in the hydrolysates. Each class of protease contains specialized catalytic sites that provide substrate specificity and guide their cellular localization to cleave distinct peptide bonds (Lopez and Bond, 2008). Enzymes can be endoproteases, exoproteases or a combination of both. Commercial enzymes such as alcalase, flavourzyme, neutrase, pepsin, and papain are some examples of proteases typically used. These proteases contain catalytic sites that provide substrate specificity to cleave distinct peptide bonds. Exo-proteases (e.g. flavourzyme) selectively cleave peptides at either the N- or C- terminus of the protein, whereas, endo-proteases (e.g. alcalase, neutrase, and pepsin) catalyze hydrolysis of non-terminal amino acids (Kristinsson and Rasco, 2000). Some proteases such as papain, display a broad range of



**Figure 1** General procedure for enzymatic protein hydrolysis.

functions with both exo- and endo-peptidase activity. **Table 1** gives a summary of the most commonly used commercial proteases reported in literature for preparation of hydrolysates from different protein sources.

The extent of hydrolysis also depends on various factors applied during the enzymatic reaction such as temperature, pH, enzyme to substrate ratio and substrate concentration (Alder-Nissen, 1986). After hydrolysis, the enzyme is inactivated by pasteurization and/or pH adjustment. The hydrolysate slurry is cooled down, separated (i.e. centrifugation, ultrafiltration) and stored (i.e. freezing, spray drying). Aliquots of the hydrolysate are used to determine the extent of hydrolysis or degree of hydrolysis, measuring the amount of peptide bonds broken compared to the total amount of peptides in the un-hydrolyzed proteins (Alder-Nissen, 1986). Therefore, a higher degree of hydrolysis is indicative of more peptide bonds cleaved resulting in lower molecular weight peptides.

### Nutritional Quality of Protein Hydrolysates

It is established knowledge that the human body requires a regular supply of protein and amino acids to maintain a normal adult nutritive state. Inadequate amounts of dietary protein causes catabolism in which the body uses its own tissue protein as a nitrogen source (Wu, 2016). In addition to quantity, protein quality is a determinant factor. Protein quality is determined by its digestibility, release of amino acids, di- and tri-peptides that can be absorbed in the small intestine, and its amino acid pattern. Hence, the presumption that amino acids from hydrolysates are more easily and quickly absorbed compared with intact protein, which has proven to give hydrolysates a nutritional advantage over intact dietary proteins (Bilsborough and Mann, 2006; Koopman et al., 2009). For example, Koopman et al. (2009) have illustrated this phenomena; they observed that enzymatically hydrolyzed casein increased protein digestion and absorption rate from the gut and postprandial amino acid availability which results in an increased rate of amino acids into skeletal muscle protein.

The protein digestibility-corrected amino acid score (PDCAAS) is considered the best method to evaluate dietary protein quality and is used as the standard by the Food and Drug Administration (FDA) to determine the percentage daily value for protein. It is estimated based on the mg of the limiting amino acid in 1 g of test protein divided by the mg of the same amino acid in 1 g of reference protein, and multiplied by the fecal true digestibility percentage (Schaafsma, 2000).

For decades, protein hydrolysates have been used in medical and clinical diets for people with food allergies or patients with digestive ailments that affect nutrient absorption. Clemente (2000) provides a detailed overview of some of the most common clinical applications of protein hydrolysates. Other common applications include protein supplementation in sports nutrition products. Athletes frequently consume protein hydrolysate products to increase plasma amino acid levels with the hopes of maximizing muscle protein anabolism and post-exercise recovery (Manninen, 2009).

Animal feeding studies have corroborated the nutritional advantages of protein hydrolysates. For example, rice bran protein hydrolysates improved insulin resistance in rats, compared with rats fed a carbohydrate-fat diet for twelve weeks (Boonloht et al., 2015). In another study, rats were fed a protein-free diet to induce muscle loss and then administered daily a whey protein diet (intact protein, amino acids, or whey protein hydrolysates). It was observed that feeding whey protein hydrolysates protected muscle mass loss better than the free amino acids and intact whey protein diets (Kobayashi et al., 2016). Martínez-Alvarez et al. (2015) provided a compilation of works that illustrate the benefit of protein hydrolysates in animal feeding. Based on the positive effects protein hydrolysates have on animals, it is expected that they will have a positive effect on humans as well. Several human clinical trials have shown that ingesting protein hydrolysates versus intact protein accelerates protein digestion and absorption which can augment amino acid availability and thus increase the rate of amino acids to skeletal muscle proteins (Morifuji et al., 2010; Deglaire et al., 2009).

**Table 1** Summary of Functional and Bioactive properties of protein hydrolysates derived from different protein sources and commercial enzymes reported in literature

<i>Protein source</i>	<i>Enzyme</i>	<i>Functional properties</i>	<i>Bioactive properties</i>	<i>References</i>
<b>Marine protein</b>				
Round scad ( <i>D. maruadsi</i> )	Flavorzyme	Solubility Emulsifying, Foaming	Antioxidant	Thiansilakul et al. (2007)
Smooth hound ( <i>M. mustelus</i> )	Pepsin from <i>M. Mustelus</i> Stomach Alkaline Protease, Trypsin-like protease, Crude enzyme extract from <i>M. Mustelus</i> , Intestine, Bovine Trypsin	–	Antioxidant	Bougatef et al. (2009)
Rainbow trout ( <i>O. Mykiss</i> ) frames	Microwave treatment, Alcalase	Solubility, Emulsifying, Foaming	Antioxidant, Allergenicity	Ketnawa and Liceaga (2017), Nguyen et al. (2017)
Tilapia ( <i>O. niloticus</i> )	Flavourzyme, Cryotin-F, Protease A Amano, Protease N Amano, Neutrase	–	ACE Inhibition, Antioxidant	Raghavan and Kristinsson (2008, 2009)
Atlantic salmon ( <i>S. salar</i> ) skin gelatin	Alcalase, Bromelain, Flavourzyme	–	DPP-IV Inhibition	Li-Chan et al. (2012)
Silver carp ( <i>H. molitrix</i> )	Trypsin, Neutrase, Alcalase, Papain, Pepsin, Flavorzyme	Solubility	Antioxidant, DPP-IV Inhibition	Dong et al. (2008), Zhang et al. (2016)
Tuna ( <i>T. tonggol</i> ) frame	Alcalase, Neutrase, Pepsin, Papain, A-Chymotrypsin, Trypsin	–	Antiproliferate activity, Antihypertensive properties in rats	Lee et al. (2010), Hsu et al. (2011)
Sardine ( <i>S. aurita</i> ) head and visera	Alcalase, Crude enzyme preparation from <i>Aspergillus clavatus</i> ES1, Alkaline proteases from B. Licheniformis NH1, Crude enzyme extract from viscera	Solubility, Emulsifying, Foaming, Fat Absorption	Antioxidant	Souissi et al. (2007), Bougatef et al. (2010)
<b>Plant protein</b>				
Rapeseed Meal	Alcalase, Proteinase K, Thermolysin, Flavourzyme, Pesin + Pancreatin	–	Antioxidant, ACE Inhibition, Rennin Inhibition, Antihypertensive Activity in spontaneous hypertensive rates	He et al. (2013a,b)
Chickpea	Alcalase	Solubility, Oil adsorption, Emulsifying, Foaming	Antioxidant	Li et al. (2008b), del Mar Yust et al. (2010)
Flaxseed	Papain, Trypsin, Pancreatin, Alcalase, Flavorzyme	–	Antioxidant	Karamac et al. (2016)
Hemp Seed	Pepsin, Pancreatin	–	Antioxidant, ACE Inhibition	Girgih et al. (2011a,b)
Peanut	Alcalase	Solubility, Emulsifying, Foaming	Antioxidant, ACE Inhibition	Jamdar et al. (2010)
Pea	Alcalase	–	ACE, Renin, Calmodulin (Cam)-Dependent Cyclic Nucleotide Phosphodiesterase(Campd) Inhibition	Li and Aluko (2010)



Quinoa	Pepsin, Pancreatin with bile extract	–	DPP-IV, A-Amylase, A-Glucosidase Inhibition	Vilcacundo et al. (2017)
Corn	Alcalase, Papain, Microbial protease, Validase® FP concentrate, Alkaline protease, Neutral protease		Antioxidant	Kong and Xiong (2006), Zhou et al. (2012)
Amaranth Seed	Pronase, Papain, Trypsin, Chymotrypsin, Alcalase	–	ACE Inhibition, Antihypertensive Properties in rats	Fritz et al. (2011)
<b>Animal protein</b>				
Bovine Casein	Pepsin	–	ACE Inhibition, Antihypertensive Properties in rats	Sinha et al. (2007), Miguel et al. (2009)
Porcine Plasma	Alcalase	Solubility, Emulsifying, Foaming	Antioxidant	Sinha et al. (2007)
Bovine Whey	Corolase Pp, Papain, Fungal Protease, C. Cardunculus Protease	Water Absorption, Foming, Emulsifying	Dpp-Iv Inhibition, ACE Inhibition	
Egg	Trypsin, Alcalase, Pepsin, Pancreatin, Thermolysin	Solubility, Emulsifying And Foaming	Antioxidant, ACE Inhibition, A-Glucosidase, A-Amylase Inhibition	Park et al. (2001), Yi et al. (2003), Yu et al. (2011), Chen et al. (2012a,b)
Poultry	Alcalase, Flavourzyme, Protamex, Liquipanol, Pepsin, Trypsin, Protease FP, Protease N	–	ACE Inhibition	Cheng et al. (2008), Saiga et al. (2008), Nchienzia et al. (2010)
<b>Other (alternative) protein</b>				
Cricket ( <i>G. sigillatus</i> )	Alcalase	Solubility, Emulsifying and Foaming		Hall et al. (2017)
Cotton Leafworm, ( <i>Spodoptera littoralis</i> )	Alcalase, Thermolysin, Porcine mucosal peptidases, Pepsin +, Trypsin, Alpha-chymotrypsin	–	Antioxidant, ACE Inhibition	Vercruysse et al. (2009)
Mealworm Larvae ( <i>T. molitor</i> ), Locusts ( <i>S.gregaria</i> ) and Crickets ( <i>G. sigillatus</i> )	Gastrointestinal Enzymes (A-Amylase, Pepsin, Pancreatin, And Bile Extract Solution)	–	Antioxidant, Anti-Inflammatory	Zielińska et al. (2017)
Algae	Pepsin, Amyloglucosidase	–	Anticancer, Antioxidant, Antiproliferate	Athukorala et al. (2006b), Sheih et al. (2009a,b)

## Functional Properties of Protein Hydrolysates

Apart from the nutritional quality, the functionality of the protein in a food system during processing, storage and consumption are very important factors. Proteins are highly functional molecules used in the chemical, pharmaceutical and food industries for processes such as emulsification, foaming, viscosity enhancement, encapsulation and gelation. Protein functionality refers to the properties that contribute to the flavor, texture, stability, and appearance of the final food (Kristinsson and Rasco, 2000). In many instances, intact proteins are molecularly heavy and have intramolecular bonds that prevent them from producing stable emulsions, foams and solutions (Foegeding, 2015). Minimally hydrolyzing food proteins has proven beneficial to their food industry applicability. Research shows that protein hydrolysates derived from enzymatic hydrolysis can have improved functionality. Functional properties of proteins hydrolyzed with various types of proteases and under varying hydrolysis conditions have been compared and described in literature (Hoyle and Merritt, 1994; Liceaga-Gesualdo and Li-Chan, 1999; Nilsang et al., 2005; King et al., 2007; Humiski and Aluko, 2008; Pacheco-Aguilar et al., 2008; Cheung et al., 2009; Nchienzia et al., 2010; Ktaria et al., 2012; Zhao et al., 2013; Hall et al., 2017; Nguyen et al., 2017).

Protein solubility is the most the important functional property because it will influence other properties such as emulsification and foaming, making it a good indicator of overall functionality (McCarthy et al., 2013). A protein's solubility is dependent upon its affinity to water or other polar solvents, which is dictated by non-covalent forces such as hydrogen bonding and hydrophobic and ionic interactions (Damodaran et al., 2007). Hydrophobic interaction decreases protein solubility as it promotes protein-protein interactions while ionic forces increase solubility by promoting protein-water interactions and repulsion between protein molecules (Wilding et al., 1984). Enzymatic hydrolysis results in a major structural change where enzyme cleavage of the protein results in smaller peptides and exposed ionizable amino and carboxyl groups of the amino acids, which increases the solubility of the protein (Kristinsson and Rasco, 2000).

In literature, solubility of wheat protein hydrolysate was reported as strongly influenced by pH with the highest level of solubility observed at pH 4.0 and lowest at pH 6.0 (Claver and Zhou, 2005). Soy protein hydrolysates were found to also be pH-dependent and almost completely soluble in the pH 2.0–9.0 range (McCarthy et al., 2013). Solubility of whey protein hydrolysates was influenced by hydrolysis time, with solubility increasing with increasing hydrolysis. This was attributed to the degradation of the protein into smaller peptide units which have more binding sites as hydrolysis continues (Sinha et al., 2007). Pacific hake (*Merluccius productus*) protein hydrolysates also showed improved solubility when compared to un-hydrolyzed fish protein (Pacheco-Aguilar et al., 2008). Generally, most native food proteins have hydrophobic amino acids in the interior of their tertiary structure. Hydrophobic interactions decrease protein solubility by promoting protein-protein interactions (Wilding et al., 1984). Enzymatic hydrolysis decreases protein molecular size which affects hydrophobicity, as well as polar and ionizable groups. The resulting hydrophilic interactions increase solubility by promoting protein-water interactions and repulsion between protein molecules (Wilding et al., 1984). Similarly, protein hydrolysates may also have increased water-holding capacity, which influences texture and integrity of food products like frozen fish fillets or meat (Cheung et al., 2009).

The amphiphilic nature of proteins allows them to act as emulsifying and foaming agents by lowering the surface tension between the phases. This requires favorable intermolecular interactions between the protein and the phases in the colloidal system (e.g. lipid, gas, aqueous, etc.). Limited enzymatic hydrolysis of proteins can release peptides containing hydrophobic groups as well as increase hydrophilic carboxylic and amino groups to contribute to its amphiphilic nature. Thus, a balance of hydrophilic and hydrophobic groups, that will support protein suspension in solution, are obligatory for good emulsifying and foaming properties (Hettiarachchy and Ziegler, 1994).

Since protein size influences functionality, hydrolysis time and protease specificity play a key role during preparation of functional protein ingredients. While the protein's size and structure are modified by hydrolysis, other characteristics of the native protein such as amino acid sequence, conformation, and hydrophilic-hydrophobic balance have a strong influence on functionality. For example, soy protein hydrolysates with larger molecular weight peptide fractions showed higher emulsification (Barca et al., 2000). Other hydrolysates from various protein sources, fish (Pacheco-Aguilar et al., 2008; Nguyen et al., 2017), wheat gluten (King et al., 2007), hemp (Yin et al., 2007), egg white (23), and chickpea (del Mar Yust et al., 2010) have shown improved emulsifying and foaming activity compared to unhydrolysed protein. Moreover, limited enzyme hydrolysis can partially denature proteins to aggregate and forms gels (Ni et al., 2015). For instance, partially hydrolyzed oat protein formed stronger gels compared to oat protein isolate under the same conditions (Nieto-Nieto et al., 2014). Similarly, soy protein hydrolysates formed a gel with better network structure than that of the intact soy protein isolate (Tsumura et al., 2005).

Since functionality depends on different protein characteristics, that may not be achievable simultaneously, enzymatic hydrolysis can be controlled in such a way that specific protein modifications may be attainable. It is important to note that depending on the type of protein, hydrolysis exceeding a certain level can result in peptides that are too small (higher degree of hydrolysis) to interact with multiple components in a food matrix. This has been proven by the consistent decline in foaming and emulsion stability of extensively hydrolyzed proteins (van der Ven et al., 2001; Guan et al., 2007). Other authors have also shown the color and aroma of protein hydrolysates to be mostly conserved and somewhat neutralized, respectively, when incorporated into a food matrix, though the flavor may be adversely affected (Mackie, 1982; Hoyle and Merritt, 1994). Hydrolysis reactions, coupled with the conditions to inactivate the enzyme and inactivation of spoilage and pathogenic organisms, can release oligopeptides with high nonpolar amino acid contents (Adler-Nissen, 1984; Gildberg et al., 2002). In an aqueous system, this is perceived as bitter-metallic flavorings, and it is dependent on factors that are intrinsic and extrinsic to the enzyme and source protein which are often collectively difficult to control (Kristinsson and Rasco, 2000). Thus, attaining the desired functionality of the

resulting hydrolysate may generate unpalatable characteristics, which can interfere with successful marketability as a value-added ingredient.

### Bioactive Properties of Protein Hydrolysates

Based on structural properties, amino acid composition and peptide sequence, protein hydrolysates have also been studied for their biological activity in human health (Hartmann and Meisel, 2007; Möller et al., 2008; Chalamaiah et al., 2012; Udenigwe and Aluko, 2012). Peptides from various sources of hydrolysates have also been indicated to have other bioactive potential such as anticancer (Yi et al., 2003; Picot et al., 2006; Kannan et al., 2009; Sheih et al., 2009b; Alemán et al., 2011; Suarez-Jimenez et al., 2012), antimicrobial (Liu et al., 2008; Di Bernardini, Harnedy et al., 2011; Chalamaiah et al., 2012; Najafian and Babji, 2012), and anticoagulant (Koyama et al., 1998; Jung et al., 2002; Rajapakse et al., 2005; Athukorala et al., 2006a; Jung and Kim, 2009) activity. **Table 1** summarizes some of the literature available on biological activities of protein hydrolysates, while a brief overview of some of the most commonly studied biological activities of protein hydrolysates is given below.

Cardiovascular disease (CVD) is the leading problem in developed countries and high blood pressure is one of the risk factors for CVD onset. Angiotensin-I converting enzyme (ACE) plays a key role in regulating blood pressure by converting inactive angiotensin-I into its active form angiotensin-II, a vasoconstrictor, resulting in high blood pressure. Several food proteins and peptides have shown potent ACE inhibitory activity, which is a major target in regulating hypertension. Milk proteins have been the most studied source of antihypertensive peptides (Udenigwe and Aluko, 2012). Casein and whey hydrolysates have been identified to have strong antihypertensive activity, by way of ACE inhibition (Miguel et al., 2009; Tavares et al., 2011; Nongonierma et al., 2016). Other potent antihypertensive peptides have been identified from protein hydrolysates of plants, nuts, seeds (Li et al., 2007; Jamdar et al., 2010; Li and Aluko, 2010; Fritz et al., 2011), egg (Miguel and Aleixandre, 2006; Majumder and Wu, 2010), fish (Lee et al., 2010; Chalamaiah et al., 2012), insects (Vercruysse et al., 2005; Vercruysse et al., 2009), and other meat proteins (Ahhmed and Muguruma, 2010).

Type II diabetes is a long term metabolic disorder which is also recognized as a major risk factor for (CVD) as there is a link between insulin resistance and high blood pressure (Mannino et al., 2008). Similarly, protein hydrolysates provide a natural therapeutic strategy for the management of type-2 diabetes. Dipeptidyl peptidase-IV (DPP-IV) protease activity degrades incretin hormone involved in blood glucose regulation (Barnett, 2006). Other modes of a diabetic regulation may include  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibition. Peptides derived from protein hydrolysates contain structural characteristics that allow them to exert these antidiabetic properties (Yu et al., 2011; Lacroix and Li-Chan, 2012; Li-Chan et al., 2012; Nongonierma and FitzGerald, 2013; Zhang et al., 2016; Vilcacundo et al., 2017).

Antioxidant activity is among the most researched bioactive functions of protein hydrolysates. Numerous reports and publications have confirmed that enzymatically-derived protein hydrolysates, can serve as a natural source of antioxidants (Sarmadi and Ismail, 2010). Antioxidant peptides from protein hydrolysates contain certain amino acid residues that aid in hindering lipid-oxidation, as well as chelating oxidant metal ions. Antioxidant hydrolysates have been studied from a plethora of protein sources including fish (Dong et al., 2008; Chalamaiah et al., 2012; Malaypally et al., 2015), fish by-products (Bougatef et al., 2010; Di Bernardini et al., 2011; Ketnawa and Liceaga, 2017), corn (Kong and Xiong, 2006; Li et al., 2008a; Zhou et al., 2012), nuts/seeds/legumes (Li et al., 2008b; Jamdar et al., 2010; Girgih et al., 2011a,b; He et al., 2013a; Ambigaipalan et al., 2015; Karamać et al., 2016), egg (Park et al., 2001; Davalos et al., 2004; Sakanaka and Tachibana, 2006; You and Wu, 2011; Chen and Chi, 2012; Chen et al., 2012a,b; Shi et al., 2014), milk (Hernández-Ledesma et al., 2005; Pihlanto, 2006; Nongonierma et al., 2016), insects (Nongonierma and FitzGerald, 2017), and algae (Athukorala et al., 2006b; Sheih et al., 2009b; Samarakoon and Jeon, 2012).

### Conclusion

Protein hydrolysates have been widely used for their nutritional, functional and bioactive activities. Although hydrolysates can be obtained from chemical hydrolysis, a controlled enzymatic hydrolysis, targeting specific peptide bonds, is the preferred method. Research over the years has allowed for the study of the impact that different proteases have on the quality, functionality and bioactivity of protein hydrolysates. The superior protein quality of many hydrolysates has allowed for their application in food formulation destined for individuals with diet restrictions or those seeking a higher protein intake (e.g. athletes). The improved functionality in protein hydrolysates is dependent on the hydrolysis conditions used and the extent of hydrolysis. Protein hydrolysates have an important role in the food industry applications as emulsifying, foaming and gelling ingredients. More recently, the interest in protein hydrolysates has shifted towards their biological activities with benefits to human health.

There are, however some challenges that need to be further investigating with regards to the application of protein hydrolysates. The primary drawback for using protein hydrolysates in the food industry is the possibility of producing a bitter taste due to release of bitter hydrophobic peptides. Industry efforts have led to bitter taste masking using other flavors or using a de-bittering process such as treatment with activated carbon, extraction with alcohol isoelectric precipitation, chromatography on silica gel and hydrophobic interaction chromatography (Saha and Hayashi, 2001). In addition, although protein hydrolysates tend to have lower antigenic activity, studies have shown that in some situations, allergenicity can still be present. The use of more extensive hydrolysis or pre-hydrolysis treatment of the protein (e.g. heat, microwave and ultrasonication) as a mean for lowering the antigenicity continues to be explored.

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## Omega-3 Fatty Acids

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### Introduction

The omega-3 fatty acids (n-3) are a group of polyunsaturated fatty acids (PUFAs) with the first double bond at the third carbon from the methyl end group. Human body lacks the ability to produce PUFAs, including n-3 fatty acids, hence it is necessary to obtain adequate n-3 fatty acids from various food sources such as plants, fish and fish oils. Omega-3 fatty acids are derived from alpha-linolenic acid (ALA; 18:3 n-3), an essential fatty acid, and include stearidonic acid (SDA; 18:4 n-3), eicosapentaenoic acid (EPA; 20:5 n-3), docosapentaenoic acid (DPA; 22:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3). Several researchers have shown the beneficial effect of n-3 fatty acids on cardiovascular function, fetal development and age-related diseases (Alzheimer disease and dementia) (Finley and Shahidi, 2001; Lopez et al., 2011; Shahidi and Ambigaipalan, 2018). This contribution provides a cursory account of their chemistry, sources and health effects.

### Structure and Synthesis of Omega-3 Fatty Acids

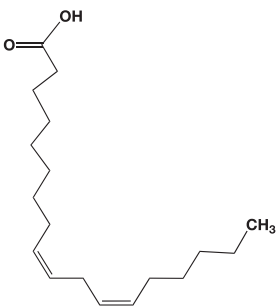
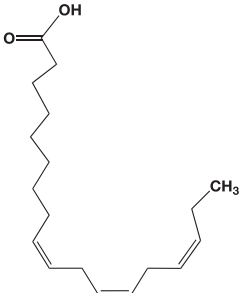
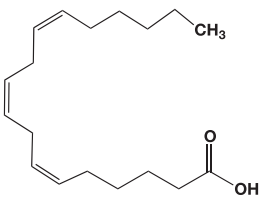
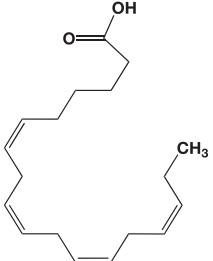
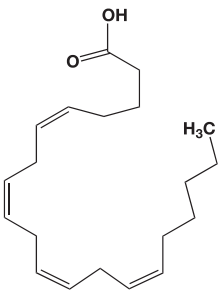
Omega-3 fatty acids are the long chain polyunsaturated fatty acids (PUFAs) with 18 to 22 carbon atoms in their chain. The term omega-3 refers that the first methylene interrupted double bond being located at the third carbon atom from the methyl end of the fatty acid chain, which include alpha-linolenic acid (ALA; *cis*-9, *cis*-12, *cis*-15-octadecatrienoic acid, 18:3<sup>Δ<sup>9,12,15</sup></sup>), stearidonic acid (SDA; *cis*-6, *cis*-9, *cis*-12, *cis*-15-octadecatetraenoic acid, 18:4<sup>Δ<sup>6,9,12,15</sup></sup>), eicosapentaenoic acid (EPA; *cis*-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17-eicosapentaenoic acid, 20:5<sup>Δ<sup>5,8,11,14,17</sup></sup>), docosapentaenoic acid (DPA; *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19-docosapentaenoic acid, 22:5<sup>Δ<sup>7,10,13,16,19</sup></sup>) and docosahexaenoic acid (DHA; *cis*-4, *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19-docosahexaenoic acid, 22:6<sup>Δ<sup>4,7,10,13,16,19</sup></sup>). However, linoleic acid (LA; *cis*-9, *cis*-12-octadecadienoic acid, 18:2<sup>Δ<sup>9,12</sup></sup>), gamma-linolenic acid (GLA; *cis*-6, *cis*-9, *cis*-12-octadecatrienoic acid, 18:3<sup>Δ<sup>6,9,12</sup></sup>), and arachidonic acid (AA; *cis*-5, *cis*-8, *cis*-11, *cis*-14-eicosatetraenoic acid, 20:4<sup>Δ<sup>5,8,11,14</sup></sup>) are omega-6 PUFAs, where the first methylene interrupted double bond starts at the sixth carbon atom from the methyl end of the fatty acid chain. The structures of omega-3 and omega-6 polyunsaturated fatty acids are shown in Table 1. Omega-3 fatty acids generally exist in a curved form (Table 1) due to the presence of *cis* configured double bonds that result in a low melting point hence being liquid at room temperature.

The metabolic pathway of synthesis of omega-3 and omega-6 PUFAs from dietary LA and ALA is shown in Fig. 1. Dietary ALA, which is the precursor of omega 3 series could be converted into SDA, EPA, DPA and DHA using series of microsomal enzymes from endoplasmic reticulum (ER) mainly in the liver of mammals. These enzymes are desaturase and elongase, which introduces new double bonds or lengthen acyl chains, respectively. DHA synthesis involves peroxisomal β-oxidation of tetracosahexaenoic acid (24:6<sup>Δ<sup>6,9,12,15,18,21</sup></sup>) that removes 2-carbonyl acetyl-CoA. Similarly, omega 6 fatty acids, especially arachidonic acid are synthesized from dietary LA (precursor of omega-6 series) in human body. Arachidonic acid and EPA could be either metabolized to a fatty acid via elongase or produce eicosanoids that are key mediators and regulators of inflammatory responses such as prostaglandins, thromboxanes, leukotrienes, resolvins and lipoxines via cyclooxygenase (COX) and lipoxygenase (LOX). There is a competition between the omega-3 and omega-6 fatty acids for the same set of enzymes for their metabolism. Thus, the ratio of omega-6 to omega-3 plays a vital role in the human body. Wall et al. (2010) reported that the optimum ratio of omega-6 to omega-3 as being 4:1. However, the intake of omega-6 is generally 15- to 16-fold higher than that of omega-3 in the Western countries due to the higher intake of vegetable oils, hence omega-3 supplementation is required (Simopoulos, 2003). Arachidonic acid is the major substrate for eicosanoid synthesis due to its higher concentration in inflammatory cells in comparison to EPA (Calder, 2001). Omega-6 fatty acids serve as precursors for the synthesis of series-2 prostaglandins (E<sub>2</sub>) that are potent mediator of inflammation and cell proliferation while omega-3 fatty acids produce series-3 prostaglandins (E<sub>3</sub>), which are generally anti-inflammatory (Bagga et al., 2003). Tilley et al. (2001) reported that prostaglandins-E<sub>2</sub> cause pain and vasodilation via promoting the production of the pro-inflammatory cytokine interleukin-6 in macrophages, while prostaglandin-E<sub>3</sub> is less potent in producing cytokine interleukin-6. Thus, the increased ratio of omega-6 to omega-3 plays a vital role in the development of chronic diseases.

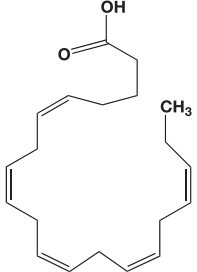
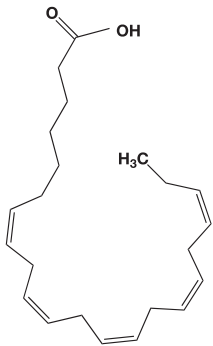
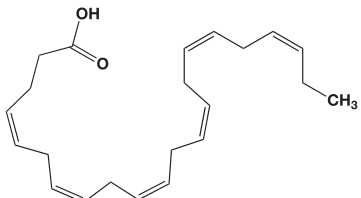
### Sources of Omega-3 Fatty Acids

Aquatic species are the major source of omega-3 fatty acids. Omega-3 fatty acids are primarily synthesized in algae and phytoplankton and transferred to fish and marine mammals via the food web. Flesh of fatty fish (mackerel, tuna, herring, sardines, menhaden and salmon), liver of lean fish (cod and halibut) and blubber of marine mammals (seals and whales) are the main sources of omega-3s, especially EPA and DHA (Shahidi, 1998). Crustaceans, bivalves and cephalopods are also good sources of omega-3s. In addition, plant seeds such as flax, chia, echium and canola as well as walnuts are good sources of ALA, which is the precursor of omega-3s in the human body (Shahidi and Ambigaipalan, 2015). Furthermore, microalgae, namely *Cryptocodinium cohnii* and

**Table 1** Structures of omega-3 and omega-6 polyunsaturated fatty acids

<i>Omega-3 fatty acid</i>	<i>Structure</i>	<i>IUPAC name</i>
Linoleic acid (LA; 18:2 <sup>Δ9,12</sup> )		<i>cis</i> -9, <i>cis</i> -12-Octadecadienoic acid
Alpha-linolenic acid (ALA, 18:3 <sup>Δ9,12,15</sup> )		<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-Octadecatrienoic acid
Gamma-linolenic acid (GLA, 18:3 <sup>Δ6,9,12</sup> )		<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12-Octadecatrienoic acid
Stearidonic acid (SDA; 18:4 <sup>Δ6,9,12,15</sup> )		<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-Octadecatetraenoic acid
Arachidonic acid (AA; 20:4 <sup>Δ5,8,11,14</sup> )		<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14-Eicosatetraenoic acid

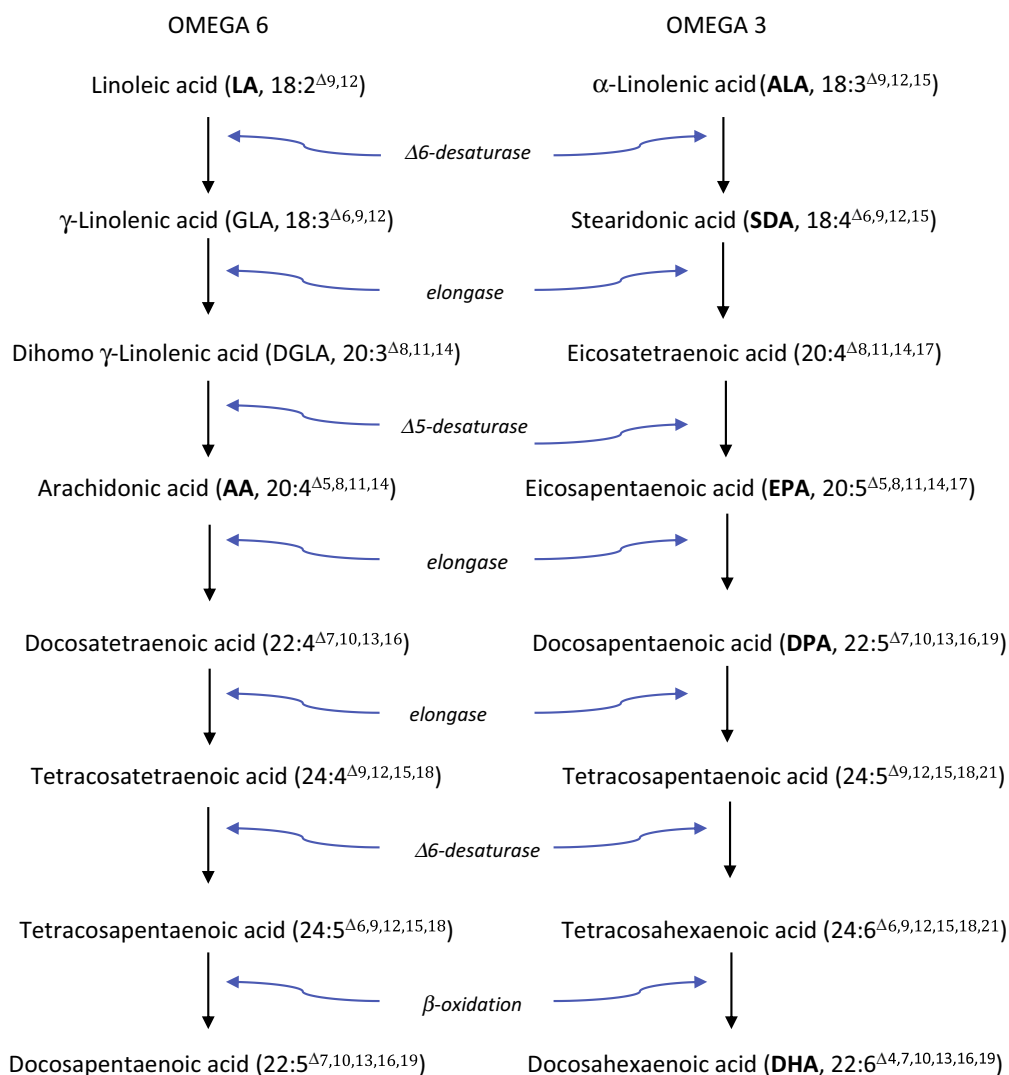
**Table 1** Structures of omega-3 and omega-6 polyunsaturated fatty acids—cont'd

<i>Omega-3 fatty acid</i>	<i>Structure</i>	<i>IUPAC name</i>
Eicosapentaenoic acid (EPA; 20:5 $\Delta^{5,8,11,14,17}$ )		<i>cis-5, cis-8, cis-11, cis-14, cis-17-eicosapentaenoic acid</i>
Docosapentaenoic acid (DPA; 22:5 $\Delta^{7,10,13,16,19}$ )		<i>cis-7, cis-10, cis-13, cis-16, cis-19-Docosapentaenoic acid</i>
Docosahexaenoic acid (DHA; 22:6 $\Delta^{4,7,10,13,16,19}$ )		<i>cis-4, cis-7, cis-10, cis-13, cis-16, cis-19-Docosahexaenoic acid</i>

*Schizochytrium* spp. are the two major algal sources of DHA at levels of 55% and 40%, respectively (Senanayake and Fichtali, 2006). Another omega-3 fatty acid SDA, has been found in seeds from *Boraginaceae* family, such as borage, *Echium* (Viper's bugloss), and *Buglossoides* (Corn gromwell), and hemp oil. Guil-Guerrero (2007) reported that dietary supplementation of SDA could increase the EPA level to a higher degree than ALA supplementation, hence its recent attraction to researchers. Some novel approaches such as genetic modification (soy modified with omega-3) and synthesis of structured lipids (incorporation of borage oil and evening primrose oil) have also been used to enhance the level of omega-3s in various plant sources (Senanayake and Shahidi, 2002; Hamam and Shahidi, 2006; Shahidi and Zhong, 2015). Safflower, sunflower, corn and soybean oil are the primary sources of omega-6 fatty acids, especially linoleic acid (Shahidi and Miraliakbari, 2004; 2005). Evening primrose is a good source of gamma-linolenic acid. Dietary sources of omega-3 and omega-6 PUFAs are shown in Table 2.

## Health Benefits

Bang et al. (1980) reported that traditional Greenlandic diet (rich in marine mammals and fish) significantly retarded the incidence of cardiovascular disease in the Inuit population and Danish settlers based on the earliest reports and epidemiological studies that is known as "Eskimo Paradox". Omega-3 PUFAs, especially EPA and DHA, play a beneficial role against cardiovascular disease and inflammation. A recent review suggests that the death rate was perhaps higher for Eskimos from myocardial infarction than the Danes based on book keeping (Fodor et al., 2014). However, this short-sighted approach and misconception that ignored different genetic background and lifestyle was criticized by Shahidi (2015). American Heart Association recommends intake of 1 g of omega-3 per day to lower the triacylglycerol levels and to prevent arrhythmias and atherosclerosis. Many clinical studies have shown that the omega-3 PUFAs could be beneficial to cardiovascular disease by preventing sudden cardiac death as well as heart failure outcomes (Burr et al., 1989; Hu et al., 2002; Harris et al., 2007; Tavazzi et al., 2008; Mozaffarian and Wu, 2011; Mozaffarian et al., 2013; Macchia et al., 2013; O'Connell et al., 2016; Siscovick et al., 2017; Shahidi and Ambigaipalan, 2018).



**Figure 1** Metabolism of omega-3 and omega-6 polyunsaturated fatty acids from dietary linoleic acid (LA) and alpha-linolenic acid (ALA) in the human body.

**Table 2** Sources of omega-3 and omega-6 polyunsaturated fatty acids

Source	g of fatty acid/100 g of sample					
	ALA	EPA	DPA	DHA	Total n-3	Total n-6
<b>Fish</b>						
<i>Mackerel</i>						
Atlantic	0.159	0.898	0.212	1.401	2.650	0.390
King	0.018	0.136	0.017	0.177	0.328	0.066
Pacific and Jack	0.050	0.509	0.123	0.932	1.600	0.190
Spanish	0.034	0.329	0.101	1.012	1.460	0.250
<i>Tuna</i>						
Bluefin	—	0.283	0.125	0.890	1.290	0.090
Skipjack (aku)	0.013	0.071	0.013	0.185	0.273	0.030
Yellowfin	0.002	0.012	0.004	0.088	0.230	0.028
<i>Herring</i>						
Atlantic	0.103	0.709	0.055	0.862	1.710	0.190
Pacific	0.057	0.969	0.172	0.689	1.860	0.280
Cisco (lake herring, tullibee)	0.020	0.120	0.050	0.510	0.70	0.62

**Table 2** Sources of omega-3 and omega-6 polyunsaturated fatty acids—cont'd

Source	g of fatty acid/100 g of sample					
	ALA	EPA	DPA	DHA	Total n-3	Total n-6
<b>Oil</b>	0.763	6.273	0.619	4.206	1.420	11.840
<i>Sardines</i>						
Canned	0.235	0.532	0.061	0.864	1.680	0.310
Oil	1.327	10.137	1.973	10.656	24.070	3.760
<i>Menhaden</i>						
Oil	1.490	13.168	4.915	8.562	28.120	3.310
<i>Salmon</i>						
Atlantic, Farmed	0.167	0.862	0.393	1.104	2.490	1.020
Atlantic, Wild	0.295	0.321	0.287	1.115	2.000	0.430
Chinook, Spring	0.089	1.008	0.301	0.944	2.320	0.260
Chum (Keta)	0.036	0.278	0.080	0.583	0.977	0.118
Coho, Farmed	0.075	0.385	0.190	0.821	1.460	0.430
Coho, Wild	0.035	0.389	0.114	0.695	1.233	0.122
Pink (Humpback)	0.070	0.547	0.134	0.859	1.610	0.229
Sockeye (Red)	0.111	0.348	0.143	0.681	1.280	0.470
Oil	1.061	13.023	2.991	18.232	35.300	2.210
<i>Cod</i>						
Atlantic (Scrod)	0.001	0.064	0.010	0.120	0.191	0.025
Pacific (Gray)	0.001	0.034	0.004	0.096	0.216	0.016
Lingcod	—	0.101	0.008	0.101	0.210	0.070
Stable fish (Black cod)	0.095	0.677	0.169	0.718	1.630	0.260
Oil, Liver	0.935	6.898	0.935	10.968	19.710	1.860
<i>Halibut</i>						
Atlantic or Pacific	0.011	0.066	0.016	0.128	0.510	0.160
Greenland (Turbot)	0.043	0.526	0.089	0.393	1.030	0.180
Haddock	0.002	0.042	0.005	0.089	0.182	0.029
<b>Marine mammals</b>						
<i>Seal</i>						
Blubber, Ringed seal	0.200	5.800	2.900	4.600	13.5	0.2
Oil	0.790	3.930	3.440	6.520	14.68	1.5
<i>Whale</i>						
Meat	0.002	0.004	0.003	0.006	0.015	—
<b>Plant sources</b>						
<i>Flaxseed (linseed)</i>						
Whole, Ground	22.813	—	—	—	22.813	6.423
Oil, Cold pressed	53.368	—	—	—	53.368	14.274
<i>Chia seed</i>						
Dried	17.83	—	—	—	17.83	5.780
<i>Canola</i>						
Oil	9.137	—	—	—	9.137	19.005
<i>Walnuts</i>						
English/Persian, Dried	9.080	—	—	—	9.080	38.090
Oil	10.400	—	—	—	10.400	52.900
<i>Hemp seeds</i>						
Hulled	8.560	—	—	—	8.560	27.67
<i>Soybean</i>						
Oil	6.789	—	—	—	6.789	50.950
<i>Mustard</i>						
Seed, Yellow	3.792	—	—	—	3.792	5.921
Oil	5.899	—	—	—	5.899	15.330

Modified from Canadian Nutrient File (2016).

The cardiovascular effects of omega-3 PUFAs are attributed to their ability to compete with arachidonic acid for cyclooxygenase enzyme that generates prostaglandins and thromboxane (Shahidi and Miraliakbari, 2006). DeFilippis et al. (2010) suggested serving of fish oil (900 mg of EPA + DHA/day) and ALA rich diet for cardiovascular and congestive heart failure patients because of their beneficial health effect. A review summarizing all epidemiological and clinical studies from the period of 1957 to 2013 established a clear link among diet, atherosclerosis, and cardiovascular events (Dalen and Devries, 2014). These authors suggested that the Mediterranean-style diet consisting of vegetables, fruit, fish, whole grains, and olive oil, reduces cardiovascular events to a greater extent than that of low-fat diets (Dalen and Devries, 2014).

High consumption of omega-3 PUFA rich diet for 8 weeks has been shown to increase insulin sensitivity and decrease serum C-reactive protein in older individuals (Tsitouras et al., 2008). However, there is limited clinical evidence available for the effect of omega-3s on type 2 diabetes mellitus. Anti-carcinogenic effect of omega-3s has also been studied; however, omega-6 and saturated fatty acids have been shown to promote cancer development. Prener et al. (1996) reported that the rate of prostate cancer incidents among Inuit population was less (70%–80%) than that of non-Inuit population due to their intake of omega-3 rich seafood diet. It has been suggested that omega-3 PUFAs could inhibit the growth of tumour cells by decreasing cyclooxygenase COX-2 and prostaglandins PGE<sub>2</sub> levels, thus could serve as a natural cyclooxygenase (COX) inhibitor (Kobayashi et al., 2006). Increased consumption of omega-6 fatty acids has been shown to be associated with breast, prostate and colon cancers due to the lipid peroxidation, generation of carcinogen after 17-beta-estradiol (E2) epoxidation and acting as co-carcinogen by enhancing the genotoxic effect of other compounds (chromatin) (Yu et al., 2004; Sakai et al., 2012).

DHA is a known primary element of membrane phospholipids in the brain (cerebral cortex, mitochondria, synaptosomes and synaptic vesicles) (Connor, 2000). Hence, consumption of omega-3 PUFAs is associated with decreased risk of cognitive decline or dementia, especially for Alzheimer disease and depression (Cole et al., 2009). Dijk-Brouwer et al. (2005) reported that lower fetal DHA, AA and essential fatty acid levels negatively influence neurological condition of early postnatal. Mathai et al. (2004) suggested that early neonatal deficiency in omega-3s is associated with the risk of developing Huntington's disease, Schizophrenia, high blood pressure and increased appetite signaling during adult life. In addition, Newberry et al. (2016) reported that intake of omega-3 PUFAs is associated with the length of gestation, preterm birth, birth weight, peripartum depression, gestational hypertension/preeclampsia, postnatal growth patterns, visual acuity, neurological development, cognitive development, autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), learning disorders, atopic dermatitis (AD), allergies and respiratory disorders. However, there is still insufficient evidence for several potential health effects of omega-3's, hence more clinical trials are required to shed light on these aspects.

## Summary

The intake of omega-3 PUFAs is associated with various health benefits such as cardiovascular health, cancer, cognitive and neurological function. However, there are insufficient clinical trials to allow making solid statements in some cases. Thus, more studies, including clinical trials are needed in the future on certain aspects that have not yet been fully explored.

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## Bioactives From Agricultural Processing By-products

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### Glossary

**Bioactive compounds** Bioactive compounds are chemical compounds found in small amounts in foods such as fruits, vegetables, nuts, whole grains, oils etc, that have an impact on physiological functions in the human body. In the nutrition field, bioactive compounds are separately studied from the macro and micro nutrients.

**Agricultural waste** Waste material produced due to various agricultural and farming operations. These wastes can be generated at any step of the agricultural operations.

**By-products** An incidental or intentional secondary product generated in the production or processing of a primary product of interest.

**Dietary fibre** Dietary fibre is a type of carbohydrate that the human body can not digest through gastrointestinal enzymes. It can be classified into soluble, insoluble or resistant starch depending on their physicochemical properties.

**Bioactive peptides** Bioactive peptides are the specific protein fragments with several amino acids resulting from physical, chemical or enzymatic protein hydrolysis that have a positive impact on physiological functions or conditions leading to improved health.

**Phytochemicals** Phytochemicals are biologically active chemical compounds found in plants, mainly their secondary plant metabolites used by the plants to compete and thrive within their environment.

### Overview

Agriculture and food processing industries generate several billions of metric tons of by-product biomass worldwide (Santana-Méridas et al., 2012; Kumar et al., 2017a). These by-products and waste streams can be generated in every phase of their lifecycle including agricultural production, industrial manufacturing, processing and distribution at different levels (Kumar et al., 2017a). According to the physicochemical and biochemical nature of the agricultural processing waste and by-products, it can be broadly classified into animal-derived materials and plant-derived materials (Ravindran and Jaiswal, 2016). Agricultural processing waste and by-products are considered to be one of the cheapest and abundantly available sources of biomass (Singh nee Nigam, 2009). However, proper techniques are essential in managing agricultural wastes and by-products to avoid potential environmental pollution (Liang et al., 2011). Therefore, it is essential to develop improved methods to enhance the use of agricultural residues. Several value-added applications have been explored recently ranging from animal feeding, fertilizers, platform chemicals and extraction of bioactive compounds, etc (Schieber et al., 2001; Kumar et al., 2017a).

Bioactive compounds have gained tremendous research and industrial interest in the recent past due to their ability in prevention and management of human chronic diseases (Joana Gil-Chávez et al., 2013). The growing trend in developing novel functional food and nutraceutical products have reinvigorated the search of alternative sources for extracting bioactive compounds, rather than relying on traditional food materials. In this context, academia and industry are looking into the possibility of using agricultural and food industry by-products as a source of bioactive compounds, mainly due to the abundant availability, low price and the absence of competing interest with food production (Joana Gil-Chávez et al., 2013; Ravindran and Jaiswal, 2016). Several agricultural by-product sources including meat processing, marine food processing, fruit processing wastes such as grape, apple pomace, citrus fruits, and vegetable and potato processing wastes such as tomato, carrot, olives and onions have been extensively studied for extracting different forms of bioactive compounds, among others (Schieber et al., 2001; Naumovski et al., 2017).

### Bioactive Compounds From Animal Derived By-products

Livestock processing industries generally produce byproduct streams such as skin, hide, blood, horns, wools, hoofs, offal, mechanically separated meat residue and feathers (Lafarga and Hayes, 2014; Suleria et al., 2016). In addition to the regular livestock processing by-products, marine processing industry by-products have gained increased attention, mainly due to the presence of broad spectrum bioactive compounds in abundant levels (Suleria et al., 2016). Researchers and industry have made continuous efforts to develop value-added applications for these by-products, where major efforts were made on identifying, extracting and purifying bioactive compounds with specific benefits (Mullen et al., 2017).

## Meat Processing By-products

The increased global demand for high quality, high value meat and poultry products has increased the production of low value, non-edible meat products such as offal, blood, skins, horns, and feathers, almost up to 54%–56% on total animal weight for bovine meat and up to 48% for porcine (Marti et al., 2012; Mullen et al., 2017). These by-products are rich in nutrients such as proteins, lipids, minerals and certain bioactive molecules; however, the food value has been limited due to the flavour, texture, and socio-logical barriers (Mora et al., 2014; Toldrá et al., 2016). Therefore, extraction of individual components for specific uses can become a useful methodology for value-added applications in meat industry by-products. The major research focus on extracting bioactives from meat industry by-products were targeted towards extraction of protein hydrolysate, collagen, and developing bioactive peptides with different functionalities (Ravindran and Jaiswal, 2016; Hong et al., 2018).

### Collagen/Gelatin

Collagen and collagen peptides are one of the primary bioactive compounds of focus from meat industry by-products (Fu et al., 2018). Collagen is a triple helical fibrous structural protein present in most parts of the animal body. However, extraction and purification process of collagen will hydrolyse the triple helical structure and produce gelatin (Mullen et al., 2017; Fu et al., 2018). Even though skins and bones have been used as primary source of collagen/gelatin extraction in the past (Mullen et al., 2017), recent studies focus on using other underutilized by-products such as meat & poultry processing waste (Khiari et al., 2014), spent hen (Hong et al., 2017, 2018) and mechanically separated meat residue (Du et al., 2014). Moreover, the recent research on collagen/gelatin has been mainly focused on extracting functional bioactive peptides using novel processing and purification methods (Khiari et al., 2014; Hong et al., 2017, 2018).

### Bioactive Peptides and Protein Hydrolysate

Bioactive peptides are short fragments of proteins with 2 to 30 amino acid residues and display several health promoting bioactivities in addition to their nutritional value (Ryan et al., 2011; Mullen et al., 2017). All meat by-products are rich in protein; therefore, traditionally proteins were extracted from the by-products and then subsequently hydrolysed to release small peptide fragments with different bioactivities (Lemes et al., 2016). Compared to the other protein rich by-products, research on meat by-product based bioactive peptides are limited to few specific types of by-products; however, peptides with different bioactivities such as antioxidant, antimicrobial, antihypertensive, anti-diabetic, anti-hypercholesterolaemic or mineral binding have been reported with different meat products (Sharma et al., 2011). Meat trimmings and cuttings are one of the meat by-products explored for bioactive preparation. Angiotensin converting enzyme (ACE)-inhibitory peptides MNPPK and ITNP from porcine skeletal muscle hydrolysate (Arihara et al., 2001), antioxidant peptide DAQEKLE from pork myofibrillar protein (Saiga et al., 2003), and anti-fatigue peptides DLYA, SLYA and VW (Arihara, 2006) are some of the bioactive peptides extracted from meat trimming and cuttings. Animal bones, horns, and hooves resulting from meat processing are mainly used in animal feeding; however, recent studies have extracted antioxidant peptides QYDQGV, YEDCTDCGN, AADNNELFPPN from these by-products (Liu et al., 2010). Animal blood and their hydrolysates were also used in preparing bioactive peptides such as GFPTTKTYFPHF, KLLSHSL, and LVVYPWT etc, with ACE-inhibitory, antioxidant and opioid activity (Yu et al., 2006; Adje et al., 2011). Most recent studies have focused on using non-conventional livestock industry by-products such as spent hen in bioactive peptide preparation (Hong et al., 2017, 2018; Yu et al., 2018), specifically with immunomodulatory, antihypertensive (Udenigwe et al., 2017) and anti-inflammatory activities (Chalamaiah et al., 2018; Yu et al., 2018).

## Marine Processing Waste/By-products

Marine capture fisheries account for more than 50% of total world fish production. Marine fish processing industry generates a great quantity of by-products as waste every year. These by-products include head, skin, blood, trimmings, fins, frames, roes and viscera (Rustad et al., 2011; Chalamaiah et al., 2012; Sila and Bougatef, 2016). Additionally, crustaceans and shellfish processing also produce considerable quantity of shells as by-products. About 20 million tons of marine processing by-products are discarded as waste every year, which accounts for 25% of the total annual marine capture (Hou et al., 2016). Currently, marine processing by-products are majorly utilized for the production of fishmeal, marine oil, pet food, fertilizer, and fish silage, which have low market value (Rustad et al., 2011). Recently, several bioactive compounds have been identified and isolated from marine processing by-products for human use. Proteins, lipids, chitin and minerals are the major sources of bioactive compounds from marine processing by-products (Kim and Mendis, 2006).

### Fish Oil

Marine processing by-products are an excellent source of high quality fish oil that can be used for human consumption (Rustad et al., 2011). Fish oil is the major product produced from leftovers of fatty fish processing. Fish contains fat in the range of 2%–30%, which depends on the species, age and seasonal changes. Several methods are currently used to extract the fish oil from fish processing by-products. Fish oil is conventionally produced by cooking and pressing followed by centrifugation. Recently, proteolytic hydrolysis of fish by-products has been applied to extract the fish oil with high yield and better quality (Liaset et al., 2003; Rustad et al., 2011). The major fatty acids of fish oil are omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Omega-3 fatty acids are essential nutrients for good health and several studies have showed that

consumption of fish oil (EPA and DHA) twice a week is linked with a lowered risk of developing cardiovascular disorders. Fish oil has been proved to lower blood pressure, reduce triglycerides, decrease chance of abnormal heart rhythm, reduce the progress of plaque in the arteries and lower the chances of heart attack and stroke (Kromhout et al., 2012).

### **Fish Collagen and Gelatine**

Fish skins, scales and bones contribute to ~30% of total by-products from fish-processing industry. Fish skins, scales and bones are good sources to separate collagen and gelatin. Collagen is a most abundant and fibrous structural protein composed of three chains forming a tight triple helix (Shoulders and Raines, 2009). Gelatin is produced by partial hydrolysis of collagen. Heat treatment or treatment with dilute solutions of acid or alkali transforms collagen into gelatin. Marine collagen from fish skin, scales and bones is widely used in biomedical and pharmaceutical industries as a scaffold and a drug carrier due to its bioactive properties such as non-antigenic, biodegradable, bioreabsorbable, non-toxic and biocompatible, compatible with synthetic polymers, synergic with bioactive components, and cell growth potential (Lee et al., 2001; Jeevithan et al., 2013; Subhan et al., 2015). Several clinical studies reported that consumption of collagen hydrolysate reduces the pain in osteoarthritis patients (Bello and Oesser, 2006). Peptides prepared from gelatin have been shown to contain antioxidant, ACE-inhibitory, immunomodulatory and antiproliferative effects (Kim and Mendis, 2006; Chalamaiah et al., 2012; Chalamaiah et al., 2018).

### **Bioactive Peptides**

Marine processing industry generates large quantities of proteins as by-products. Recently, several attempts have been made to produce bioactive peptides from marine wastes. Marine processing by-products such as viscera, heads, skin, bones, dark muscle, scales, frames, cephalothorax, cut offs, and shells from various species successfully used to produce peptides with variety of bioactivities (Sila and Bougatef, 2016). Bioactive peptides are generally obtained from marine processing wastes by using proteolytic hydrolysis or fermentation. Pepsin, chymotrypsin, trypsin, Alcalase, Flavozyme, Protamex and papain are routinely employed enzymes for the generation of bioactive peptides from marine waste proteins. Marine by-product derived peptides showed various bioactivities such as antioxidant, anticancer, ACE-inhibitory, antimicrobial, immunomodulatory, hypocholesteremi, anti-diabetic and mineral binding activities (Harnedy and FitzGerald, 2012). Bioactive peptides from marine processing by-products can be used in food and pharmaceutical applications.

### **Chitin and Chitosan**

Crustacean shells and shellfish wastes are an important group of by-products from marine processing industry. The main component of these wastes is chitin, a bioactive and abundant polysaccharide, after cellulose. By-products from shrimp, lobster, krill and crab processing plants contain large quantities of chitin. The amount of chitin in exoskeleton wastes varies from 20%–30% depending on the species and seasons. The chitin by-product from shellfish waste accounts for about 10,000 tons per year (Hamed et al., 2016). Chitin is a linear polymer of N-acetyl-D glucosamine units. Removal of some acetyl groups from chitin results in the formation of its derivative, chitosan (water soluble form). Demineralization and deproteinization are commonly used processes to obtain chitin from exoskeletons of crustaceans. Chitin and chitosan have been widely used in biomedical and pharmaceutical industries for drug release and wound dressing due to its biocompatible and biodegradable properties in the body (Younes and Rinaudo, 2015). In food industry, they are used as edible films for food preservation. Chitin and chitosan showed various bioactivities such as antioxidant, anti-microbial, anti-cancer, and anti-coagulant activities (Vongchan et al., 2003; Yen et al., 2008; Goy et al., 2009; Salah et al., 2013; Hamed et al., 2016).

### **Minerals**

Bone by-products from marine processing industry are important source of minerals. Calcium is major mineral in the fish bones and accounts for about 60% of the total minerals of the fish bones (Rustad et al., 2011). Calcium from bone by-products of Atlantic salmon and Atlantic cod was reported to be well absorbed in healthy men and can be used as natural Ca source for preparation of functional foods or as supplements (Malde et al., 2010). Calcium from fish bones should be modified into an edible form by using hot water and hot acetic acid treatments before its application in food fortification (Kim and Mendis, 2006). In addition to calcium, fish bone by-products are also considerable sources of the inorganic constituent, hydroxyapatite ( $\text{Ca}_5(\text{PO}_4)_3\text{OH}$ ). Hydrothermal alkaline hydrolysis and high temperature (~1300 °C) treatments are commonly used methods to isolate hydroxyapatite from fish bones (Ferraro et al., 2010). Hydroxyapatite from fish bones is most suitable for bone reconstruction or substitution due to its biocompatibility with human bones.

## **Bioactives From Plant-Derived By-products**

Plant-based agricultural by-products have been considered as one of the major source for extracting different bioactive compounds over the past few decades. In reality, fruits and vegetables can be considered as the most simple forms of functional food due to their composition enriched with different bioactive compounds (Kumar et al., 2017a). Therefore, the by-products generated from fruit and vegetable processing industries provide a great potential for extracting and developing different bioactive molecules. Different groups of phenolic compounds, carotenoid & other vitamins, bioactive polysaccharides, lignin, tannin and dietary fibres are some of the examples of potential bioactive compounds (Kumar et al., 2017a). In addition to the traditional fruit and vegetable

processing industries, the wine and brewing industries generate by-products such as grape pomace and brewers spent grains respectively, that are enriched with a wide array of bioactive compounds (Barba et al., 2016). Cereal and oilseed processing is another important agricultural operation that produces a variety of by-products such as protein meals, bran, and husk which can potentially use in extracting bioactive compounds (Ravindran and Jaiswal, 2016). Table 1 summarizes some of the key plant based agricultural by-products and their major bioactive compounds.

### Fruit, Vegetable and Wine Processing By-products

Most of the recent research works on fruit, vegetable and wine industry by-products were focused at extracting valuable phytochemicals (e.g. sterols, tocopherols, carotenes, terpenes, & other polyphenolic compounds), bioactive polysaccharide & dietary fibre, industrial enzymes and bioactive proteins and peptides (Schieber et al., 2001; Ravindran and Jaiswal, 2016; Kumar et al., 2017a). Table 1 shows some of the major phenolic compounds present in the fruit and vegetable by-products.

#### Phenolic Compounds

Phenolic compounds and other secondary plant metabolites are the major class of bioactive compounds studied and used in research or commercial level applications. Phenolic compounds extracted from plant-based by-products have been studied for their capacity to scavenge free radicals in both food systems and *in-vivo* models (Deng et al., 2012; Banerjee et al., 2017). Therefore, majority of the plant-based phenolic compound research were focused on finding their antioxidant activity in *in-vitro*, *in-vivo* and in animal models (Banerjee et al., 2017). Anticancer and anti-proliferative activities of phenolic compounds are another area of research interest. Terpenoids isolated from apple peel (He and Liu, 2007) and sea buckthorn (sea berry) fruit phenolics (Olas et al., 2018) have shown both antiproliferative and anticancer activity in *in-vivo* studies. Antihypertensive activity of plant based phenolic compounds is another promising area of research in food and nutritional science. Several plant phenolics extracted from fruits such as blueberries (Ahrén et al., 2015) and guava fruit (Ademiluyi et al., 2016) have shown antihypertensive activity *in-vivo*. Some of the bioactive phenolics extracted from plant based agricultural by-products have reached up to the commercialization stage. Resveratrol extracted from wine industry by-product grape pomace and olive oil processing waste is a prime example of commercialized phenolics from agricultural processing by-products in recent history (Banerjee et al., 2017).

#### Bioactive Polysaccharides

The recent research interest on fruit & vegetable processing by-products as a lignocellulosic biomass has directed the efforts to extract bioactive polysaccharides (Liu et al., 2015; Banerjee et al., 2017). Cellulose extracted from fruit & vegetable industry by-products and their derivatives showed potential uses in diagnostic, pharmaceutical and nutraceuticals applications such as sugar lowering effect, antibody immobilization, hydrogel forming ability, and water retention (Credou and Berthelot, 2014; Liu et al., 2015). Hemicellulose extracted from fruits and vegetable by-products have been used to extract xylans and their derivatives such as xylooligosaccharides with dietary fibre applications (Aachary and Prapulla, 2011). In addition, mango kernels and banana peels have been used in producing resistant starches (Banerjee et al., 2017).

#### Bioactive Proteins and Peptides

Fruit and vegetable seeds are rich in protein; therefore, they can be used in preparing protein hydrolysates with functional and bioactive applications. Several fruit and vegetable seeds such as date (*Phoenix dactylifera*), chilli pepper (*Capsicum annum*), and passion fruit (*Passiflora edulis*) have been explored in preparing peptides and or protein hydrolysate with antioxidant, antimicrobial, anti-fungal, and ACE-inhibitory activities (Pelegri et al., 2006; Ribeiro et al., 2007; Ambigaipalan and Al-Khalifa, 2015; Ambigaipalan and Shahidi, 2015). The presence of aromatic amino acids and histidine also contribute towards some of the bioactivities such as quenching metal ions and reactive oxygen species (Deng et al., 2012). Extraction of vicilin-like protein from watermelon seeds (Wani et al., 2008), and leptin from jackfruit seeds (Devalaraja et al., 2011) are some of the other examples of potential bioactives that can originate from fruit and vegetable seeds.

#### Bioactive Lipids

Both fruit and vegetable processing wastes are rich sources of lipids and their derivatives, mainly in the form of free fatty acids, waxes, isoprenoid hydrocarbons, acylglycerols, sterols and vitamins exhibiting different bioactivities (Banerjee et al., 2017). Citrus fruit peels, mango kernels, apricot seeds, pomegranate seeds, and tomato seeds are some of the plant-based by-products that are rich in lipids and their derivatives (Lisichkov et al., 2011; Banerjee et al., 2017). Especially, pomegranate seed oil has shown antioxidant, anti-obesity, anti-asthmatic and inhibition of breast cancer cell proliferation due to the presence of a conjugated omega-5-fatty acid "punicic acid" (Grossmann et al., 2010; Goula and Adamopoulos, 2012; Patterson et al., 2012; Goula, 2013). Lipids extracted from mango kernels (mango butter) has shown emollient and anti-aging properties; thus can be used in skin healing cosmetic applications (Mandawgade and Patravale, 2008).

**Table 1** Bioactive components present in different plant-based agricultural and food industry by-products

Source	Residue	Bioactive components	References
<b>Fruits</b>			
Apple	Peel and pomace	Epicatechins, catechins, anthocyanins, quercetin glycoside, chlorogenic acid, hydroxycinnamates, procyanidins	Lu and Foo, 1997; Yeap Foo and Lu, 1999; Wolfe and Liu, 2003
Avocado	Peel and seed	Epicatechins, catechins, gallic acid, chlorogenic acid, cyanidin 3-glucoside, homogentisic acid	Deng et al., 2012
Banana	Peel	Gallocatechin, anthocyanins, delphinidin, cyaniding, catecholamine	Kanazawa and Sakakibara, 2000; Someya et al., 2002; González-Montelongo et al., 2010
Citrus fruits	Peel	Hesperidin, naringin, eriocitrin, narirutin	Coll et al., 1998
Grapes & grape pomace	Seed and skin	Coumaric acid, caffeic acid, ferulic acid, chlorogenic acid, cinnamic acid, neochlorogenic acid, <i>p</i> -hydroxy-benzoic acid, vanillic acid, gallic acid, proanthocyanidins, quercetin, resveratrol	Shrikhande, 2000; Negro et al., 2003
Guava	Skin and seeds	Catechins, gallic acid, cyanidin 3-glucoside, galangin, homogentisic acid, kaempferol	Deng et al., 2012
Litchi	Pericarp, seeds	Cyanidin 3-glucoside, cyanidin 3-rutinoside, malvidin-3-glucoside, gallic acid, epicatechin-3-gallate	Lee and Wicker, 1991; Duan et al., 2007
Mango	Kernel	Gallic acid, ellagic acid, gallates, gallotannins, condensed tannins	Arogba, 2000; Puravankara et al., 2000
Palm	By-product of palm milling	Tocopherols, tocotrienols, sterols, phenolic antioxidants	Choo et al., 1996; Tan et al., 2007
Pomegranate	Peel and pericarp	Gallic acid, cyanidin-3,5-diglucoside, cyanidin-3-diglucoside, delphinidin-3,5-diglucoside	Gil et al., 2000; Noda et al., 2002
<b>Vegetables</b>			
Carrot	Peel	Phenolic compounds, beta-carotene	Chantaro et al., 2008
Cucumber	Peel	Chlorophyll, pheophytin, phellandrene, caryophyllene	Zeyada et al., 2008
Potato	Peel	Gallic acid, caffeic acid, vanillic acid	Zeyada et al., 2008
Tomato	Skin and pomace	Carotenoids	Strati and Oreopoulou, 2011
<b>Cereals &amp; Oilseeds</b>			
Barley	Bran & brewers spent grain	$\beta$ -glucan	Sainvitu et al., 2012
Rice	Bran	$\gamma$ -oryzanol, rice bran oil	Oliveira et al., 2012; Perretti et al., 2003
Wheat	Bran and germs	Phenolic acid, antioxidants	Wang et al., 2008
Canola	Canola meal	Bioactive proteins and peptides, phenolic compounds	Yoshie-Stark et al., 2006; Chandrasekara et al., 2016
Soybean	Soy meal	Bioactive proteins and peptides, flavonoids, phenolics	Singh et al., 2014

Adopted with permission from Kumar et al. (2017a).



## Cereal, Oilseed Processing and Brewing Industry By-products

The cereal and oilseed industry generates great deal of by-products, mainly in the form of oilseed meal (soy meal, canola meal, sunflower meal etc.), bran and germ, and brewers spent grain resulting from brewing industries (Naumovski et al., 2017; Vanamala et al., 2017). Cereal grains and oilseeds in general are rich in phenolics, protein and lipids with different biological activities. Especially, processing operations can enrich the composition of these bioactive compounds due to removal of one or more high value components; thus, making them a valuable source for bioactive compounds extraction.

### Bioactive Proteins and Peptides

Cereal and oilseed industry by-products have become one of the primary sources of bioactive proteins and peptides in the past few decades (Maestri et al., 2016). Soy protein is one of the key substrates for developing hydrolysate and peptides with a wide array of bioactivities including antioxidant, ACE inhibition, hypocholesterolemic, anti-cancer, anti-obesity, and immunomodulatory activities (Singh et al., 2014; Maestri et al., 2016). Canola protein is the major by-product generated from canola oil processing and gained increased attention recently in value-added utilization (Aider and Barbana, 2011). ACE-inhibitory activity, antioxidant activity, anti-thrombotic activity, and bile acid binding are some of the bioactivities reported for peptides extracted from canola proteins (Aider and Barbana, 2011). Hemp seed meal (Girgih et al., 2014), sunflower seed meal (Zhao et al., 2017), and camelina meal (Kumar et al., 2017b) are the emerging minor oil seed meals with high protein contents that can be used in extracting bioactive peptides. The extraction of bioactive peptides from cereal proteins are mainly done with the extracted protein from cereal grains rather than their by-products, except in the case of brewers spent grain (BSG). BSG resulting from brewing industry are rich in fibre and protein (70% and 20% respectively) (Guido and Moreira, 2017), and has been used for developing ACE-inhibitory peptides (Connolly et al., 2015), glycaemic response modulating peptides (Connolly et al., 2017), immunomodulatory peptides (Crowley et al., 2015), antioxidant peptides (Vieira et al., 2016), etc.

### Bioactive Lipids

Bioactive lipids are another class of active compounds present in the cereal and oilseed industry by-products. Rice bran oil is a prime example of bioactive lipid generated in the rice processing industry with a wide array of bioactivities (Khoei and Chekin, 2016). Rice bran oil especially contain  $\gamma$ -oryzanol which has shown biological activities in serum cholesterol lowering, anti-oxidation, anti-carcinogenic and attenuating allergic inflammations (Nagasaka et al., 2011; Khoei and Chekin, 2016).

### Dietary Fibre

Cereal processing by-products such as bran and BSG are rich in fibre content, that can be used for extraction of biologically active materials such as dietary fibre (Gangopadhyay et al., 2015).  $\beta$ -glucan is one of the key form of dietary fibre extracted from both oat and barley bran and BSG due to their superior bioactivities in reducing serum cholesterol level, reducing blood glucose level and potential antihypertensive effect (Gnai et al., 2012; Gangopadhyay et al., 2015). In addition,  $\beta$ -glucan has shown positive effects on body weight management (Koh-Banerjee et al., 2004).

## Conclusions

There is a growing demand and interest on bioactive compounds for human health applications due to their potential effects on reducing and managing chronic disease conditions. In this context, exploring new sources for extraction of bioactives hold a special interest among the scientific community. Agricultural by-products and waste materials are a valuable biomass that contains a wide array of biologically active materials at different concentrations. Extraction of these bioactives from by-product biomass can provide benefits to both agricultural and nutraceutical industries. However, the bioactive materials present in by-product biomass may possess their own challenges due to structural and functional changes that can occur during various steps of processing. Therefore, further research should be carried out to explore non-traditional agricultural by-products for bioactive extraction, developing new extraction techniques for customized applications in by-product biomass. In addition, a detailed comparative study on the biological activity of bioactives extracted from agricultural by-products Vs bioactives extracted from traditional (native materials) feedstock should be carried out to assess the value of agricultural by-product based bioactives.

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# Bioactives From Land-Based Animal Processing By-Products

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## Introduction

The slaughter of livestock animals generates considerable amounts of by-products each year. According to Regulation EC 1069/2009, animal by-products are defined as materials of animal origin that people do not consume in a direct way (Lafarga and Hayes, 2014; Toldrá et al., 2012). In the European Union, it is estimated that over 20 million tons of animal by-products are produced annually (Henchion et al., 2016). These by-products are mainly composed of blood, meat trimmings, offal and bone/skin (Toldrá et al., 2016); however, they are normally used for production of low-value products, such as animal feed, fertilizers and pet food (Alao et al., 2017). Animal by-products are abundant in proteins, essential amino acids, minerals and vitamins, which can provide a great valorization opportunity for meat processing industry (Toldrá et al., 2016). Therefore, utilization of animal by-products for production of high value-added ingredients not only diminishes environmental impact but also provides new food ingredients and improves the potential revenue for food industry (Henchion et al., 2016). For instance, animal by-products rich in high-quality protein can serve as starting materials for generation of protein hydrolysates/peptides with relevant functional properties or bioactivities (Mora et al., 2014; Toldrá et al., 2016). Bioactives cover a wide range of compounds, including peptides, oligosaccharides, fatty acids and enzymes (Lafarga and Hayes, 2014; Mora et al., 2014). Bioactive peptides are defined as short peptides with approximately 2–20 amino acids that can exert health-promoting effects on humans. They are inactive when present within the primary sequence of the parent protein but exert beneficial effects on body systems once they are released (Udenigwe and Aluko, 2012). In recent years, there is an increasing interest in exploration of bioactive peptides/hydrolysates from animal by-products (Mora et al., 2014; Toldrá et al., 2012, 2016). Therefore, this chapter aims at providing some updated information on the composition and nutritional characteristics of animal by-products and bioactive peptides/hydrolysates recovered from these by-products.

## Composition and Nutritional Characteristics of Animal Processing By-Products

Blood is a major by-product representing approximately 4% of live animal weight (Bah et al., 2013). Currently, blood generated in slaughterhouses are under-utilized because they are processed mainly for use as animal feed and fertilizer (Bah et al., 2016; Mora et al., 2014). Blood is normally separated into two fractions during the slaughter process, namely blood cells and plasma (Lynch et al., 2017). The blood cell fraction contains red and white blood cells and platelets, while plasma includes several proteins such as albumins, globulins and fibrinogen (Bah et al., 2013; Lynch et al., 2017). Red blood cells represent the most abundant cells, containing hemoglobin, an iron-containing protein that can facilitate binding of oxygen to increase solubility and transportation (Bah et al., 2016; Lafarga and Hayes, 2014). Heme iron is more easily absorbed from meat into the body, compared with non-heme iron (Buzala et al., 2016). In addition, heme has been applied as a natural color enhancer for meat products in the food industry (Ofori and Hsieh, 2012). Plasma, on the other hand, mainly contains serum albumins, globulins and fibrinogen, among which albumins are the most abundant proteins responsible for regulation of fluid distribution, osmotic pressure and transport of small metabolites in animals (Lafarga and Hayes, 2014; Ofori and Hsieh, 2012).

Trimming is defined as the remaining portions of meat after chopping of primal cuts from the animal carcass and contain meat, fat, cartilages and mechanically recovered meat (Mora et al., 2014). However, portions of head meat, internal organs, major tendons, or ligaments are not considered trimmings (Mora et al., 2014; Toldrá et al., 2016). The protein content in meat trimmings may vary from 14% to 17% in pork and from 7% to 12% in beef (Mora et al., 2014).

Slaughtering offal is described as the entrails and internal organs of the slaughtered animal excluding muscle and bone (Pereira and Vicente, 2013). Offal can be further divided into two categories, namely red and white offal. Red offal (e.g. heart, liver and lung) are normally employed in many processed meat products or serve as food ingredients in sausages, while edible white offal, such as intestines and stomach, requires further processing (Florek et al., 2012). The average protein content in offal ranges from 15% to 18%, which is a good source of essential and non-essential amino acids (Pereira and Vicente, 2013). In contrast, the contents of protein and fat in offal tend to be lower than that of meat cuts (Florek et al., 2012).

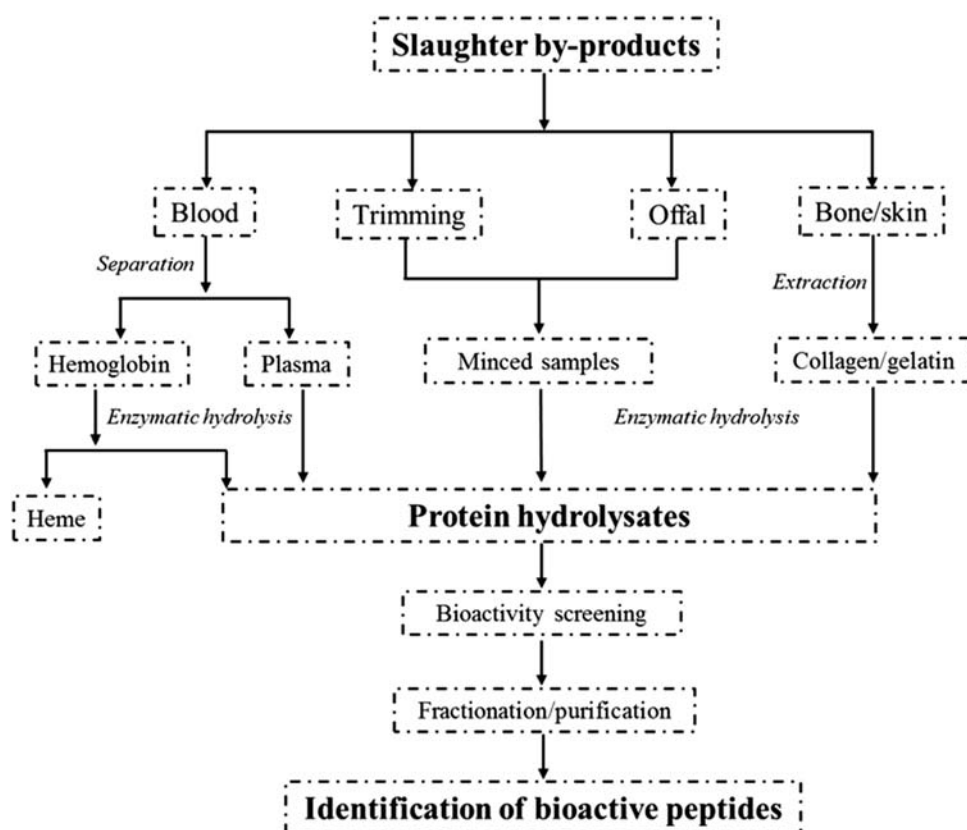
Bones and hides act as the major meat by-products during meat processing, which are typically used for feeding purposes or production of collagen/gelatin (Mora et al., 2014; Toldrá et al., 2016). Collagen constitutes approximately 30% of all vertebrate body proteins. Collagen molecule is composed of three almost identical  $\alpha$ -chains, namely collagen triple helix. This triple helix consists of a repetitive motif, Gly-X-Y, where X is proline and Y is hydroxyproline, which can contribute to stability of the helical structure. Collagen can be extracted from meat by-products through chemical or biochemical treatment, followed by extraction process using water (Fu et al., 2018). This process can cleave covalent and hydrogen bonds, leading to breakdown of triple helix and transformation to soluble collagen (Fu et al., 2018).



## Production of Bioactive Peptides Derived From Animal Processing By-Products

Animal processing by-products rich in proteins constitute a good substrate for proteolysis (Mora et al., 2014). Enzymatic hydrolysis of by-products by commercial proteases is an effective approach to recover protein-containing part from by-products (Lafarga and Hayes, 2014). Prior to enzymatic hydrolysis, the pre-treatment procedures are imperative for certain raw materials. For example, collagen is normally subjected to pre-treatment at high temperature or pressure so that the structure is partially or completely destroyed, making it easier for enzyme access (Zhang et al., 2013). Schematic diagram for production of bioactive peptides from animal processing by-products is illustrated in Fig. 1. Following optimal pH and temperature adjustments, animal by-products are usually minced and diluted with water. Hydrolysis reaction usually lasts for a few hours either in batch reactors or in continuous reactors. Once the desired degree of hydrolysis is achieved, the reaction is terminated by heat treatment to inactivate the enzymes. Afterwards, the digests are subjected to centrifugation to recover supernatant after removal of fat and pellet. The resultant hydrolysates (supernatants) can be submitted to bioactivity screening, followed by fractionation and purification with the aid of chromatographic techniques to identify potent bioactive peptides (Fig. 1). In terms of blood or hemoglobin samples, after heat treatment, the pH of hydrolysate is lowered to precipitate heme (Vatsyayan and Goswami, 2011). Subsequently, the hydrolysate mixture is centrifuged to separate insoluble heme from the aqueous solution.

Although bioactive peptides can be naturally released by endogenous enzymes in meat during postmortem aging (Fu et al., 2017), the activities of endogenous enzymes are limited so that the resultant peptides may be inactive or have low bioactive potencies. In this regard, the most common approach to release bioactive peptides is use of exogenous proteases (Udenigwe and Aluko, 2012). An array of commercial food-grade proteases have been used for generation of bioactive peptides from animal by-products, including Alcalase, Neutrase, Flavourzyme, Bromelain, Papain, Pepsin, Trypsin and Protamex (Lafarga and Hayes, 2014). After enzymatic hydrolysis, thousands of peptides can be released, but a limited number of them possess promising bioactivity. Therefore, a follow-up purification procedure needs to be implemented, including a series of chromatography, membrane filtration or electrodialysis technologies (Udenigwe and Aluko, 2012). However, fractionation and purification are time-consuming and the yield is relatively low. In order to optimize screening process, *in silico* approaches have been recently employed to search for certain bioactive peptides from animal by-products (Fu et al., 2016a,b; Lafarga et al., 2014), which involves screening of the optimal protein/enzyme combination, followed by virtual prediction of the potential bioactivity of peptides (Fu et al., 2016a,b).



**Fig. 1** Schematic diagram for production of bioactive peptides from animal processing by-products.



## Health Beneficial Potentials of Bioactive Peptides From Animal By-Products

As the major component of animal by-products is protein, they are excellent precursors for production of protein hydrolysates that can be extracted, purified and commercialized as high value-added ingredients (Mullen et al., 2017). Bioactive peptides recovered from animal by-products were reported to possess antihypertensive, antioxidant, antidiabetic and antimicrobial activities (Lafarga and Hayes, 2014). A number of examples of bioactive peptides/hydrolysates from animal processing by-products are summarized in Tables 1–3.

### Antihypertensive Activity

Hypertension is a major risk factor responsible for most cardiovascular diseases (Padwal et al., 2001). Angiotensin-I converting enzyme (ACE) and renin, two main elements in the renin-angiotensin-aldosterone system, play a pivotal role in regulation of blood pressure and fluid homeostasis (Manrique et al., 2009). Simultaneous inhibition of these two enzymes has been shown to exert a synergistic effect on treatment of hypertension. However, long-term administration of synthesized drugs may provoke several side effects on human health (Manrique et al., 2009). Therefore, there is an increasing interest in development of natural ACE- and renin-inhibitory peptides with fewer side effects. In recent years, several ACE- and renin-inhibitory peptides have been identified from animal processing by-products, such as collagen, heart, viscera, hemoglobin, plasma and whole blood (Table 1).

Although a considerable number of *in vitro* experiments have confirmed ACE- or renin-inhibitory activity of peptides/hydrolysates from animal by-products, their *in vivo* efficacy has not been adequately documented. Chicken skin protein hydrolysates with ACE- and renin-inhibitory activities have been reported to exhibit antihypertensive effects *in vivo* based on metabolomics data and animal models (Onuh et al., 2013, 2014; Onuh et al., 2015; Onuh et al., 2016). Fu et al. (2015) produced collagen hydrolysates with potent ACE-inhibitory activities and confirmed their stability towards temperature, pH and digestive enzymes. The most active ACE-inhibitory peptides were identified to be VGPV and GPRGF that can be transported across the monolayer of human intestinal epithelial Caco-2 cells through paracellular pathway and retained their ACE-inhibitory activities (Fu et al., 2016a, 2016b). Recently, the *in vivo* antihypertensive effect of papain hydrolysates from bovine globulins and bovine serum albumin (BSA) were verified (Lafarga et al., 2016a). A decrease in systolic blood pressure of 14.3–36.2 mm Hg was observed at the oral dosage of 200 mg kg<sup>−1</sup> (Lafarga et al., 2016a, 2016b).

**Table 1** ACE- or renin-inhibitory peptides/hydrolysates identified from animal processing by-products

ACE-inhibitory activity	Hydrolysate/Sequence	Source	Enzyme	IC <sub>50</sub>	In vivo bioactivity	References
Renin-inhibitory activity	TY, FL	Bovine hemoglobin	Pepsin, Trypsin	96.43 and 290.66 μM	N/A	Wang et al. (2017)
	VGPV, GPRGF	Bovine collagen	Alcalase, Papain	405.1 and 200.9 μM	Transported across monolayers of human intestinal epithelial Caco-2 cells through paracellular pathway	Fu et al. (2016a,b)
	Whole hydrolysates	Bovine connective tissue	Pepsin, Pancreatin	N/A	N/A	Ryder et al. (2016)
	YY	Bovine serum albumin	Papain	180 μM	Antihypertensive effect on SHR over 24 h at the dosage of 200 mg/kg of body weight	Lafarga et al. (2016a,b,c)
	SLR	Bovine serum albumin	Papain	171.9 μM	Antihypertensive effect on SHR over 24 h at the dosage of 200 mg/kg of body weight	Lafarga et al. (2016a)
	HF, KR	Bovine hemoglobin	Papain	221.2 and 383.7 μM	N/A	Lafarga et al. (2016c)
	YF	Bovine fibrinogen	Papain	94.6 μM	N/A	Lafarga et al. (2015)
	HR, YR, HLP	Bovine hemoglobin	Papain	7.0, 8.7 and 6.4 mM	N/A	Lafarga et al. (2016c)
	SLR	Bovine fibrinogen	Papain	7.2 mM	N/A	Lafarga et al. (2015)

**Table 2** Antioxidant hydrolysates from animal processing by-products

Samples	Source	Enzyme	Assays	References
Whole hydrolysate	Porcine tissues (colon, appendix, rectum, pancreas, heart, liver and lung)	Protamex, Alcalase	DPPH, ABTS, inhibition of oxidation, iron chelation capacity	Damgaard et al. (2014, 2015)
Whole hydrolysate	Bovine fibrinogen and $\gamma$ -globulins	Papain	FRAP and ORAC assays	O'Sullivan et al. (2017)
Whole hydrolysate	Bovine connective tissue proteins	Pepsin and Pancreatin	ORAC	Ryder et al. (2016)
Whole hydrolysate	Bovine plasma proteins	Papain, bromelain, FP400 and FPII	DPPH, FRAP	Bah et al. (2016)
Whole hydrolysate	Bovine plasma proteins	Alcalase	Reduced TBARS and peroxide values	Seo et al. (2016)

**Table 3** Antidiabetic peptides/hydrolysates from animal processing by-products

Hydrolysate/Sequence	Source	Enzyme	IC <sub>50</sub>	References
PPL	Bovine serum albumin	Pepsin or ficain	0.3 mM	Lafarga et al. (2014)
ER	Bovine serum albumin	Papain	4.4 mM	Lafarga et al. (2016a)
PPG	Bovine collagen	Papain, ficain, bromelain	2.2 mM	Lafarga et al. (2014)
Whole hydrolysate	Bovine hemoglobin	Pepsin and pancreatin	1.62 mg mL <sup>-1</sup>	Caron et al. (2017)

### Antioxidant Activity

Oxidative stress is an imbalance between production of reactive oxygen species (ROS) and antioxidant defenses, which is implicated in a series of chronic diseases, such as cardiovascular disease, neurodegenerative disorders, inflammation, gastric ulcers, diabetes mellitus and cancer (Samaranayaka and Li-Chan, 2011). Therefore, a substantial attention has been paid to search natural antioxidants to protect against ROS and other free radicals present in the food system (Samaranayaka and Li-Chan, 2011). In the past years, protein hydrolysates with antioxidant activities are of great interest, as it is more cost- and time-saving to prepare, compared with purified antioxidant peptides (Lafarga and Hayes, 2014). In this respect, several recent studies have focused mainly on antioxidant activities of the whole hydrolysates from animal by-products (Damgaard et al., 2015; Damgaard et al., 2014). Antioxidant hydrolysates have been produced from porcine tissues through enzymatic hydrolysis from colon, appendix, rectum, pancreas, heart, liver, and lung (Damgaard et al., 2015). The relevant information on antioxidant hydrolysates and peptides derived from animal by-products are listed in Table 2.

Antioxidant activity can be monitored by a variety of assays with different mechanisms, including hydrogen atom transfer (HAT), single electron transfer (ET), reducing power and metal chelation (Shahidi and Zhong, 2015). The commonly used antioxidant assay include DPPH (2,2-Diphenyl-1-picrylhydrazyl), FRAP (ferric reducing antioxidant power), ORAC (oxygen radical absorbance capacity) and metal ion chelating ability assays (Shahidi and Zhong, 2015). In addition, some cell models have been applied to assess antioxidant activities *in vitro* against inducers of oxidative stress (e.g. H<sub>2</sub>O<sub>2</sub>). The main assays include measurement of cell proliferation, cycle arrest and apoptosis and cellular antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase (Shahidi and Zhong, 2015).

### DPP-IV Inhibitory Activity

Type 2 diabetes (T2D) is a chronic metabolic disorder characterized by hyperglycemia, insulin resistance and pancreatic beta-cell dysfunction (Patil et al., 2015). Inhibition of dipeptidyl peptidase-IV (DPP-IV) activity is one of the latest strategies for prevention and treatment of T2D (Nongonierma and FitzGerald, 2016). DPP-IV inhibitors can play a key role in regulation of glucose level through retarding degradation of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (Power et al., 2014). Enzymatic hydrolysis of animal by-product proteins can release DPP-IV inhibitory peptides (Table 3), such as ER and PPL from hemoglobin (Lafarga et al., 2016c) and PPG from bovine collagen (Lafarga et al., 2014). Furthermore, *in vivo* animal studies demonstrated that hydrolysates from porcine skin gelatin with DPP-IV inhibitory activities exhibited antidiabetic effects (Nongonierma and FitzGerald, 2016). For instance, ingestion of gelatin hydrolysates (300 mg per day) from porcine skin can improve glucose tolerance in streptozotocin-induced diabetic rats after 21 and 42 days (Huang et al., 2014).

### Antimicrobial Activity

Antimicrobial peptides (AMPs) are oligopeptides with the ability to inhibit growth of certain pathogen bacteria (Pellegrini, 2003). Compared with conventional antibiotics, AMPs can exhibit antimicrobial activity against a broad-spectrum of microorganisms, including gram-positive and gram-negative bacteria and fungi (Pellegrini, 2003). Overall, the antimicrobial mechanisms can be

categorized into the barrel-stave mechanism, the toroid pore or wormhole mechanism and the carpet mechanism (Guilhelmelli et al., 2013).

To date, antimicrobial peptides have been isolated and characterized from animal blood proteins. Daoud et al. (2005) isolated antimicrobial peptides from bovine hemoglobin through hydrolysis and the most active peptide sequences were characterized to be VTLSHLPSTFT PAVHASLDKFLANVSTVL. A recent study by Przybylski et al. (2016) revealed that an antimicrobial peptide (TSKYA) can serve as meat preservative (0.5%, w/w) to reduce the lipid oxidation and inhibit bacterial growth during 14-day refrigeration storage.

### The Other Bioactivities

Apart from bioactivities mentioned previously, hydrolysates/peptides from animal by-products have been reported to exhibit some other physiological functions. An iron-binding peptide derived from porcine plasma protein hydrolyzed by Flavourzyme was characterized to be DLGEQYFKG (Lee and Song, 2009). Recently, O'Sullivan et al. (2017) produced hydrolysates from  $\alpha/\beta$  globulin, serum albumin,  $\gamma$ -globulin and fibrinogen and confirmed their anti-cancer potentials against several cancer cell lines, such as lymphoma cells, breast cancer cells and epithelial colorectal adenocarcinoma cells. The high safe profiles combined with the bioactive potentials of hydrolysates/peptides from animal by-products makes it interesting to explore new bioactive potentials in future research activities.

### Future Perspectives

Currently, tons of land-based animal processing by-products are generated annually. From the perspectives of economic benefits, by-product management and sustainability, these protein-rich by-products are, undoubtedly, ideal precursors of hydrolysates/peptides with an enormous variety of bioactivities. In this regard, transforming by-products into valuable bio-functional ingredients, which improves individual health by alleviating the burden of metabolic diseases, will be achievable. In spite of their great potentials, there are scarce studies on *in vitro* and *in vivo* studies aiming to fully unlock and confirm bioactive potencies of animal protein-derived peptides.

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## Pancreas-Stimulating Foods: Cholecystokinin Enhancers

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### Glossary

**Anorexigenic** the ability of a compound to suppress appetite leading to reduced food intake.

**Cholecystokinin** a gastrointestinal hormone that stimulates the secretion of pancreatic enzymes and emptying of the gallbladder.

**Cholecystokinin receptors** a group of receptors on which cholecystokinin binds to activate signalling processes leading to appetite suppression.

**Cholecystokinetic** to cause the contraction and emptying of the gallbladder in order to release bile into the intestine.

**Gut hormones** a group of peptides secreted by the enteroendocrine cells of the gastrointestinal tract that regulate appetite and the digestion process.

**Orexigenic** the ability of a compound to stimulate appetite leading to increased food intake.

**Pancreozymic** to cause the release of pancreatic enzymes for food digestion.

### Gastrointestinal Processing of Food

Consumption of energy-dense food, digestion and energy metabolism are important contributors to the development of obesity. The mouth, stomach, small intestine and large intestine are major locations for food digestion, in addition to other contributors such as the salivary glands, liver, gallbladder, and pancreas. Bacteria present in the gastrointestinal tract, also known as the gut microbiota, also play an important role in this process. Digestion typically involves the interaction of ingested food components with digestive enzymes, bile salts, bacteria, and hormones (Wallace, 2013). In general, food intake is regulated through processes that are coordinated *via* the gut–brain axis. The gut hormones play significant roles in this process by activating different signalling pathways that control appetite (Cummings and Overduin, 2007; Morton et al., 2006; Sternini et al., 2008; Wren and Bloom, 2007). In fact, pharmacological approach for body weight and obesity management includes the alteration of the gut hormones, namely orexigenic (appetite-suppressing) ghrelin and anorexigenic (appetite-suppressing) cholecystokinin (CCK), glucagon-like peptide-1, pancreatic polypeptide, peptide YY, and oxyntomodulin (Hameed et al., 2009; Wren and Bloom, 2007). This article discusses the structure and roles of CCK, as well as the effects of foods and food-derived compounds in stimulating CCK secretion in the gastrointestinal tract and in controlling appetite.

### Cholecystokinin, a Gut Peptide Hormone

Cholecystokinin is a gut hormone that controls the secretion of pancreatic enzymes (pancreozymic action) for food digestion, and emptying of the gallbladder (cholecystokinetic action); the latter was discovered first, thus, the hormone was named on the basis of its gallbladder function (Liddle et al., 1985; Rehfeld, 2004). CCK is a part of the gut hormone triad, with secretin and gastrin, and functions as an endogenous anorectic, i.e. appetite-suppressing, factor (Rehfeld, 2004). The identification of the gut peptide depends on the number of amino acid residues that it contains. For instance, CCK-58 contains 58 amino acid residues in its structure (Reeve et al., 1996). The various structural forms, including bioactive CCK-5, CCK-8, CCK-22, CCK-33, CCK-39, CCK-58 and CCK-83, are derived from posttranslational modifications of preprocholecystokinin, a 115-amino acid residue CCK precursor, mostly by prohormone convertase activity (Chaudhri et al., 2006; Rehfeld, 2004). All CCK forms have the sequence -Tyr-Met-Gly-Trp-Met-Asp-Phe at their C-terminal, with  $\alpha$ -amidation at Phe and O-sulfation at Tyr residues. The latter modification is introduced by the activity of tyrosylprotein sulfotransferase and is important in determining the affinity of CCK for its cell surface receptors in the body (Rehfeld, 2004; Rehfeld et al., 2007).

The physiological activity of CCK starts when it binds its G-protein-coupled receptors, CCK1R and CCK2R, formally known as CCK-A and CCK-B receptors, respectively. CCK1R selectively binds sulphated CCK with high affinity and is responsible for pancreatic enzyme secretion and gastric emptying, whereas CCK2R is predominant in the brain and binds both sulphated and non-sulphated CCK (Rehfeld, 2004). Although produced by several gastrointestinal and neuronal cells, CCK is mainly secreted from enteroendocrine I cells located in the proximal regions of the duodenum and jejunum. In the small intestine, CCK dose-dependently stimulates physiological effects that lead to appetite suppression, which can lead to food intake reduction in humans (Larsson and Rehfeld, 1978; Tripathi et al., 2015; Zhang et al., 2015). Therefore, CCK and its receptors and signalling pathways have

become important physiological targets for exogenous factors intended for use in controlling body weight and obesity (Witkamp, 2011).

### Models for Studying the Effects of Food on CCK Secretion

Several studies have investigated the effects of food and its components on CCK secretion in healthy, overweight and obese human subjects (Bourdon et al., 2001; Feltrin, 2004; Pasman et al., 2008; Schwartz et al., 1994). Such studies typically measure plasma CCK levels in different subjects before and after treatments with single or multiple doses of the food component by direct ingestion, intake with meals, or by intra-duodenal infusion. In the studies, postprandial plasma CCK level serves as an indication of appetite status. However, a study involving humans that consumed high fat or high carbohydrate meals could not associate the increased postprandial CCK-8/-33(S) levels with satiety and satiation (Gibbons et al., 2016). This suggests that CCK may not be working independent of the other gut hormones in controlling appetite and food intake. Similarly, rodents have been used as models for studying the effects of food compounds on CCK secretion (Nishi et al., 2003, 2001; Sharara et al., 1993). Although evidence from *in vivo* studies is important for practical applications, it is challenging to determine which components of the food are bioactive and to elucidate effects on molecular signalling processes *in vivo*. In this instance, cultures of murine enteroendocrine cell lines and primary intestinal mucosal cells have been used to study the effects of food compounds on CCK-activated signalling pathways (Nishi et al., 2001; Choi et al., 2007; Pasman et al., 2008; Nakajima et al., 2010, 2011; Kim et al., 2013; Park et al., 2014; Al Shukor et al., 2016). Particularly, murine intestinal secretin tumour cell line (STC-1) has emerged as an ideal model for this type of study. STC-1 cell expresses similar features as regular enteroendocrine cells, including the expression of genes and proteins involved in CCK production, secretion of CCK, and CCK receptor-mediated signalling processes; for a detailed discussion, see McCarthy et al. (2015). The cellular model is important for studying molecular events that may have occurred *in vivo*, but would be insufficient if used as the sole means of establishing bioactivity of the food compounds.

### Effects of Food Compounds on CCK Secretion

Foods contain compounds that affect many physiological processes in the body, including biochemical pathways involved in energy metabolism. Therefore, some food-derived compounds can play a role in obesity management, for instance, *via* regulation of gut hormone secretion. In this case, food products can be strategically formulated to contain well-defined components that are capable of modulating appetite signalling processes (Prinz and Stengel, 2017; Serrano et al., 2017). CCK is secreted when dietary components reach the duodenum (Gribble, 2012). A number of foods and their components activate CCK secretion, and this is relevant for controlling appetite and body weight. For instance, consumption of spinach thylakoid, with high fat or high carbohydrate meals, dose-dependently elevated plasma CCK level in healthy and obese humans compared to control groups that did not consume the thylakoid (Roberts and Moreau, 2016). This activity was attributed to the thylakoid membrane content, which has multiple components, and was proposed to be responsible for the anti-obesity property of spinach. As discussed below, fatty acids, proteins, peptides, dietary fibres and phenolic compounds present in foods have particularly demonstrated CCK-secreting effects in humans, animals and cell cultures (Tables 1–3).

#### Free Fatty Acids

In humans, the ingestion of lipids, and subsequent presence of intraluminal lipids, stimulates the secretion of CCK. Although the mechanism of the interaction has yet to be clarified, the length and degree of unsaturation of the fatty acid chain affect the magnitude of the CCK response. Several studies demonstrated that only fatty acids with 12-carbon chain length or higher are able to trigger a significant increase in plasma CCK level (Barbera et al., 2000; Feltrin, 2004; Hopman et al., 1984; McLaughlin et al., 1999). Maher and Clegg (2018) suggested that the differential response could be because the digestion of medium-chain and short-chain fatty acids does not require emulsification with bile acids, which are secreted by CCK activity. Degree of unsaturation of fatty acids is also an important factor in triggering CCK release. Unsaturated fats, especially polyunsaturated fatty acids, significantly increase plasma CCK levels compared to saturated fatty acids (Shall et al., 1989; French et al., 2000; Pasman et al., 2008). Moreover, administering polyunsaturated fatty acids in the free fatty acid (FFA) form, rather than as a part of triglycerides (TG), led to a more pronounced effect on CCK secretion (Pasman et al., 2008). This could be because of the release of only two FFA during TG digestion, thus the administration of FFA would contribute to a higher amount of FFA in the gastrointestinal tract when compared to TG intake.

#### Proteins, Protein Hydrolysates and Peptides

There is ample evidence that intake of dietary proteins or their hydrolysates affects CCK secretion in cultured cells, animal models and humans. Plant-derived and synthetic trypsin-inhibitors have also shown stimulatory effects on CCK release. The compounds act indirectly by competitively inhibiting trypsin activity thereby preserving trypsin-sensitive CCK-releasing peptides in the lumen (Herzig, 1998). Studies with potato proteins, which have a high content of protease inhibitors, reported significant increases in CCK



**Table 1** Some examples of food-derived free fatty acids and their CCK-stimulating effects

Sample	Model	Dose	Treatment	Duration	Outcome	References
Long Chain Fatty Acids (LCFA)	Human (upper intestinal infusion in 7 healthy male volunteers, aged 18–35, with body mass index of 20–25)	One dose	5 duodenal infusions: (i) 0.9% saline (control), (ii) 20% Intralipid (mixture of linoleic (C18:2) and linolenic (C18:3) acid), (iii–V) 20% emulsions of oils enriched with stearic (C18:0), oleic (C18:1), linoleic (C18:2) acids	Rate of 1 mL/min over a 100 minutes period	All lipid infusion conditions significantly triggered CCK response; no differences between the modified FAs composition of the oils.	<a href="#">French et al., 2000</a>
Long Chain (LCT) vs. Medium Chain (MCT) Triglycerides	Human (ingestion in 6 healthy male volunteers, aged 22–38)	60 mM	Ingestion of 60 mM LCT (corn oil: 7% palmitic acid (C16:0), 3% stearic acid (C18:0), 44% oleic acid (C18:1), 39% linoleic acid (C18:2)) or MCT (Ceres-MTC-dietary oil: 58% octanoic acid (C8:0), 39% decanoic acid (C10:0)).	Single intake	Ingestion of LCT triggered significant increase in plasma CCK; ingestion of MCT did not trigger any significant increase in CCK.	<a href="#">Hopman et al., 1984</a>
Unsaturated vs. Saturated Fatty Acids	Human (6 healthy volunteers, aged 20–41, 1 woman and 5 men)	Five meals with at least one week between meals	Ingestion of five meals with different fat contents (2 meals containing 3.5 g pure FAs, 3 meals containing 30 g triglycerides)	Single intake	Unsaturated fats triggered a significantly higher CCK release than saturated fats; diunsaturated fatty acids showed higher CCK release than monounsaturated fatty acids.	<a href="#">Shall et al., 1989</a>
Medium (MCT) vs. Long (LCT) Chain Fatty Acids	Human (9 healthy subjects, aged 20–56, 3 women and 6 men)	Two doses on two different days	Duodenal infusion of saline followed by LCTs (26% oleic acid, 52% linoleic acid) and MCTs (56% octanoic acid, 40% decanoic acid) in a randomised order on two different days.	Rate of 1 mL/min	Significant increase in plasma levels of CCK after LCT infusion; no significant increase in plasma levels of CCK after MCT infusion.	<a href="#">Barbera et al., 2000</a>

(Continued)

**Table 1** Some examples of food-derived free fatty acids and their CCK-stimulating effects—cont'd

<i>Sample</i>	<i>Model</i>	<i>Dose</i>	<i>Treatment</i>	<i>Duration</i>	<i>Outcome</i>	<i>References</i>
C12 vs. C10 Fatty Acids	Human model (12 healthy male subjects, aged 19–47, normal body weight). 4 subjects studied in a nonrandomised study, 8 subjects in the randomised study.	Three doses separated by 3–10 days	Intraduodenal infusion of fatty acid solutions containing 5.3 g of FAs (lauric acid (C:12) or decanoic acid (C10:00)).	Rate of 2 mL/min for 90 min	Both C:12 and C:10 triggered increase in plasma CCK levels; C:12 increased CCK to a greater extent than C:10.	<a href="#">Feltrin, 2004</a>
C12 vs. C10 Fatty Acids	Human (9 healthy male subjects, aged 18–35)	Single dose	Infusion in the upper gut of the three test solutions (0.10 mol/L C10 or C12 emulsions, or vehicle solution)	2 minutes infusion	C12 significantly increased plasma CCK level; C10 did not have an effect on CCK.	<a href="#">McLaughlin et al., 1999</a>
Korean pine nut FAs, Italian stone pine nut FAs, oleic acid, linoleic acid, $\alpha$ -linolenic acid, capric acid	STC-1 cell culture model	Single dose	50 $\mu$ M FAs added to STC-cells, then media harvested after 60 min.	60 min incubation	Korean pine nut FAs triggered the highest CCK release (8 folds more potent than the others FAs tested).	<a href="#">Pasman et al., 2008</a>
Korean pine nut FFA, Korean pine nut TG	Human (18 female subjects, aged 54–56, post-menopausal, overweight, BMI 27).	Single dose	Gel capsules containing 3 g Korean pine nut FFA (15.2% pinolenic acid, 45.1% linoleic acid, 26.8% oleic acid, 7.2% saturated FAs), or 3 g Korean pine nut TG (15.3% pinolenic acid, 45.6% linoleic acid, 26.4% oleic acid, 6.9% saturated FAs), or 3 g live oil (0.7% linolenic acid, 5.6% linoleic acid, 77.4% oleic acid, 11.7% saturated FAs). Randomised, placebo-controlled, double-blind cross-over trial	Single intake	Pine nut FFA had a stronger effect than pine nut TG on CCK-8 release.	

**Table 2** Some examples of food proteins, protein hydrolysates and peptides, and their CCK-stimulating effects

Sample	Model	Dose	Treatment	Duration	Outcome	References
Protein POTII	Human (6 type II diabetic subjects, aged 34–62, five men and one woman)	Single administration	Oral ingestion of a glucose/protein solution (control) or glucose/protein solution with addition of 1.5 g POTII.		Significant increase in plasma CCK by POTII.	<a href="#">Schwartz et al., 1994</a>
Potato extract containing trypsin inhibitor proteins	STC-1 cells		Potato extract (Potein) and digested form were incubated with STC-cells for 60 minutes	60 minutes	Dose-dependent increase in CCK secretion in STC-cells supernatant; CCK releasing ability comparable to effect of positive control; digested sample triggered higher CCK response than the untreated.	<a href="#">Nakajima et al., 2011</a>
Proteins, protein hydrolysates, amino acids	Rat model	Single administration	5 mL of liquid containing the different formulations was fed to rats <i>via</i> orogastric tube		Intact proteins had strong stimulatory effect on CCK secretion; carbohydrates and amino acids did not trigger any CCK response.	<a href="#">Sharara et al., 1993</a>
Casein, soybean protein isolate (SPI), egg white, wheat gluten hydrolysates, SPI-like amino acid mixture	Rat intestinal mucosal cells	Single exposure	15 mL of hydrolysates containing solution was incubated 5 minutes with the mucosa	5 minutes	All hydrolysates stimulated CCK release in cells; SPI hydrolysates triggered the highest CCK response; SPI-like amino acids mixture did not trigger any CCK response.	<a href="#">Nishi et al., 2001</a>
Peptone and lysophosphatidic acid (LPA)	STC-1 cells		6.25–50 mg/mL peptones exposure for 6 hours	6 hours	Peptone activated GPR93, which in turn induced CCK release; peptone induced higher cAMP level (from GPR93 overexpression) than LPA.	<a href="#">Choi et al., 2007</a>
$\beta$ -51–63 peptide (from soybean $\beta$ -conglycinin)	Rat model	Single exposure	Intraduodenal infusion of 3 $\mu$ M hydrolysates (flow rate 0.5 mL/min for 5 minutes) followed by 1 hour of basal diet (15 g)	5 minutes	Intraduodenum infusion of $\beta$ -51–63 peptide significantly increased plasma CCK concentration.	<a href="#">Nishi et al., 2003</a>
$\beta$ -51–63 peptide (from soybean $\beta$ -conglycinin)	STC-1 cells	Single exposure	60 minutes exposure of STC-1 cells with 1.2 mM $\beta$ -51–63 peptide	60 minutes	Exposure to $\beta$ -51–63 peptide caused dose-dependent CCK secretion in STC-1 cells.	<a href="#">Nakajima et al., 2010</a>
0.5%–1% w/v Protein hydrolysates (meat, casein, soybean, ovalbumin)	STC-1 cells	Single exposure	STC-1 cells exposed to peptones for 2 hours	120 minutes	Protein hydrolysates caused dose-dependent increase in CCK release; peptone-induced CCK release was lost by the addition of extracellular and intracellular $\text{Ca}^{2+}$ chelating agents.	<a href="#">Némoz-Gaillard et al., 1998</a>

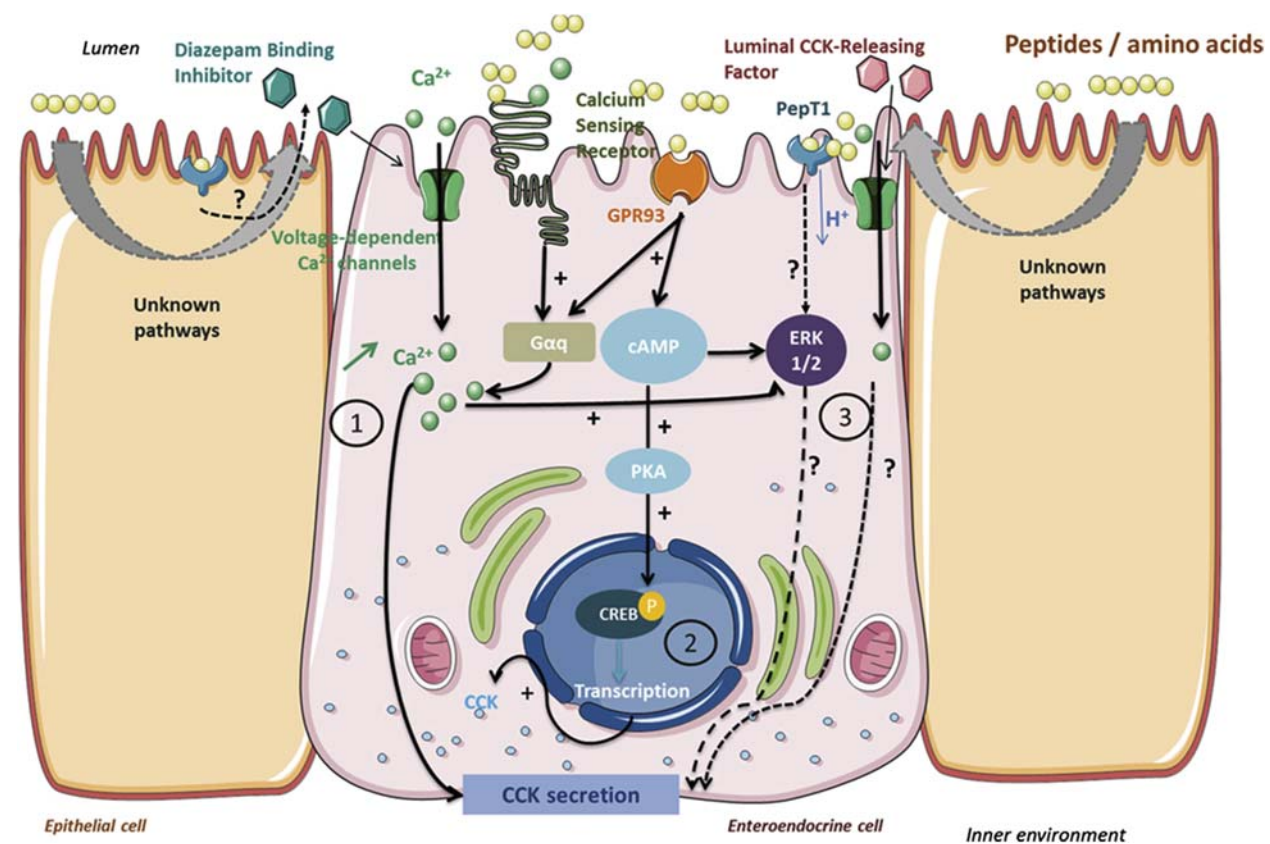
**Table 3** Some examples of dietary fibres and phenolic compounds, and their CCK-stimulating effects

<i>Sample</i>	<i>Model</i>	<i>Duration</i>	<i>Treatment</i>	<i>Dose</i>	<i>Outcome</i>	<i>References</i>
<b>Fibres</b>						
Guar Gum	Human, lean health men (n = 12)	1 week	Liquid Formula diet	27 g/L of fibre per day	Fasting plasma CCK level increased	<a href="#">Meier et al., 1993</a>
	Human, postmenopausal, obese women (n = 15)	5 weeks	800 kCal meal	20 g fibre per day	Postprandial plasma CCK level increased	<a href="#">Heini et al., 1998</a>
White Bean Flakes	Human, healthy men (n = 10)	Single administration	Meal containing 60 g white bean flakes	11.8 g	Postprandial plasma CCK level increased	<a href="#">Bourdon et al., 2001</a>
<b>Phenolic compounds</b>						
Hesperetin and hesperidin	Cell culture, STC-1 cell	1 hour	Cells were washed with HEPES buffer and incubated with hespetin and hespeidin at 37 °C for 60 min. CCK concentrations measured using commercial EIA kit.	0.1 mM, 0.5 mM, 1 mM	Hesperetin significantly and dose-dependently increased CCK secretion; hesperidin (glycoside) showed no effect on CCK release.	<a href="#">Kim et al., 2013</a>
Naringenin and naringin	Cell culture, STC-1 cells	1 hour	Cells incubated with naringenin and naringin at 37 °C for 60 min. CCK concentrations measured using commercial EIA kit.	0.1 mM, 0.5 mM, 1 mM	Naringenin significantly increased CCK secretion; naringin (glycoside) did not show any significant effect on CCK secretion.	<a href="#">Park et al., 2014</a>
Quercetin, kaempferol, apigenin, rutin and baicalein	Cell culture, STC 1-cells	2 hours	Incubation of cells with flavonoid samples at 37 °C. CCK measured by RIA.	20 µM	Significant increase in CCK secretion by quercetin, kaempferol and apigenin; rutin showed a lower activity compared to its aglycone, quercetin.	<a href="#">Al Shukor et al., 2016</a>

release in humans and cellular model as a result of the treatments (Nakajima et al., 2011; Schwartz et al., 1994). Gastrointestinal digestion is regarded as a major step in the CCK stimulatory effect of dietary proteins. This was confirmed by the observation that only intact or moderately hydrolysed proteins triggered a notable CCK response, while extensively ( $\geq 60\%$ ) hydrolysed proteins, or the corresponding amino acid mixtures, did not cause any effect on CCK level (Sharara et al., 1993; Nishi et al., 1999, 2001). The digestion process determines the release of bioactive peptides in the lumen, where they can interact with the brush border barrier and stimulate gut hormone release. A number of peptide-cell membrane interactions can lead to increase in CCK secretion, as shown in Fig. 1. Dietary peptides could trigger the release of CCK directly (Nakajima et al., 2010), or through intermediate elements such as luminal CCK-releasing factor (LCRF), calcium-sensing receptor (CaSR) or G-protein coupled (GPCR) receptors, and calcium ion channels (Caron et al., 2017; Némóz-Gaillard et al., 1998). Moreover, peptides are also capable of influencing CCK gene transcription, by stimulating membrane elements such as GPR93, which in turn induces increase in cAMP level and activation of pathways involved in CCK expression and secretion, including the extracellular signal-regulated protein kinases 1 and 2, phosphokinase A, and calmodulin-dependent protein-kinase signalling pathways (Caron et al., 2017; Choi et al., 2007). Although the mechanism is not clear yet, dietary arginine-containing peptides are also important stimulators of CCK secretion (Nishi et al., 2003, 2001). Furthermore, proteolysis occurs during food processing, e.g. fermentation, meat ripening and exogenous protease treatments (Udenigwe and Aluko, 2012), and it is possible to produce appetite-suppressing peptides as a result. There is also an opportunity in exploring if some food-derived peptides are structurally similar to CCK1R binding motif of CCK, and if these peptides can act as agonists for appetite stimulation.

### Dietary Fibres

Several studies have established the effects of dietary fibres on appetite, satiety and gastrointestinal functions (Anderson, 2009; Haber et al., 1977; Poppitt et al., 2002). Although the mechanism of action is not well established yet, the intake of some dietary fibres can elevate and sustain postprandial plasma CCK levels. In a study by Meier et al. (1993), the addition of 27 g/L guar gum to a liquid formula diet consumed by healthy men led to an increase in fasting plasma CCK level and a decrease in colonic transit time.



**Figure 1** An overview of intestinal signalling pathways activated by food peptides for cholecystokinin (CCK) secretion. The activation of calcium-sensing receptor or G protein-coupled receptor (GPR93) by protein hydrolysates results in an increase of intracellular calcium level, initiation of calcium-mediated signalling and CCK gene transcription, leading to CCK synthesis and secretion. Figure reprinted from Caron, et al., 2017. Protein digestion-derived peptides and the peripheral regulation of food intake. *Front. Endocrinol.* 8, 85. <https://doi.org/10.3389/fendo.2017.00085>, © 2017 Caron, Domenger, Dhulster, Ravallec and Cudennec.

A study with obese women supported this observation, as the consumption of high-fibre, low-calorie diet resulted in an elevated postprandial plasma CCK level (Heini et al., 1998). Moreover, a study on the effect of low vs. high fibre meals reported a significant increase in postprandial CCK after ingestion of meals containing 11.8 g of bean flakes (Bourdon et al., 2001). It was also found that plasma CCK remained above the baseline level for a longer duration (4 h) after the intake of the bean flake meal in comparison to 180 min recorded after low fibre meal intake (Bourdon et al., 2001). According to the authors, the physiological responses associated with dietary fibres, such as slower colonic transit time and lower absorption efficiency in the small intestine, are possibly responsible for the prolonged CCK secretion. There is also the potential activity of other components of the flakes, including phytochemicals, as discussed below.

### Phenolic Compounds

Foods rich in phenolic compounds have demonstrated anti-obesity effects in animal and human studies (Chan et al., 1999; Lai et al., 2015; Mohamed et al., 2014). For this reason, several studies have investigated the interaction of plant-derived phenolic compounds with appetite-regulating intestinal hormones, including CCK. For example, the release of CCK from cultured STC-1 cells was elevated in the presence of flavonoids, hesperetin or 3',5,7-trihydroxy-4'-methoxyflavanone (Kim et al., 2013), naringenin or 4',5,7-trihydroxyflavanone (Park et al., 2014), and quercetin or 5,7,3',4'-flavon-3-ol (Al Shukor et al., 2016). In contrast, their respective glycosides, hesperidin, naringin and rutin, did not stimulate CCK secretion in the cells. Although the flavonoids are mainly found in their glycoside forms, they are enzymatically converted into their aglycone forms (hesperetin, naringenin and quercetin) in the gastrointestinal lumen (Li et al., 2010), prior to their transport to the enteroendocrine cells. Other flavonoids, kaempferol and apigenin, increased CCK release in STC-1 cells, but no activity was observed for baicalein (Al Shukor et al., 2016). Interestingly, baicalein is the only aglycone without a B-ring hydroxyl group, of all the flavonoids studied. This underscores the relevance of B ring substituents in determining the CCK releasing activity of phenolic compounds. Detailed mechanisms of bioactivity is still scanty. A proposed mechanism involves the activation of transient receptor potential (TRP) ankyrin 1 channels (TRPA1), which are non-selective cation channels, leading to an influx of extracellular calcium ions into the enteroendocrine cells and subsequent dose-dependent increase of CCK release (Kim et al., 2013; Park et al., 2014). Activation of TRP and TRPA1 channels increases intracellular calcium level, which mediates the release of CCK (Purhonen et al., 2008). This mechanism was confirmed in the studies by removing calcium ions from the media, and in the presence of TRP and TRPA1 antagonists, which resulted in inhibition of the hesperetin- and naringenin-induced CCK releasing effects (Kim et al., 2013; Park et al., 2014).

In addition to direct effect on CCK secretion, there is interest in investigating compounds that can mimic CCK in binding their receptors and activating the appetite-suppressing signalling processes. On the basis of this approach, eight food-derived phenolic compounds, including tannic acid, gallic acid, benzoic acid, hydroxybenzoic acid, protocatechuic acid, quercetin, kaempferol and resveratrol, were found to have no effect in activating the CCK1R signalling pathway in cultured CHO epithelial cells (Al Shukor et al., 2014). In fact, the compounds had anti-CCK1R activities. In the study, CCK sequestration occurred due to its interaction with one of the compounds, tannic acid, and this could be responsible for the antagonistic effect. If translated *in vivo*, the anti-CCK1R effect of the compounds can lead to a loss of the appetite-suppressing capability of CCK. Considering the lack of effect on CCK1R pathway activation, it is possible that the aforementioned mechanisms, and the other satiety hormones, receptors and pathways play a role in mediating the satiety-inducing effects of polyphenol-rich foods.

### Concluding Remarks

CCK plays a major role in appetite and food intake control, and an increase in its secretion from enteroendocrine cells is an important factor in energy metabolism. This article highlights some examples of the effects of food components on CCK release based on cellular, animal and human studies. Proteins and fatty acids are the most potent dietary CCK stimulants, and their structures are the major determinants of bioactivity. The processes triggered by polyphenols leading to CCK secretion in cells could explain, at least in part, the anti-obesity activity of polyphenol-rich foods. There is however limited information on the molecular mechanisms of action and structure-function relationships, especially for dietary fibres. Future studies need to investigate the CCK-stimulating effects of the various food components when consumed as food products, as the beneficial compounds can become more or less bioaccessible in the food matrix thereby influencing their bioactivity.

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# Bioactives From Spices and Herbs

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## Introduction

For centuries, spices and herbs are not only excellent sources of flavors and food colors but are also excellent sources of bioactive compounds. The term bioactive came from the phrase biologically active, meaning the compound has a positive effect on living organisms, tissues or cells. Bioactive molecules are food substances that play an important role in human health and growth, biochemical reactions, mechanisms and development and have proven health benefits. They also amend disease risks by alleviating these conditions. The National Institutes of Health (NIH) defines bioactive food components as “constituents in foods or dietary supplements, other than those needed to meet basic human nutritional needs, which are responsible for changes in health status”. Bioactives are present in foods in small quantities and they occur naturally in both plants and animals in low concentrations. The major sources of bioactive food components are plants and many come from spices and herbs. Some examples of bioactive substances are phytosterols, lutein, lycopene, fatty acids and peptides. Bioactives also include antioxidants such as polyphenols. Antioxidants protect the human body from oxidation brought about by free radicals, superoxide, oxygen radicals and other substances that trigger oxidation. Some bioactive compounds such as piperine can enhance bioavailability of other bioactives or nutritional substances. Black pepper is a good source of piperine. Piperine and capsaicin belong to the vanilloid family of compounds, which provides the pungent taste in foods. Piperine also has putative anti-inflammatory activity and may be active at promoting digestive processes. This chapter will provide the recent advances in bioactives derived from spices and herbs, their chemistry and mechanisms of action. It will also include the current and emerging extraction technologies employed for the extraction of bioactives from spices and herbs.

## Spices and Herbs

Spices and herbs are plant materials that have been used for thousands of years as food flavorings and as sources of substances for medicinal purposes. In fact, spices established and enriched empires centuries ago due to their functional properties. Spices were an important part of the ancient commercial trade before the 15<sup>th</sup>-century. The spice trade was monopolized by the middlemen from the Middle East and North Africa for more than 5000 years and at the time, they protected and secured their valuable Asian sources of spices. These middlemen became very rich from the spice trade, which was used for flavoring food, as preservatives for meat, in making perfume and as sources of substances for traditional medicine. For this reason, European countries tried ways of finding new routes to the sources of spices, Spain being one of them, which set forth voyages to find new paths for sources of spices. During that time, the spices were very expensive. In Europe, a pound of nutmeg was believed to be more valuable than gold. Spices were among the most valuable commodities in the ancient as well as medieval times. The search for spices from the East by European explorers such as Ferdinand Magellan, Vasco da Gama, Bartholomeu Dias, and Christopher Columbus led to the great Age of Exploration and the discovery of the New World. One of the successful voyages was led by Da Gama who found the route to India through the tip of Africa and returned from his voyage with nutmegs, cinnamon, ginger and peppercorns for the king of Portugal, King Manuel. During this period, the dominance of European countries came about and the monopolies of the Portuguese, Dutch, French, Spanish and English were established in various parts of the spice trades. This started and enriched the European empires.

Spices and herbs are harvested from different parts of the plant. Herbs are the leafy materials while spices are the seeds, bark, fruit/berries, pods and flowers of the plant. [Table 1](#) summarizes what parts of the plant the spices come from. Saffron, vanilla and cardamom are the most expensive spices in the world. [Table 2](#) shows the global primary and secondary producers of spices. India is the main source of spices followed by Indonesia then China. For the herbs, [Table 3](#) summarizes the sources of herbs in the world.

## Chemistry of Bioactives From Spices and Herbs

Bioactives are secondary metabolites that perform important functions in plants. Bioactives are produced in the plants to function as protectants, attractants or signaling compounds. The flavonoids are examples of bioactives that protect the plant from free radicals generated during photosynthesis. Alkaloids on the other hand fend off insect attacks while terpenoids attract pollinators. Plants produce these substances to defend themselves against various agents in the environment for their survival and for adaption. The roles of phenolic compounds and flavonoids include structural functions in different supporting or protective tissues, involvement in defense strategies, as attractants for pollinators and seed-dispersing animals, as allelopathic agents, ultra violet (UV) protectants and signal molecules in the interactions between plants and their environment ([Jaganath and Crozier, 2010](#)). There are other functions of bioactives in plants, which have yet to be discovered or explained.

**Table 1** Sources of spices and herbs

<i>Part of the plant</i>	<i>Spice/Herb</i>
Leaves	Basil, oregano, bay leaf, thyme, tarragon
Bark	Cinnamon, cassia
Seed	Fennel, fenugreek, dill mustard
Flower/bud, pistil	Clove, saffron
Fruits/berries	Clove, chilli, black pepper, allspice
Bulbs	Onion, garlic, leek
Root	Ginger, turmeric
Aril	Mace

**Table 2** Main production areas of spices in the world

<i>Spice</i>	<i>Largest producer</i>	<i>Second largest producer</i>
Cardamom	Guatemala	India
Ginger	India	China
Turmeric	India	Pakistan
Chili pepper	India	China
Cumin	India	Turkey
Vanilla	Indonesia	Madagascar
Nutmeg	Indonesia	Grenada
Cinnamon	Indonesia	China
Cloves	Indonesia	Madagascar
Saffron	Iran	Spain
Pepper	Vietnam	India

Source: <http://www.rosemaryinstitute.com/general-knowledge/2669-general-knowledge-list-of-largest-producing-countries-of-agricultural-commodities>

**Table 3** Main world production areas of herbs

<i>Country</i>	<i>Herbs</i>	<i>Volume of main traded products (metric tons)</i>
Egypt	Basil	4.8
	Marjoram	3.8
	Parsley	—
	Dill	—
	Cilantro	—
	Spearmint	—
	Peppermint	—
Morocco	Thyme	4.6
	Rosemary	5.8
Turkey	Oregano	12.0
	Sage	3.9
	Laurel	9.0
Albania	Sage officinalis	2.2

Source: 11th World Spice Congress Herbs Market Report (2012).

The main groups of plant bioactive compounds are classified based on their functions, molecular structures or sources. One classification includes the following:

*Glycosides* - secondary metabolites bound to a carbohydrate, mono- or oligosaccharide or to uronic acid) – cardiac glycosides, cyanogenic glycosides, glucosinolates, saponins and anthraquinone glycosides;

*Flavonoids and proanthocyanidins* – pigment containing substances with phenol groups;

*Tannins* – large polymers of flavonoids and hydrolysable tannins;

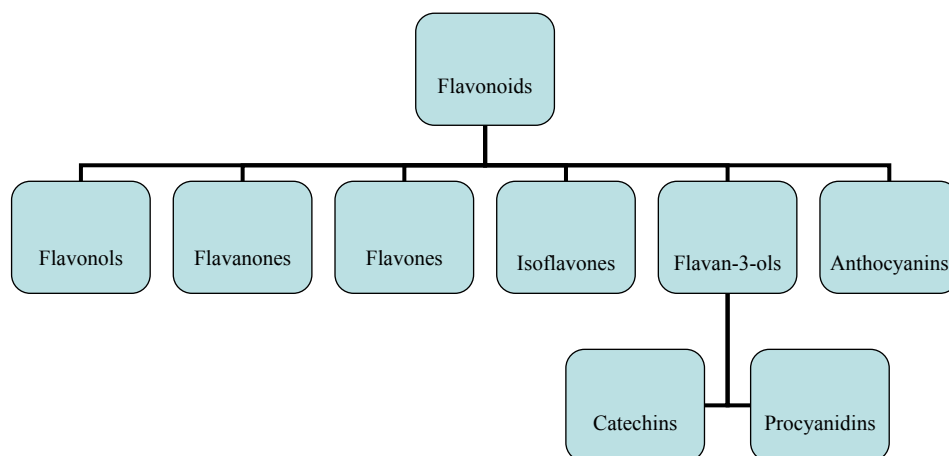
*Mono- and sesqui-terpenoids and phenylpropanoids* – synthesized from 5-carbon building blocks isoprene;

*Diterpenoids* – composed of 4 isoprene units and are hydrophobic in nature;

*Resins* – complex lipid-soluble mixtures that may consist of both diterpenoid and triterpenoids and mono- and sesquiterpenoids;

*Lignans* – lipophilic phenylpropanoids that form an 18-carbon skeleton, which have special functions in the plant cell membranes;

*Alkaloids* – heterocyclic compounds which contain nitrogen



**Figure 1** Different classes of flavonoids.

Fig. 1 shows the categories of flavonoids while Fig. 2 illustrates their basic structures. The chemical structures of bioactives of commonly used spices are shown in Fig. 3, illustrating the complexity and common features of the different structures that can be correlated to their functions. There are more than 6000 different flavonoids that have been described in literature and this list of bioactives continues to grow (Harborne and Williams, 2000) as new ones are discovered from different plant materials. Table 4 summarizes the different groupings that are important in spices and herbs, examples of bioactive compound from each group and spices/herbs sources. Tables 5 and 6 show a summary of the diverse health benefits derived from spices and herbs bioactives that includes blocking low-density lipoproteins and cholesterol, antioxidant activity, anticancer, improving cardiovascular functions, joint and digestive health as well as strengthening the immunity system. It is of great benefit to include bioactives particularly from spices and herbs as an essential part of our daily diet due to the immeasurable and considerable health benefits they provide.

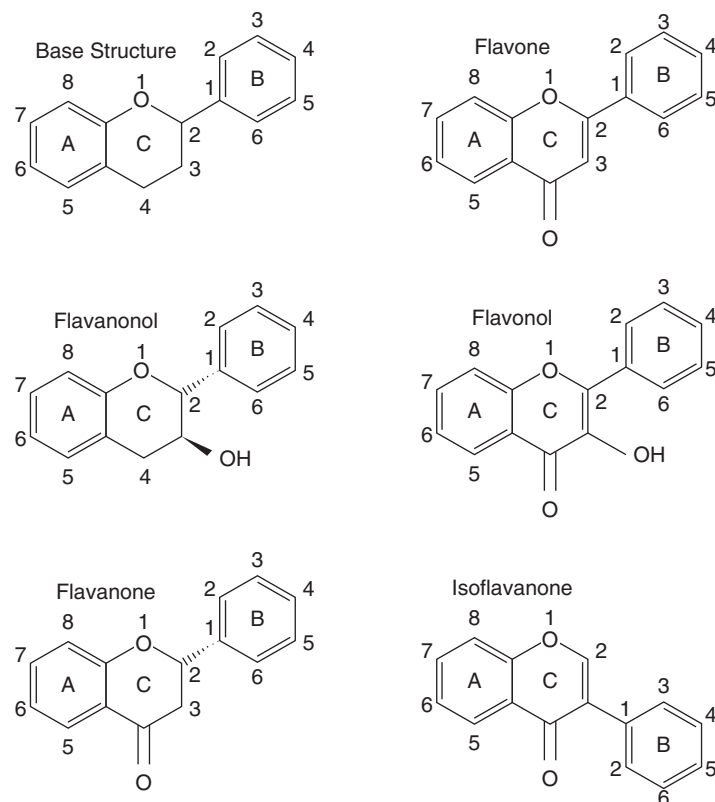
## Bioactives From Spices and Herbs for Health and Wellness - Mechanism of Action

The bioactives from spices and herbs have been studied for their efficacy in ameliorating health and wellness in man by modulating human metabolism in a manner favorable for the prevention or reduction in the risk of degenerative diseases such as cardiovascular diseases, diabetes, obesity and cancer (Anderson et al., 1999). The impact of bioactives on health from selected spices and herbs are discussed below.

### Black Pepper

Black pepper (*Piper nigrum* L.) contains a bioactive phytochemical, piperine (Fig. 4), that exhibits many physiological and pharmacological properties including antioxidant, anti-inflammatory, antimutagenic, antitumor, antiapoptotic, antigenotoxic, antiarthritic, antifungal, antidepressant, anti-HVB (hepatitis B) and gastro-protective activities. Piperine also enhances the bioavailability of phytochemicals (e.g., curcumin) and drugs. Piperine inhibits free radicals and reactive oxygen species and lipid peroxidation due to their flavonoids and phenolic contents. The antioxidant activity of black pepper was evaluated through the determination of glutathione peroxidase, catalase, superoxide dismutase including measurement of reduced glutathione content and the amount of malondialdehyde, and protein carbonyl levels in the hippocampus.

Table 7 summarizes the bioactivities and pharmacological effects of piperine from black pepper including the researchers who conducted these studies. Black pepper (*P. nigrum* L.) is a very widely used spice and is known for its pungent taste and aroma. It is also widely known that black pepper has important bioactive and preservative properties. Piperine has been shown to affect enzymes that bring about various effects such as chemoprevention, detoxification, and enhancement of the absorption and bioavailability of nutrients and drugs. It has also been shown based on modern cell, animal, and human studies that piperine has immunomodulatory, antioxidant, anticarcinogenic, and anti-inflammatory properties (Bang et al., 2009). The chemical structure of piperine has the formula of C<sub>17</sub>H<sub>19</sub>NO<sub>3</sub> and the IUPAC name 1-(5-[1,3-benzodioxol-5-yl]-1-oxo-2,4-pentadienyl). It is also known as piperoylpiperidine. It is a very weak base that hydrolyzes to piperidine and piperic acid (Pruthi, 1999; Agarwal, 2010). 1-Piperoylpiperidine (piperine) exists as 4 isomeric structures: piperine (trans-trans isomer), isopiperine (cis-trans isomer), chavicine (cis-cis isomer), and isochavicine (trans-cis isomer) as shown in Fig. 5. The 3 geometric isomers of piperine have almost no pungency (Ravindran, 2003). Since piperine constitutes about 98% of the total alkaloids in black pepper (Hirasa and Takemasa, 1998), it is considered as the main contributor of pungency. Thus piperine content is used as a measure of total pungency of black pepper (Parthasarathy et al., 2008).



**Figure 2** Basic structures of flavonoids.

## Cinnamon

There are four main species of cinnamon that are used for food. These are *Cinnamomum cassia* (cassia or Chinese cinnamon), *Curcuma burmanni* (Korintje, Padang cassia or Indonesian cinnamon), *Curcuma loureiroi* (Saigon cinnamon, Vietnam cassia or Vietnamese cinnamon) and *Curcuma verum* also known as *Curcuma zeylanicum* (Sri Lanka cinnamon or Ceylon cinnamon). The differences between species of cinnamon are shown in [Table 8](#). The composition, antioxidant properties and essential oil components of cinnamon are presented in [Tables 9](#) and [10](#).

Cinnamon has shown potent antioxidant activity ([Table 9](#)), can lower blood sugar, and inhibit inflammation and lower cholesterol and triglycerides in the blood. The antioxidant activity of cinnamon is due to their bioactive components namely the polyphenols such as flavonoids, phenolic acids, lignans, essential oils and alkaloids ([Fig. 6](#)). Eugenol, limonene, terpineol, catechins, proanthocyanidins, tannins, linalool, saffrole, pinene, methyl eugenol and benzaldehyde are some of the bioactive compounds from cinnamon.

The effect of cinnamon bioactive compounds to mitigate diabetes through various mechanisms has been under investigation for more than 15 years. [Peng et al. \(2010\)](#) investigated the inhibitory effects of proanthocyanidins, catechin, epicatechin and procyanidin B2 from cinnamon bark on the formation of specific advanced glycation end products (AGEs). The cinnamon polyphenols displayed inhibitory effects on these AGEs which were attributed to their antioxidant activities and carbonyl scavenging properties ([Peng et al., 2010](#)). AGEs accumulation has been implicated as a major pathogenic process in diabetic complications and other disorders such as atherosclerosis and Alzheimer's disease ([Peng et al., 2010](#)). According to [Peng et al. \(2010\)](#) the cinnamon polymers were composed of monomeric units with a molecular mass of 288. They isolated two trimers with a molecular mass of 864 and a tetramer with a mass of 1152.

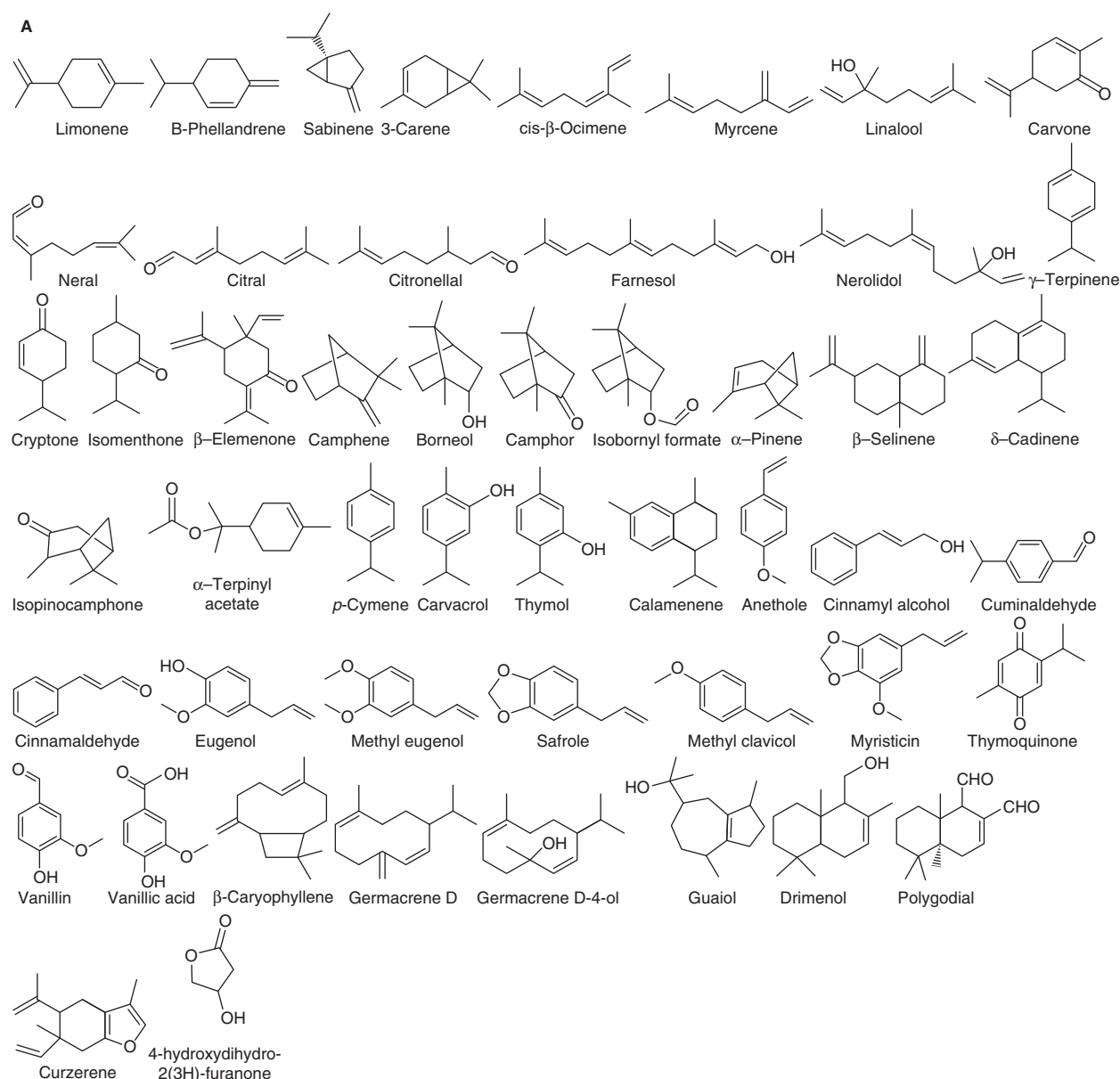
[Anderson et al. \(2004\)](#) isolated and characterized the insulin-enhancing complexes from cinnamon. They found that polyphenolic polymers are A type doubly linked procyanidin oligomers of catechins and/or epicatechins that may function as antioxidants and potentiate insulin action ([Anderson et al., 2004](#)). The cinnamon polyphenolic polymer may be beneficial in the control of glucose intolerance and diabetes ([Anderson et al., 2004](#)).

Research works have agreed that dietary factors are involved in the regulation and prevention of type 2 diabetes. A number of publications came to similar conclusions that cinnamon and its bioactive components act as antioxidants and potentiate insulin action. A summary of recent research works on cinnamon illustrating their bioactive roles in mitigating diseases is shown in [Table 11](#).



## Turmeric

Turmeric (*Curcuma longa*) belongs to the ginger family and is widely grown and used in various parts of the world particularly India, Bangladesh, China, Thailand, Cambodia, Malaysia, Indonesia and the Philippines. It has been used for centuries in India and China for treatments of infection, skin ailments, stress and depression. The main bioactive in turmeric is curcumin, the orange-yellow colored lipophilic polyphenol that is responsible for its antioxidant, anti-inflammatory and anticancer effects. It has been determined that diet will alleviate certain diseases and food is considered as a major potential source of cancer chemopreventive agents, curcumin being one of them (Fig. 7) through the modulation of inflammatory pathways. It has also been shown that at the molecular level, this multi-targeted agent exhibited anti-inflammatory activity through the suppression of numerous cell signaling pathways including NF- $\kappa$ B, STAT3, Nrf2, ROS and COX-2 (Kunnumakkara et al., 2017). The anti-inflammatory and antioxidant effects have been attributed to the hydroxyl and methoxy groups of curcumin (Deogade and Ghate, 2015; Rahman and Biswas, 2009), which includes down-regulating enzymes of inducible synthase (iNOS), cyclo-oxygenase-2 (COX-2), lipoxygenase and xanthine oxidase activity. Furthermore, curcumin has shown its effectiveness by inhibiting inflammatory cell proliferation, metastasis and angiogenesis through various molecular targets (Shehzad et al., 2013). Curcumin has been found



**Figure 3** Chemical structure of bioactive compounds of commonly used spices (A) volatile compounds (B) nonvolatile compounds. Source: Gottardi, D., Bukvicki, D., Prasad, S., Tyagi, A.K., 2016. Beneficial effects of spices in food preservation and safety. Front. Microbiol. 7, 1394 (Open access article). <https://doi.org/10.3389/fmicb.2016.01394>.

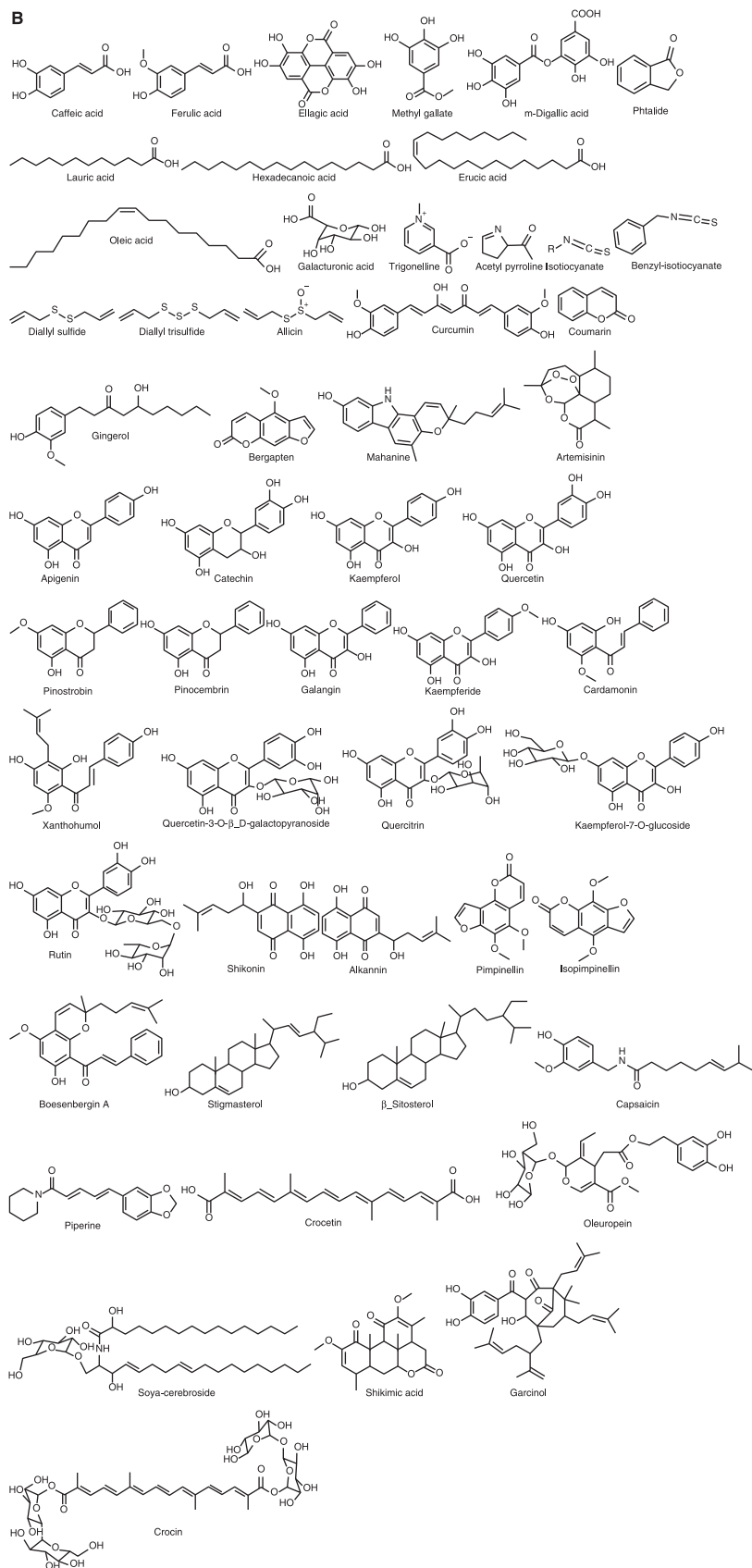


Figure 3 (continued).

**Table 4** Classification of bioactive compounds

Structural classes	Examples	Spices and herbs sources
<b>Flavonoids</b>		
Flavanols	Quercetin, quercetin-3-O-rutinoside, myricetin, kaempferol, isorhamnetin, gingerol, myricetin, quercetin, kaempferol, nobiletin, scutellarein, sinensetin, tangeretin, isoorientin, orientin	Onion, ginger, Celery, parsley, lemon grass, onion leaves, bird chili, bell pepper, fenugreek seed, peppermint, oregano, rosemary, sage
Flavones	Apigenin, luteolin, chrysin, luteolin, diosmetin	Parsley, thyme
Flavanones	Eriodictyol, hesperetin, naringenin	Cumin, peppermint
Flavan-3-ols	Catechin, epicatechin, procyanidins, gallic acid esters of catechins, epicatechins and gallic acid esters of epicatechins, theaflavins and gallic acid esters of theaflavins, thearubigins	Mint, basil, rosemary, sage, dill
Anthocyanidins and anthocyanins	Cyanidin, delphinidin, petunidin, peonidin, malvidin,	Red onions
<b>Nonflavonoid phenolic compounds</b>	Gallic acid, <i>p</i> -hydroxybenzoic acid, procatechuic acid, vanillic acid, syringic acid, ellagic acid, rosmarinic acid, tannic acid, gallic acid	Cinnamon, clove, anise, dill, fennel, caraway, parsley
Phenolic acids	<i>p</i> -coumaric, caffeic, ferulic, chlorogenic acid, curcuminoids, curcumin	Ginger, cardamon, turmeric
Hydroxycinnammates		
Carotenoids	$\beta$ -Carotene, lycopene, lutein, zeaxanthin	Cayenne pepper, chili pepper
Organosulfides, indoles, glucosinolates/sulfur compounds	Sulphoraphane, allyl methyl trisulfide, diallyl sulfide, indol-3-carbinol, sulforaphane, sinigrin, allicin, alliin, allyl isothiocyanate, piperine	Garlic, onions, leeks, chives, shallots, horseradish, mustard, wasabi, black pepper, mustard green
Terpenes	Pinene, myrcene, linalool, caryophyllene	Parsley, basil, rosemary, thyme, lemon grass, lavender, clove, rosemary, black pepper

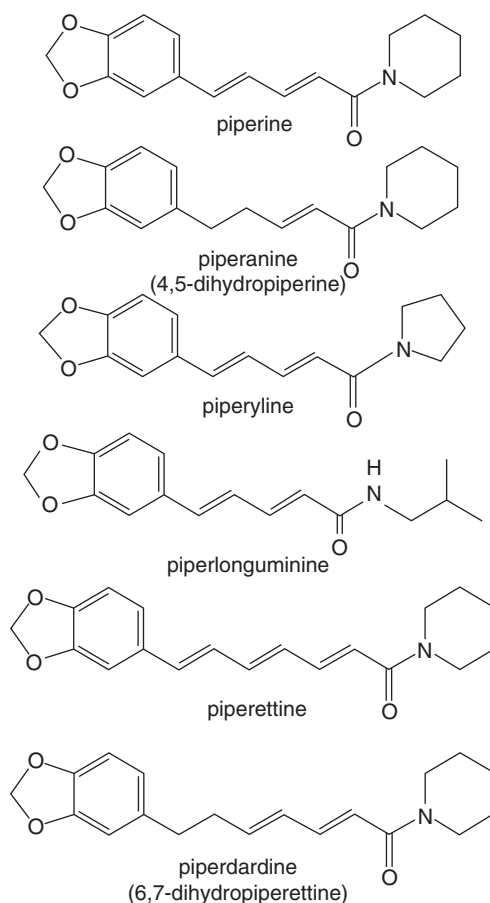
**Table 5** Classification of bioactives from spices and herbs and their associated health benefits

Class	Examples	Food source	Health benefit	References
Carotenoids	Pro-vitamin A carotenoids: $\alpha$ -carotene, $\beta$ -carotene, $\beta$ -cryptoxanthin Non-provitamin A carotenoids: lycopene, lutein, zeaxanthin, astaxanthin	Yellow and red peppers	Antioxidant, antioxidants trapping free radicals, source of Vitamin A, enhance functioning of immune system, help reproductive system properly function, antiproliferative, anticancer, prevention of cardiovascular disease, maintains healthy eyes, prevention of colon cancer, prevention of macular degeneration	Rao and Agarwal (2000) Seo et al. (2005) Burri (2000) Delgado-Vargas et al. (2000) Handelman (2001) Krinsky (2001) Wang and Bohn (2012) Ottaway (2008) Scheerens (2001) Watson and Preedy (2012) Young and Lowe (2001)
Polyphenols	Phenolic acids, anthocyanins, flavonols, flavones, flavonoids, flavanones, isoflavones, flavonones, catechin, epicatechin	Spices and herbs	Antioxidant and cardiovascular benefits, lipid-lowering, immunomodulator, anticancer antiestrogen, anti-osteoporotic, antiproliferative, lower risk of heart attack and stroke, anticarcinogenic activity, inhibit atherosclerosis, anti-inflammatory	Scheerens (2001) Manach et al. (2004) Liu (2003) Pan et al. (2009)
Organosuplhur compounds	Allicin., diallyl sulphide, diallyl disulphide, diallyl trisulphide	Garlic, onion, leek, chive, scallion, shallot	Cholesterol lowering, anti-inflammatory, improved liver function, improved immunity, antimicrobial effect	Tapsell et al. (2006) Block et al. (1992) Block et al. (1993a) Block et al. (1993b) Block and Thiruvazhi (1993) Block (1994) Scheerens (2001)

to have positive effect against many diseases such as cancer as it has been determined to suppress transformation, beginning, development and invasion of tumor, angiogenesis and metastasis (Kocaadam and Sanlier, 2017). It has been shown by extensive evaluation and preclinical tests that curcumin is a strong antioxidant and it suppresses activation of NF $\kappa$ B and other molecules that are involved in carcinogenesis (Kocaadam and Sanlier, 2017). Curcumin also prevents cancer cell proliferation and exhibits anticancer potential (Prasad et al., 2017).

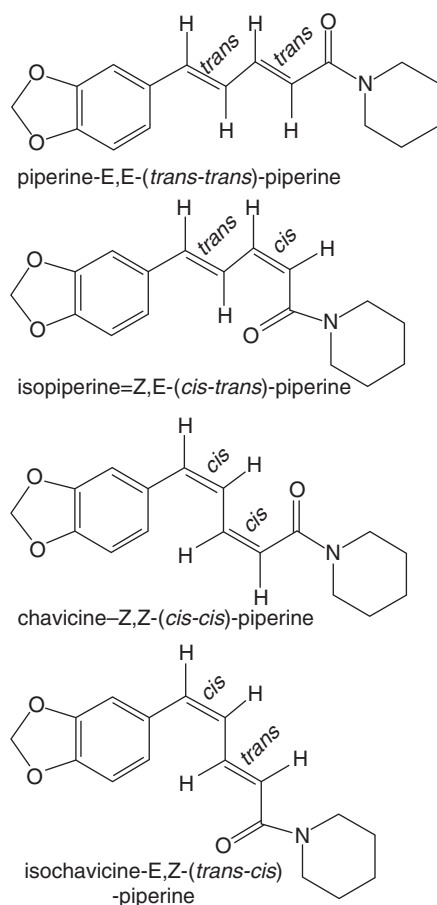
**Table 6** Bioactive functions of selected spices and herbs

Spice/Herb	Effect/function
Cayenne pepper	Capsaicin in cayenne pepper has been shown to reduce appetite and increase fat burning (weight management) Help combat lung, liver and prostate cancer
Cinnamon	Potent antioxidant activity Help fight inflammation Lower cholesterol and triglycerides in the blood
Fenugreek	Improve function of insulin Lower blood sugar levels
Garlic	Help combat sickness and cold Reduce cholesterol and LDL Help improve heart health
Ginger	Reduce blood pressure in people who are hypertensive Treat nausea caused by morning and sea sickness, and chemotherapy Strong anti-inflammatory Help pain management
Rosemary	Prevent allergies and nasal congestion Strong antioxidant
Sage	Improve brain function and memory Inhibits breakdown of acetylcholine, a chemical messenger in the brain
Turmeric	Powerful antioxidant and help fight antioxidant damage in the body Strong anti-inflammatory Fight Alzheimer's
Vanilla	Reduce the risk of heart disease and cancer Antioxidant activity Anti-inflammation Potential for lowering blood cholesterol

**Figure 4** Structures of piperamides found in commercial whole and ground peppercorns. Reprinted with permission from Friedman, M., et al. J. Agric. Food Chem. 56, 3029. Copyright 2008 American Chemical Society.

**Table 7** Effects of bioactive compound piperine from black pepper

Effects	References
Antioxidant activity -Decrease lipid peroxidation, inhibit lipoxygenase, inhibit free radicals and reactive oxygen species	Vijayakumar et al. (2004) Selvendiran and Sakthisekaran (2005) Ahmad et al. (2010)
Anti-cancer activity –alcoholic extracts exhibited effective immunomodulatory and antitumor activities; induced apoptosis	Ahmad et al. (2012) Selvendiran and Sakthisekaran (2005) Ahmad et al. (2010) Bang et al. (2009)
Anti-inflammatory activity – piperine reduce the synthesis of prostaglandin, expression of interleukin 6 and matrix-proteinase 13	Sunila and Kuttan (2004)
Immuno-modulatory and antitumor activity – piperine is cytotoxic to Ehrlich ascites carcinoma cells and Dalton's lymphoma ascites and in addition, increases white blood cell counts	
Bioavailability enhancer – piperine has shown bioavailability enhancing effects on nutrients and drugs by amending membrane structure and increasing absorption	Acharya et al. (2012)
Digestive activity – piperine enhances digestion through increasing saliva production and stimulation of the pancreatic enzymes	Hussain et al. (2011) Srinivasan (2007)


**Figure 5** Structures of four theoretically possible and characterized piperine cis–trans isomers. Reprinted with permission from Friedman, M., et al. J. Agric. Food Chem. 56, 3029. Copyright 2008 American Chemical Society.

Curcumin has been found to work synergistically with other bioactives such as resveratrol, piperine, catechins, quercetin and genistein. According to Kunnumakkara et al. (2017), there has been over 100 different completed clinical trials with curcumin, which clearly show its safety, tolerability and its effectiveness against various chronic diseases in humans. Various studies conducted using in vitro, in vivo, and in human intervention trials have indicated that curcumin may be effective in the prevention and

**Table 8** Differences between cinnamon species

Species	Source	% Volatile oil	Remarks
<i>C. burmannii</i>	Indonesia	1–3.5	High amount of coumarin With mucilage
<i>C. cassia</i>	China (Tung Hing, Sikiang)	High V.O.	Sweetest, also known as <i>C. aromaticum</i>
<i>C. cassia</i>	Northern Vietnam	3–4	
<i>C. louriero</i>	Central Vietnam	4–6	Also called Vietnamese cinnamon or Saigon cinnamon

**Table 9** Composition and antioxidant values of ground cinnamon

Nutrients	Units	Value per 100 g
Water	g	10.58
Energy	kcal	247
Protein	g	3.99
Total lipid (fat)	g	1.24
Carbohydrates, by difference	g	80.59
Fiber, total dietary	g	53.1
Sugars, total	g	2.17
Calcium, Ca	mg	1002
Vitamin C, total ascorbic acid	mg	3.8
Vitamin B-6	mg	0.158
Vitamin B-12	mcg	0
Vitamin A, RAE	mcg_RAE	15
Vitamin A, IU	IU	295
Vitamin D	IU	0
Vitamin E (alpha-tocopherol)	mg	2.32
Fatty acids, total saturated	g	0.345
Fatty acids, total monounsaturated	g	0.246
Fatty acids, total polyunsaturated	g	0.068
H-ORAC	μmol TE/100 g	143,264
L-ORAC	μmol TE/100 g	3326
Total ORAC	μmol TE/100 g	131,420
Total phenolics	mg GAE/100 g	4533

Source: United States Department of Agriculture, Agricultural Research Service, USDA Food Composition Databases.

**Table 10** Essential oil components of cinnamon species

Species	Linalool	β-Caryophyllene	α-Terpineol	Dihydro-cinnamaldehyde	Cinnamic acetate	Eugenol
<i>C. cassia</i> or <i>C. aromaticum</i> <sup>a</sup>	<0.1%		0.2%	0.7%	0.1%–3.6%	<0.1–3.6%
<i>C. loureiro</i>	0%–5%	0%–0.1%		1.1%–1.6%	0.6%–3.9%	0%–0.3%
<i>C. zeylanicum</i> or <i>C. verum</i>	2.1%–7.8%	1.6%–3.2%	1.0%–1.9%	0.2%–1.0%	1.5%–10.6%	2.9%–22.3%
<i>C. burmannii</i> <sup>a</sup>	0%–1.0%	0.1%–0.6%	1.5%–2.6%	1.6%–1.7%	0%–0.4%	0%–0.4%

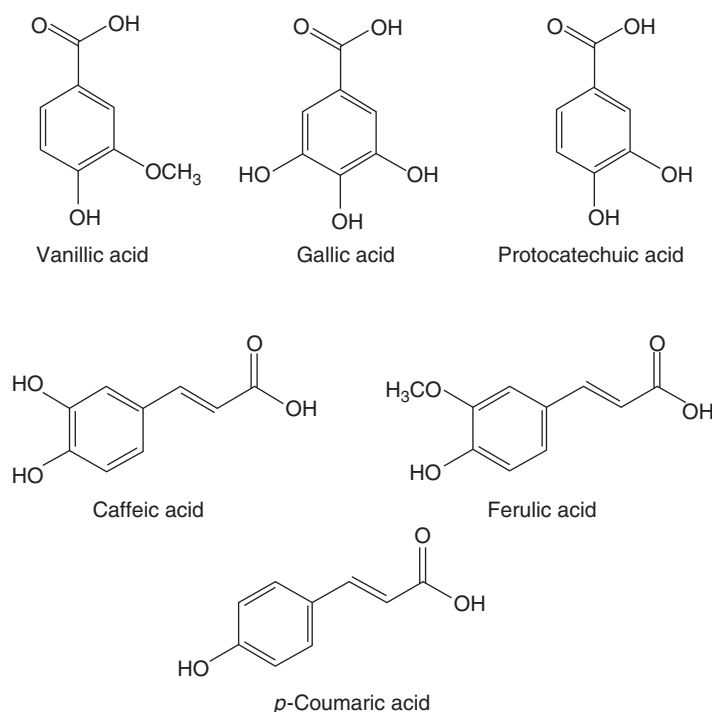
<sup>a</sup>With calcium oxalate.

treatment of many diseases through molecular targets, which further elaborate the safety of this natural product (Kocaadam and Sanlier, 2017). Table 12 summarizes the most recent studies on turmeric and curcumin. Goozee et al. (2016) elegantly described the potential clinical value of curcumin and its mechanisms of action in Fig. 8.

## Vanilla

Vanilla is extracted from cured vanilla beans. The vanilla beans come from a climbing, tropical orchid plant that is native to Mexico. There are four main commercial cultivars of vanilla. These are the Bourbon vanilla or Bourbon-Madagascar vanilla that is produced from *Vanilla planifolia* plants in Madagascar, Coromos and Réunion but is also grown in India. Mexican vanilla is extracted from *V. planifolia* in Mexico where vanilla plant originally came from. Tahitian vanilla produced from *Vanilla tahitiensis* comes from French Polynesia and was hypothesized to be a hybrid of *V. planifolia* and *Vanilla odorata* through genetic testing. On the other hand, West Indian vanilla is made from *Vanilla pompona*, which is grown in the Caribbean and Central and South America.

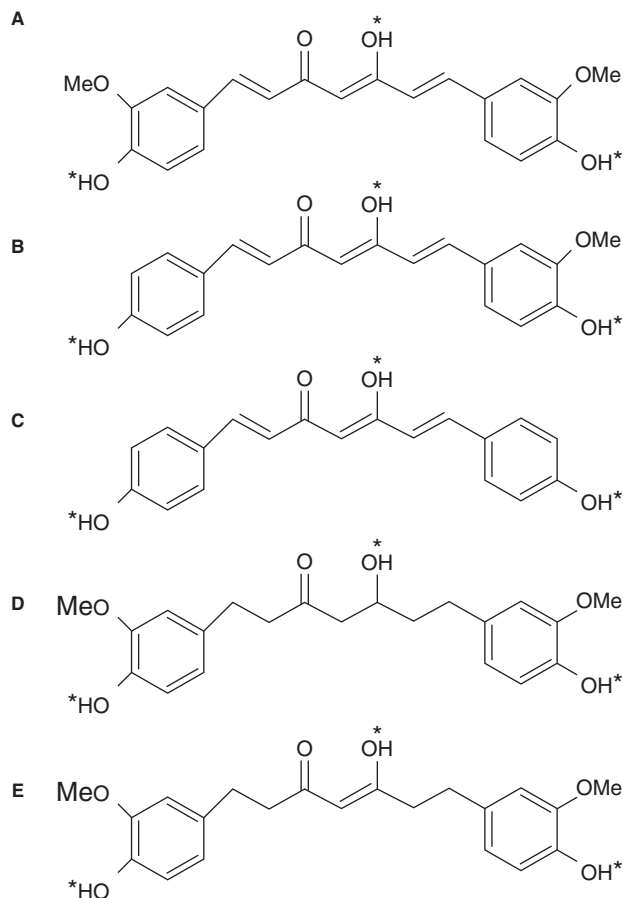




**Figure 6** Phenolic constituents of cinnamon. Source: Nabavi, S.F., et al., 2015. *Nutrients* 7, 7732 (Open access article).

**Table 11** Functions of cinnamon

Function	References
Insulin mimetic properties – bioactives enhance glucose uptake by activating insulin receptor kinase activity, autophosphorylation of the insulin receptor and glycogen synthase activity	Baker et al., 2008 Crawford, 2009 Solomon and Blannin, 2009 Safdar et al., 2004
Methyl hydroxyl chalcone polymer from cinnamon – increased insulin-dependent glucose metabolism by 20-fold in vitro	
Cinnamon polyphenols - decreased oxidative stress and improved fasting glucose	Lu et al., 2012 Hlebowicz et al., 2009 Davis and Yokoyama, 2011 Ziegenfuss et al., 2006 Qin et al., 2010 Akilen et al., 2010 Roussel et al., 2009 Wang et al., 2007 Ziegenfuss et al., 2006 Allen et al., 2013
Aqueous extract significantly improved metabolic syndromes – fasting blood sugar, systolic blood pressure and body composition	
Cinnamon is associated with a statistically significant decrease in levels of fasting plasma glucose, total cholesterol, LDL-C, and triglyceride levels, and an increase in HDL-C levels	
Leaf essential oil from cinnamon (40.24% linalool) – tested in diabetics significantly lowered fasting blood glucose and fructosamine and are concomitant with elevated plasma and pancreatic insulin levels under a fasting condition; appropriate doses linalool chemotype exhibited therapeutic potential in glycemic control in diabetics	Lee et al., 2013
Cinnamaldehyde (CAL), cinnamic acid (CA), and cinnamyl alcohol (CALC) (bioactives of Cinnamomum), aqueous cinnamon extract (ACE) - Pharmacoinformatics studies implicated that ACE and its Bioactives (CAL, CA, and CALC) exhibited comparable activity with that of Trichostatin A (TSA), a well-known anti-cancer agent and histone deacetylase (HDAC) inhibitor; based on both the experimental and computational data that cinnamon bioactives exhibited significant HDAC8 inhibitory activity, thereby suggesting their potential therapeutic implications against cancer. The altered expression of histone deacetylase family member 8 (HDAC8) has been found to be linked with various cancers making its selective inhibition a potential strategy in cancer therapy.	Patil et al., 2017



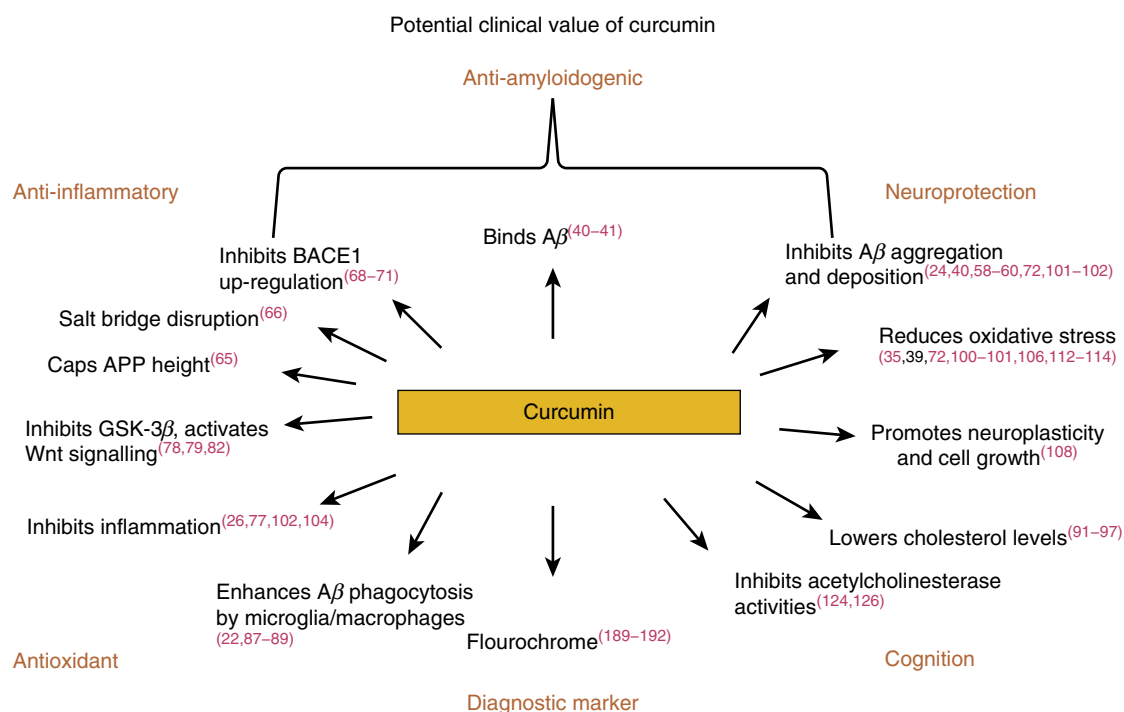
**Figure 7** Structures of curcumin (A), desmethocurcumin (B), bisdesmethocurcumin (C), hexahydrocurcumin (D), and tetrahydrocurcumin. Asterisks indicate potential sites for conjugation. Reprinted with permission from Marczylo, T. J. *Agric. Food Chem.* 57, 798. Copyright 2009 American Chemical Society.

Bourbon-Madagascar vanilla is the most popular because of its desirable aroma and flavor profile. Vanilla is the one of the most expensive spices, second to saffron, the most expensive spice in the world which is retailing from \$5000 to 10,000 per pound.

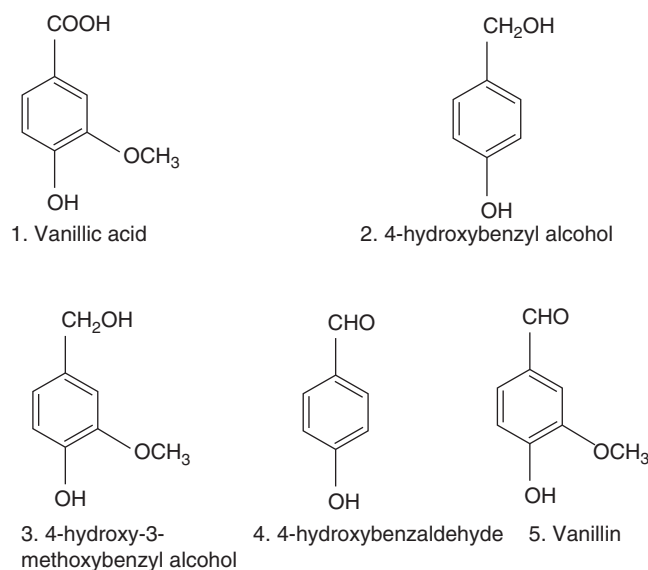
Vanilla extracts are rich in polyphenols (Fig. 9), which are also responsible for their pleasing aroma and flavor. In addition to these compounds, vanilla extracts contain more than 200 volatile constituents. Vanilla also contains complex tannins, polyphenol resins, free amino acids and waxes. It is not surprising that vanilla and its components can provide health benefits. Studies show that vanillin, one of the main polyphenols in vanilla extract, is able to inhibit early and advanced glycation modification and amyloid-like aggregation of albumin and thus exhibits protective effect toward its native conformation (Awasthi and Saraswathi, 2016). In another study, Iannuzzi et al. (2017) employed biophysical, biochemical and cell based assays, which showed that vanillin and curcumin similarly affect insulin amyloid aggregation by promoting formation of harmless fibrils and that vanillin restrains AGE formation and protects from AGE-induced cytotoxicity. Iannuzzi et al. (2017) added that their novel findings suggest that the curcumin degradation products (vanillin is one) can also provide health benefits. Vanillin was found to suppress metastasis in a mouse model as well as divanillin, apocynin and diapocynin based on the Transwell invasion assay as reported by Jantaree et al. (2017). In this study the dimeric forms exhibited potency higher than those of vanillin and apocynin in inhibiting invasion. Both monomeric and dimeric forms target regulation of the invasion process by inhibiting phosphorylation of FAK and Akt and the molecular docking studies suggested that the dimers should bind more tightly than vanillin and apocynin to the Y397 pocket of the FAK FERM domain (Jantaree et al., 2017). In another study by Lirdprapamongkol et al. (2009), they examined the antimetastatic potential of vanillin and its structurally related compounds, vanillic acid, vanillyl alcohol, and apocynin on hepatocyte growth factor (HGF)-induced migration of human lung cancer cells by the Transwell assay. They found that vanillin and apocynin could inhibit cell migration, and both compounds selectively inhibited Akt phosphorylation of HGF signaling, without affecting phosphorylation of Met and Erk. In addition, vanillin and apocynin could inhibit the enzymatic activity of phosphoinositide 3-kinase (PI3K), as revealed by an in vitro lipid kinase assay, suggesting that inhibition of PI3K activity was a mechanism underlying the inhibitory effect on cancer cell migration. They also concluded that the presence of an aldehyde or ketone group in the vanillin structure was important for this inhibition and that vanillin and apocynin also inhibited angiogenesis as determined by the chick chorioallantoic membrane assay. Furthermore, Taner et al. (2017) found that vanillic acid exhibited antioxidant activity using the

**Table 12** Functions of turmeric/curcumin

Function	References
Curcumin-free turmeric (CFT) exhibits activity against human HCT-116 color tumor xenograft and has similar in vivo activity to turmeric and curcumin in inhibiting growth promoting proteins and induces apoptosis.	Prasad et al., 2017
The anti-inflammatory activity of curcumin, demethoxycurcumin, bisdemethoxycurcumin was found to be greater than that of eugenol in relation to the COX-2 receptor obtained from silico studies. The findings therefore suggest that the combination of zinc oxide with turmeric liquid extract has a higher anti-inflammatory effect than eugenol as demonstrated by both in vivo and in silico studies.	Meizarini et al., 2018
The tumor volume within the CMG group (curcumin $\beta$ -D-glucuronide or curcumin monoglucuronide) was significantly less than that of the control group and there was no significant loss of body weight which suggest that CMG could be used as an anticancer agent without the serious side effects that most anticancer agents have.	Ozawa H et al. (2017)
Dalton's lymphoma ascites (DLA) cell viability was inhibited by the Curc-Alb conjugate (Conjugation of curcumin, Curc) to albumin, lb) in a dose dependent manner in vitro, as evidenced by the MTT assay. Significant tumor reduction was observed when the Curc-Alb conjugate was administered intraperitoneally in DLA-induced mice after 1 day (prevention therapy) and 7 days (reduction therapy) of tumor induction. There was significant reduction in both tumor volume and tumor cell numbers in the treated animals as well as a marked increase in their mean survival time and percent increase in life span. The effect was greater when the conjugate was administered soon after inducing the tumor as compared to when treatment was started after allowing tumor to grow for 7 days. Results demonstrate that curcumin albumin conjugate has immunomodulatory and tumor growth inhibition properties and has the potential to be used as an anticancer agent in affected human subjects.	Aravind SR and Krishnan LK (2016)
Evaluation of a liquid extract of turmeric roots (TEx) formulated for its in vitro, anticancer activity against several human, colorectal cancer cell lines. TEx had potently inhibited the growth of all human colon cancer cell lines tested in a dose- and time-dependent manner. TEx inhibited the formation of HCT116 spheroids when the cells were incubated with the extract. The extract also disrupted the formation of tubules formed by MDA-MB231 cells grown on Matrigel at concentrations that did not affect the overall viability of the cells, indicating a potent anti-invasive activity.	Dimas et al. (2015)



**Figure 8** Curcumin: reported mechanisms of action. BACE1,  $\beta$ -APP-cleaving enzyme-1; A $\beta$ ,  $\beta$  amyloid; APP, amyloid precursor protein. Source: Goozee, K.G., et al., 2016. Examining the potential clinical value of curcumin in the prevention and diagnosis of Alzheimer's disease. Br. J. Nutr. 115, 455 (Open access article).



**Figure 9** Chemical structures of the major flavor compounds found in cured beans of *Vanilla planifolia*. Reprinted with permission from Sun, R., et al. J. Agric. Food Chem. 49, 5162. Copyright 2001 American Chemical Society.

trolox equivalent antioxidant capacity (TEAC) assay, did not have cytotoxic and genotoxic affects and seemed to decreased DNA damage induced by  $H_2O_2$  in human lymphocytes.

### Extraction of Bioactives From Spices and Herbs

Bioactives can be extracted from spices and herbs in a variety of ways. The extraction methods can be classified in to two: conventional extraction and nonconventional extraction methods. Under the conventional extraction methods are Soxhlet extraction, maceration and hydrodistillation. The Soxhlet method was named after the German chemist Franz Ritter Von Soxhlet who proposed the said extraction technique. This method was originally designed for extraction of lipids but it is being used for a variety of products including in the extraction of bioactives. A small amount of sample is placed in a thimble which is then placed in the extraction chamber and distillation of the solvent is contained in a distillation flask then heating of the solvent starts. As the solvent distills into the thimble, the amount of solvent in direct contact with the plant material increases until it reaches the overflow level and the solvent is aspirated by a siphon action into the distillation flask. This method of extraction ensures intimate contact of the sample matrix with the extraction solvent. The extraction cycle is repeated several times to ensure complete extraction of the bioactives. The rate of cycling of solvent aspiration can be controlled by the solvent heating temperature. After the extraction is completed, the bioactives in the solvent can be used as is or can be concentrated in a rotary evaporator or by using a freeze dryer depending on the type of solvent used in the extraction process. The Soxhlet method is applicable to the extraction of nonvolatile and semivolatile organic compounds with extraction time of 2–18 h.

For the maceration extraction, the plant material is milled or ground into small particles and mixed with the solvent and sealed. At the end of the extraction process, the extract is filtered and the solid residue is pressed to remove all the extract being held by the solid. The extract is sometimes filtered again to completely remove the particulates that will tend to sediment at the bottom of the extract during storage. It is typical for the maceration technique to last from 2 to 5 days.

Hydrodistillation is another traditional method of extraction. There are three types of hydrodistillation: water distillation, water and steam distillation and direct steam distillation. For water distillation, the plant material is packed in a steel container then a measured amount of water is added and this is heated to boiling temperature. In direct steam distillation, steam is used to extract the bioactives by injecting steam directly into the plant material while in water and steam distillation a combination of boiling water and steam are used. Hydrodistillation method cannot be used if the bioactives are heat labile. For these three methods of extraction, the efficiency of the process will depend on the solvent selection. The efficiency of the extraction process is affected by the solvent polarity, affinity of the bioactives to the solvent, mass transfer/diffusion and extraction temperature.

Under the nonconventional extraction methods are: ultrasound-assisted extraction (UAE), microwave assisted extraction (MAE), pulsed-electric field extraction (PEF), enzyme-assisted extraction (EAE), pressurized liquid extraction (PLE), and supercritical fluid extraction (SFE). Ultrasound-assisted extraction uses ultrasound to facilitate extraction by disrupting the cellular material of the plant thereby freeing the bioactives from the cell walls and for the solvent to diffuse easier through the cellular material. The typical

**Table 13** The advantages and disadvantages of greener extraction methods

Method	Advantages	Disadvantages
Microwave-assisted extraction (MAE) (used with traditional methods)	Rapid extraction; small amount of solvent; relatively low additional costs	Use of high pressure and temperature; limited amount of sample; non-selective (large number of compounds extracted)
Supercritical fluid extraction (SFE) methods	Rapid extraction; small amount of organic solvent or no solvent; no solvent residue; preserves thermally labile compounds; tunable solvent (SCF) density; selective extraction (small number of compounds extracted); inexpensive to operate/run	High setup cost; technical knowledge of SCF properties required (e.g., phase behavior, cross-over region)
Mechanical extraction	Mainly for extraction of oil and juice; does not require external heat and solvent	Limited application and non-selective
Ultrasound-assisted extraction (UAE) (used with traditional methods)	Rapid extraction; small amount of solvent; relatively low additional cost	Non-selective

Source: Khaw, et al., 2017. Solvent supercritical fluid technologies to extract bioactive compounds from natural sources: a review. *Molecules* 22, 1186 (Open access article).

sound waves used are from 20 kHz to 100 mHz. The sound waves create compression and expansion that causes the disruption of the cellular components of the plant, in a process called cavitation. Bubbles produced during this process collapse at high level of energy and pressure. The factors that affect the efficiency of the process are particle size of the plant material, nature of the solvent, temperature, pressure, and frequency of sound waves used and the time of extraction.

Microwave energy has been used in several processes including sterilization, pasteurization, thawing and extraction. Microwave-assisted extraction (MAE) has been in use for several years due to the refinement of laboratory, pilot plant and commercial scale equipment. The principle is based on generation of heat through the conversion of electromagnetic energy due to ionic conduction and dipole rotation. From the change in directions of molecules, collision occurs between molecules and these collisions generate heat. The heat created facilitates diffusion of the solvent into the plant matrix and also increases the efficiency of the solvent to dissolve the bioactives. MAE offers several advantages in extraction of bioactives, including quick heating (results in quick extraction) and better yield compared to traditional extraction techniques. In addition, using MAE reduces the amount of solvent employed in the extraction process.

Pulsed-electric field extraction (PEF) uses an electric field to facilitate extraction. When an electric potential passes through the cellular membrane, there is segregation of molecules based on their charge and this brings about repulsion between charged molecules thereby creating pores and weakening of certain locations in the membrane. This increases permeability of the cellular structure that results in more efficient diffusion of the solvent through the cellular material. The efficiency of PEF depends on the field strength employed, specific energy input, pulse number, temperature and the properties of the plant material and solvent used.

Use of enzymes to breakdown the cellular materials of the plant is another novel method of extraction. Plant cellular materials are composed of structural biomolecules such as cellulose, hemicellulose, lignin and pectin which form the structure, bind and strengthen the plant cell wall. In some instances, location of the bioactives is not readily accessible to the solvent. In addition, some of these bioactives are bound to the structural polysaccharides. Employment of enzymes can facilitate the release of the bioactive by breaking down the cell walls through hydrolysis of the polysaccharides and the lipid material. Cellulases,  $\alpha$ -amylase, hemicellulases and pectinase are some of the enzymes used to hydrolyze the cell wall. Various factors affect the efficiency of the process for EAE. These factors are the type of enzyme used, concentration of enzyme, time and temperature employed, pH of the medium, particle size of the plant material and ratio of solid to solvent.

Pressurized liquid extraction (PLE) is another novel technique for the extraction of bioactives in plant materials. The principle is the application of high pressure so that the solvent will remain liquid above its boiling point during the extraction process. Several terminologies are used to describe this extraction technology in addition to PLE: accelerated fluid extraction (ASE), high pressure solvent extraction (HSPE) and enhanced solvent extraction (ESE). Employment of high pressure increases the efficiency of the extraction process. In addition, high temperatures increase the solvency of the analytes, reduces the viscosity of the solvent and increases diffusion and mass transfer.

Supercritical fluid extraction (SFE) is a technique of extraction when the supercritical state of a solvent is employed. Supercritical state is attained when a substance is subjected to temperature and pressure beyond its critical point. At this point, the fluid has different properties and acts like a gas in terms of diffusion, viscosity, surface tension, density and solvation power. Due to these properties, the supercritical fluid is more efficient in extracting substances and in a shorter time. SFE has been successfully employed in commercial extraction of volatile flavor compounds. Carbon dioxide is the ideal solvent for SFE due to its critical temperature of 31 °C and has been used in a variety of supercritical CO<sub>2</sub> extractions. Carbon dioxide is an ideal solvent for hydrophobic substances like lipids and fats but is not as effective for hydrophilic compounds. A modifier is often used to alter the low polarity of carbon dioxide thereby making it a good solvent for specific polar substances. To efficiently extract bioactives using supercritical CO<sub>2</sub> extraction, the following parameters are controlled: temperature, pressure, particle size of the plant material, time of extraction,

**Table 14** Extraction of bioactives from spices and herbs

Spice or herb	Extraction method used	Findings	References
Fresh lemon balm and peppermint leaves	Conventional and ultrasound-assisted extraction (UAE)	A significant increase in bioactives when UAE was used	<a href="#">Sic Zlabur et al. (2016)</a>
Wild garlic ( <i>Allium ursinum</i> )	UAE using response surface methodology	Successfully optimized the extraction process	<a href="#">Tomsik et al. (2016)</a>
Ginger	Conventional extraction, UAE, high pressure homogenization-assisted extraction	UAE gave the highest polyphenol content and antioxidant activities at lower extraction temperature	<a href="#">Gunathilake and Vasantha Ripasinghe (2015)</a>
Coriander	Subcritical water extraction (SWE), hydrodistillation	Extraction efficiency of SWE was higher than hydrodistillation with reduced extraction time	<a href="#">Saim et al. (2008)</a>
Chili pepper compared with tomato by-products	SFE	Extraction process from chili was faster from tomato under identical operating conditions	<a href="#">Venturi et al. (2017)</a>
Clove bud	SFE, steam distillation and MAE	The oil yield was higher in steam distillation and microwave oven extraction but oil extracted by using SFE contained higher amount of eugenol and eugenyl acetate.	<a href="#">Yasdani et al. (2014)</a>
Rosemary	SFE	SFE extracts have higher antioxidant activities than commercial and Soxhlet extracts and dibutyl hydroxytoluene (BHT)	<a href="#">Caldera et al. (2012)</a>
Lavender	Microwave-assisted hydrodistillation and enzyme pretreatment	An increase of 25% in yield in comparison with the classic extraction conditions of conventional hydrodistillation	<a href="#">Calinescu et al. (2014)</a>
Licorice and skullcap	Bifunctional enzyme	The extraction yield of liquiritigenin and isoliquiritigenin aglycones increased 6.51- and 3.55-fold increases, respectively.	<a href="#">Xu et al. (2013)</a>

flow rate of CO<sub>2</sub> and solid to solvent ratio. [Khaw et al. \(2017\)](#) outlined the advantages and disadvantages of some of the extraction methods discussed above in [Table 13](#).

[Table 14](#) summarizes recent techniques applied in the extraction of bioactives from spices and herbs. The nonconventional extraction methods have been successfully applied in bioactive extraction and surpass the traditional extraction methods in terms of quantity and quality of yield. These examples in [Table 14](#) also show that combination processes work well in specific bioactive extraction applications. This strategy is good to explore especially for hard-to-extract bioactive compounds.

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## Phlorotannins

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### Introduction

“Let food be thy medicine and medicine be thy food” is quoted from Hippocrates (Smith, 2004). Plants and animals of marine and terrestrial origins are considered to be main food resources for humans, of which the marine environment is considered to be one of the best and huge reservoirs. It has been repeatedly reported that marine sources consist of animals and plants, which can be potentially used as foods in our daily life (Kim, 2012; Kim and Taylor, 2011). Macroalgae is one of the class of marine plants that has gained much attention in the recent years for health food supplements used as food ingredients and animal feeds in East Asia. These macroalgae are rich sources of soluble dietary fibers, proteins, peptides, carotenoids, polysaccharides, minerals, and potential metabolites (Yang et al., 2016; Sánchez-Camargo et al., 2016). It has also been suggested elsewhere that metabolites derived from macroalgae have potential biological activities such as antibacterial, antioxidant, anti-inflammatory, antiadipogenic, antidiabetic, anti-HIV, and anti-cancer activities (Yang et al., 2016). Owing to ample of these medicinal properties, the marine macroalgae can be considered as a potential health benefitting medicinal food.

### Phlorotannins

“Phlorotannin” (1,3,5-trihydroxy benzene), is a group of polyphenolic compounds which are commonly present in the brown algae accounting for about 5–12% of the dry mass (Sathya et al., 2017). The development of phlorotannin can occur through polymerization of phloroglucinol oligomers via acetate-malonate (polyketide) pathway (Li et al., 2011a; Sathya et al., 2017). *Eisenia bicyclis*, *Ecklonia cava* and *Ecklonia kurome* are commonly found in Japanese and Korean coastal areas. Phlorotannin content in *Eisenia bicyclis* accounts for 3% of total dry mass of algae. HPLC results suggest that *E. bicyclis* consists of phloroglucinol (0.9%), phloroglucinol tetramer (4.4%), eckol (7.5%), phlorofucofuroeckol A (21.9%), dieckol (23.4%), and 8,8'-bieckol (24.6%), plus some 17.3% of other unknown phenolic compounds (Shibata et al., 2004). However, phlorotannin content in seaweeds can also vary through the individual species, geographic region and extraction techniques (Jégou et al., 2015). Fucodiphlorethol G was isolated from *E. cava* for the first time and acetylated to make different derivatives of the compound (Young et al., 2007).

### Extraction Procedure

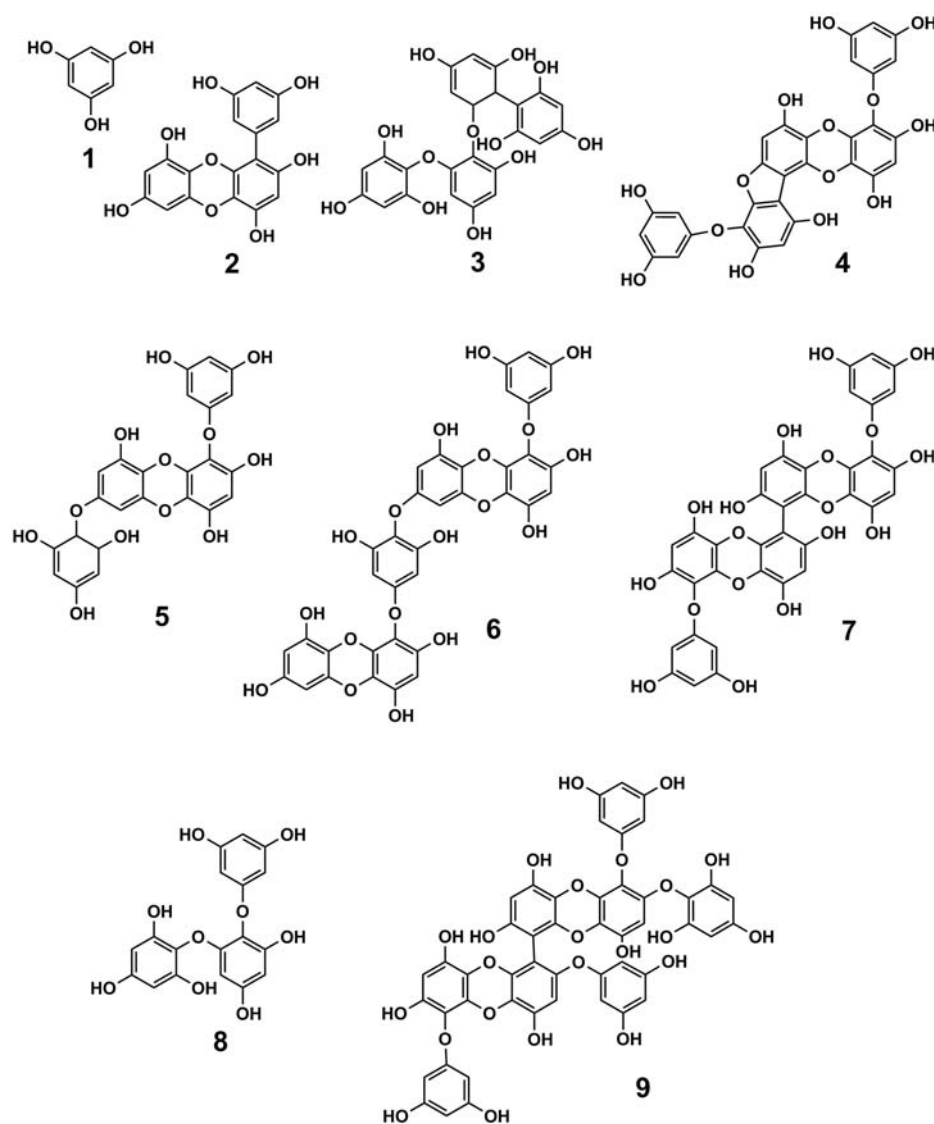
Phlorotannins (Fig. 1) are commonly isolated from the seaweeds, particularly brown algae, through conventional organic solvent isolation followed by chromatographic techniques to purify the compounds (Kim et al., 2016; Koivikko et al., 2007; Venkatesan, Kim, & Shim, 2016). Furthermore, nuclear magnetic resonance spectroscopy is widely used to characterize the structure of compounds. Supercritical carbon dioxide has also been used at times to extract the phlorotannins (Saravana et al., 2017; Sánchez-Camargo et al., 2016).

HPLC-HRMS (high resolution mass spectrometer) is the most commonly used method to characterize phlorotannins (Melanson and Mackinnon, 2015). Folin–Ciocalteu assay is a commonly used technique to quantify the phlorotannin content in a given species. Recently, <sup>1</sup>H qNMR was also used to quantify the phlorotannin content in brown algae (Jégou et al., 2015; Parys et al., 2007). Furthermore, Ultra High Performance Liquid Chromatography (UHPLC) has also been employed to study the isomeric complexity between the phlorotannins (Heffernan et al., 2015). Water-Organic solvent mixtures were also used to extract the phlorotannins. The details of step by step extraction procedure and purification were given by Gall et al. (2015). Balboa et al. (2015) developed a biorefinery process for the isolation of phlorotannin in a single method. In this method, conventional solvent extraction with 96% ethanol, supercritical CO<sub>2</sub> extraction method, and membrane microfiltration were used. Tierney et al. (2013, 2014) developed pressurized a liquid extraction method, which was used to extract the polyphenol compounds from Irish macroalgae *Ascomphylum nodosum*, *Pelvetia canaliculata*, *Fucus spiralis* and *Ulva intestinalis* and compared with the traditional solid–liquid extraction techniques in terms of their antioxidant properties. The results suggest that pressurized liquid extraction produces higher phenolic compounds when compared to traditional extraction methods (Tierney et al., 2013, 2014). Two-dimensional liquid chromatography was used to isolate phlorotannin from seaweed (Montero et al., 2014). Centrifugal partition chromatography (CPC) was used to separate the phlorotannins, which were isolated from the ethyl acetate fraction using the solvent system n-hexane:EtOAc:methanol:water (2:7:3:7, v/v). Dieckol (fraction I, 40.2 mg), phlorofucofuroeckol-A (fraction III, 31.1 mg), and

fraction II (34.1 mg) with 2,7-phloroglucinol-6,6-bieckol and pyrogallol-phloroglucinol-6,6-bieckol were isolated from the crude extract (500 mg) by a one-step CPC system (Lee et al., 2014). In another study, a macroporous adsorption resin was used for chromatographic purification of seaweed phlorotannins (Figs. 2 and 3). Four different resins (HP-20, SP-850, XAD-7HP, and XAD-2) were tested, and HP-20 resin showed the highest adsorption and desorption capacities (Kim et al., 2014). Compared to organic solvent extract, the recovery yield of dieckol from the boiling water of *E. cava*, *Ecklonia stolonifera*, *Ecklonia bicyclis* was found to be 86%, 93%, and 98%, respectively (Chowdhury et al., 2014).

Hydrophilic interaction chromatography techniques were used to extract and determine the phlorotannin content in *Eisenia bicyclis*. The yields of the phlorotannins increased 2–4 times in summer (June–October) and then were decreased to normal levels in winter (November–March). In the extraction of *E. bicyclis*, ethanol percentage in water, extraction time and washing time significantly affected the yield of the extract and the phlorotannins, whereas the temperature and the sample/solvent ratio impacted the extraction to a lesser degree (Kim et al., 2013b).

The use of a microwave-assisted extraction (MAE) method for the extraction of phlorotannins from *Saccharina japonica* Aresch has been evaluated with particular emphasis on the influential parameters, including the ethanol concentration, solid/liquid ratio, extraction time, extraction temperature, and microwave power (He et al., 2013). Biosynthesis of phlorotannins has also been explored (Bertoni, 2013). The UHPLC-HRMS method described was successful in rapid profiling of phlorotannins in brown seaweeds based on their degree of polymerisation. HILIC (Hydrophilic interaction chromatography) was demonstrated to be an effective separation mode, particularly for low molecular weight phlorotannins (Steevensz et al., 2012). Mature thalli contained

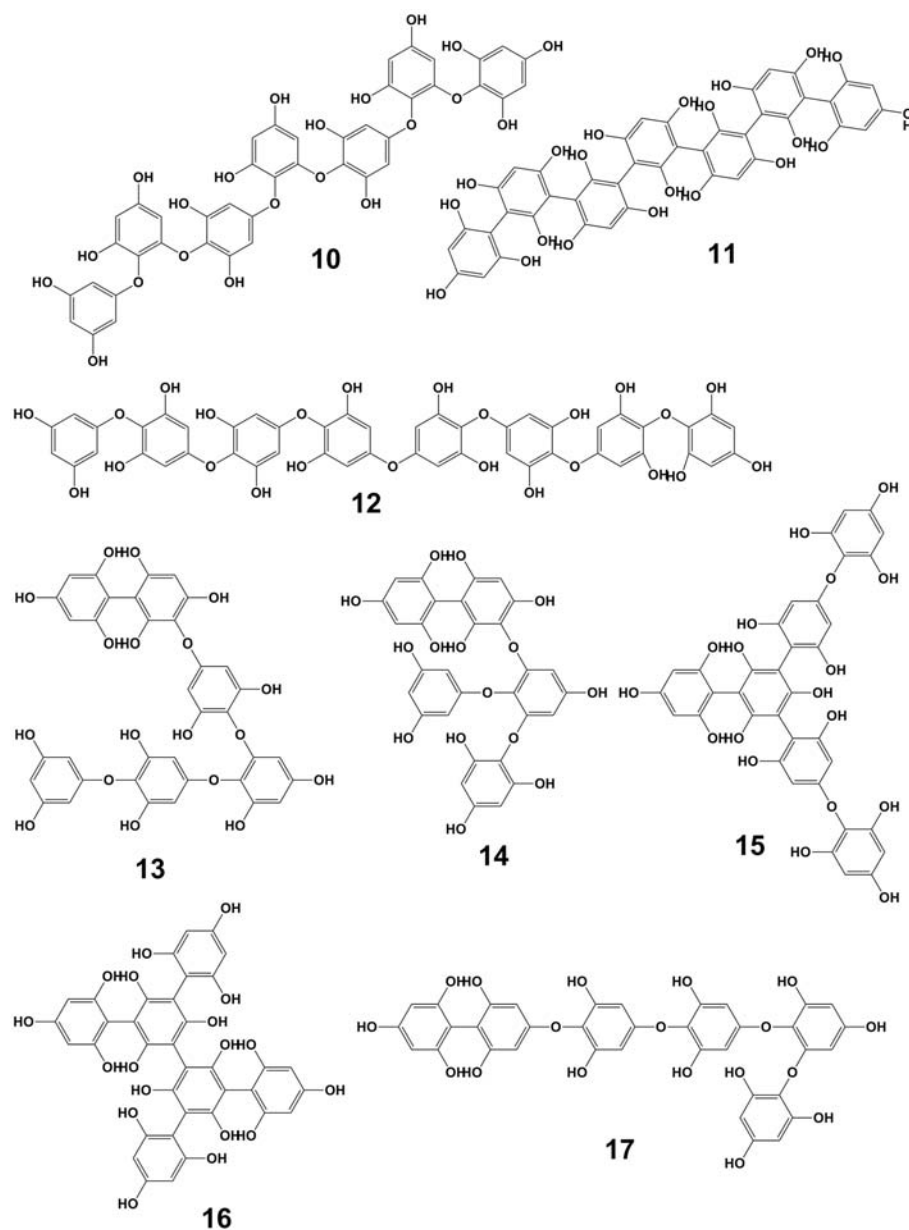


**Figure 1** Chemical structures of: (1) phloroglucinol; (2) eckol; (3) fucodiphlorethol G; (4) phlorofucofuroeckol A; (5) 7-phloroeckol; (6) dieckol; (7) 6,6'-bieckol; (8) triphlorethol-A; and (9) 2,7'-phloroglucinol-6,6'-bieckol (Venkatesan, Kim, & Shim, 2016).

1.5-fold more dieckol (1.82 mg/g-dry tissue) than young thalli. In the tissues of *E. cava*, blade tissue contained more phlorotannins than the stipe or holdfast. Among differently dried thalli, approximately 90% or more dieckol and phlorofucofuroeckol-A were extracted from shadow-dried tissue as compared with lyophilized tissue (Chowdhury et al., 2011). The 2,4-dimethoxybenzaldehyde (DMBA) assay was developed whereby the chemical specifically reacts with phlorotannins to form a coloured product (Stern et al., 1996). Phlorotannins from different regions and species of North American and Australasian marine herbivores vary significantly in terms of structure and function (Van Altena and Steinberg, 1992). Important and chemically characterised phlorotannins with HPLC and NMR techniques are shown in Figs. 1–3.

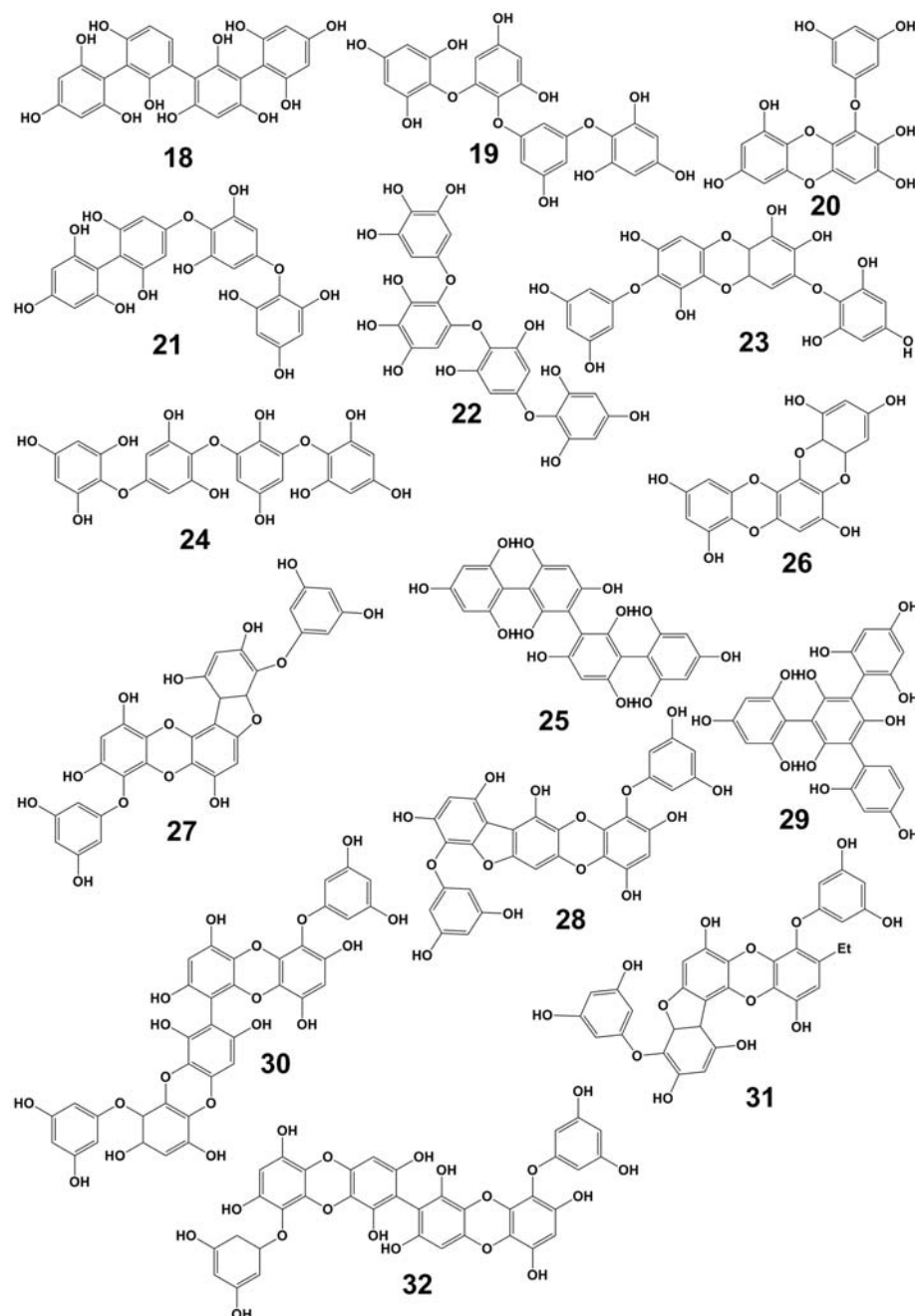
### Biological Activities of Phlorotannins

Phlorotannins show promising biological activities such as antioxidant, anti-HIV, antiproliferative, radio protective, antidiabetic, skin protection, and antiallergic activities (Wijesekara et al., 2010) due to their structural properties. Few of them will be discussed here.



**Figure 2** Important structures of phlorotannins isolated from seaweeds, (10) heptaphlorethol, (11) heptafucol, (12) Octaphlorethol A, (13) fucotetraphlorethol A, (14) fucotetraphlorethol J, (15) trifucodiphlorethol A, (16) hexafucol B, and (17) fucotetraphlorethol D (Tierney et al. 2014); (Sánchez-Camargo et al. 2016); (Lee et al. 2016).



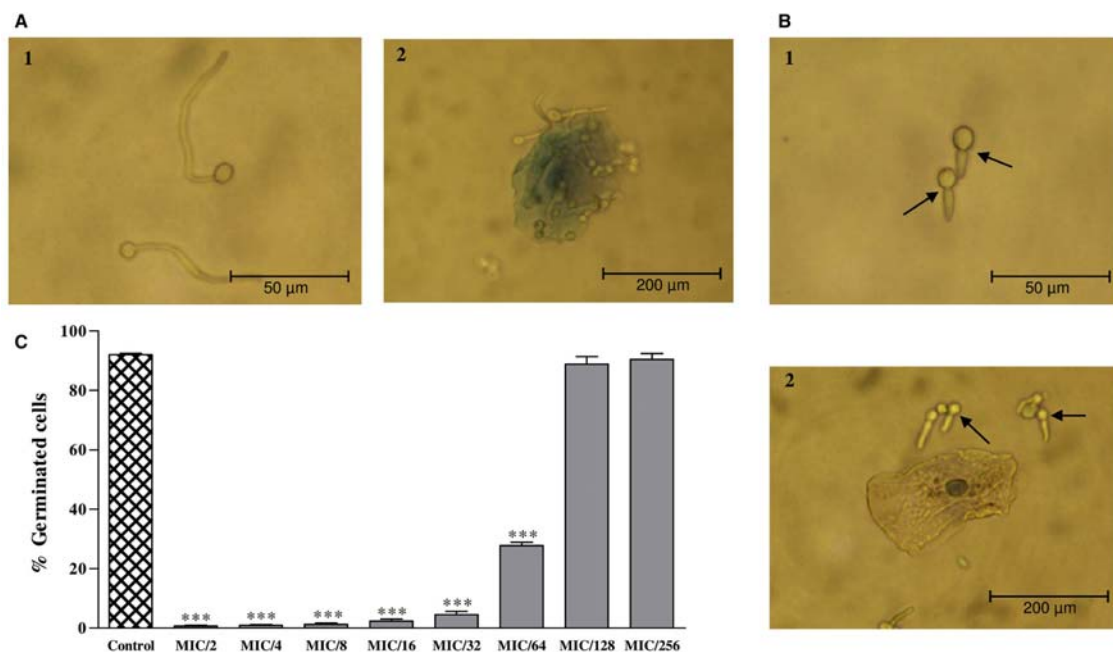


**Figure 3** Important structures of phlorotannins from seaweed, (18) Tetrafulcol A, (19) Tetraphlorethol B, (20) Eckol, (21) Fucodiphlorethol, (22) Tetrafulhalol A, (23) Diphoethohydroxycarmalol (24) Tetraisofulhalol, (25) Tetrafulcol A, (26) dioxinodehydroeckol (27) Phlorofucofuroeckol, (28) Phlorofucofuroeckol B (29) Tetrafulcol B (30) 6,8-bieckol, (31) phlorofucofuroeckol A, and (32) 8,8'-bieckol (Lopes *et al.* 2012) (Sugiura *et al.* 2007) (Parys *et al.* 2007) (Lee *et al.* 2012).

### Antimicrobial Activity

The antibacterial activities of phlorotannins have been reported in the literature by several research groups (Shannon and Abu-Ghannam, 2016), and phlorotannins have the ability to bind to the bacterial protein and cause cell lysis. The phenolic group of phlorotannins can bind with the amino group of bacterial proteins (Wang *et al.*, 2009). Eom *et al.* (2012, 2013, 2014) reported the effective ways to overcome methicillin-resistant *Staphylococcus aureus* growth by phlorofucofuroeckol-A, which is derived from *E. bicyclis* marine algae. The phlorotannins suppressed *mecl*, *mecR1*, and *mecA* gene expression in a dose-dependent manner (Eom *et al.*, 2012, 2013, 2014). Lopes *et al.* (2013) reported the antifungal activity of phlorotannins against dermatophytes and





**Figure 4** Effect of purified phlorotannins extracts from *F. spiralis* in the dimorphic transition of *C. albicans* ATCC 10231 (Untreated control cells - A1; cells treated with extract at MIC/32 - B1), in the adherence of the yeast to the epithelial cells (Untreated control cells - A2; cells treated with extract at MIC/32 - B2) and in the germ tube formation (C). The figure was adapted with permission from Lopes et al. (2013).

yeasts. Antimicrobial activity of phlorotannins against Gram positive and Gram negative bacteria was checked and the phlorotannin extracts were found to have excellent antimicrobial inhibition activity (Lopes et al., 2012) as shown in Fig. 4. Recently, marine-derived compounds have shown potential biological activity in terms of antibacterial effects as well (Lee et al., 2016).

### Antioxidant Activity

Food industry often searches for antioxidant molecules to prevent the food from spoilage. Natural derived antioxidants are considered to be safe for human health. Phlorotannins have ability to scavenge the reactive oxygen species (ROS) such as hydroxyl, peroxyl, superoxide radicals (Kirke et al., 2017). In addition to this, phlorotannins can act as total antioxidant with high reducing power activity. DPPH free radical scavenging of *Sargassum aquifolium* species were the highest, corresponding to  $6.770 \pm 0.001$  mg phlorotannin  $\text{g}^{-1}$  dry weight (DW),  $6.1290 \pm 0.0200$  mg ascorbic acid  $\text{g}^{-1}$  DW,  $19.7210 \pm 0.0300$  mg  $\text{FeSO}_4$   $\text{g}^{-1}$  DW and  $76.28 \pm 0.20\%$  of 25  $\mu\text{g}$  DPPH  $\text{mL}^{-1}$  extract (Cuong et al., 2016). It is obvious to say that higher amounts of phlorotannin content show higher antioxidant activity (Charoensiddhi et al., 2014; Wang et al., 2012). The general method to check the antioxidant activity of compound were as follows with the small modifications (Yotsu-Yamashita et al., 2013). First, 5  $\mu\text{L}$  of sample solution were mixed with 95  $\mu\text{L}$  of 1:1 volume of 0.4 M ethanolic solution of DPPH and 100 mM aqueous MES (sodium 2-(N-morpholino)ethanesulfonate) buffer (pH 6.0) for 1 min. Further, the plates were incubated for 30 min at 37 °C and read at 517 nm. The radical scavenging activity of the sample was calculated using the following formula:

$$A_{\text{sample}}/A_{\text{control}} \times 100$$

$A_{\text{sample}}$  is the absorbance of DPPH incubated with test compound;  $A_{\text{control}}$  is the absorbance of DPPH incubated without compound.

The following experiments are the most commonly used assays to measure the radical scavenging activities of the bioactive compounds.

- Ferric reducing/antioxidant power (FRAP) assay (Quéguineur et al., 2013) (Quéguineur et al., 2012)
- DCFH-DA (Dichloro-dihydro-fluorescein diacetate) assay (Yotsu-Yamashita et al., 2013)
- Trolox equivalent antioxidant capacity (TEAC) assay (Audibert et al., 2010)
- Electron spin resonance spectroscopy (Ahn et al., 2007)

The most important antioxidant activities of phenolic compounds and phlorotannins from the brown seaweed have been discussed in a previous review (Balboa et al., 2013). These reviews are also useful to get more information on antioxidant activities of other polysaccharides, proteins, peptides, lipids, terpenoids and steroids (Balboa et al., 2013; Li and Kim, 2011). Trifucodiphlorethol A, trifucotriphlorethol A as well as fucotriphlorethol A can significantly scavenge the ROS with  $\text{IC}_{50}$  range around 10.0–14.4  $\mu\text{g}/\text{mL}$

(Parys et al., 2010). Fig. 1 shows the important phlorotannins, which were isolated from *E. cava*. All the isolated compounds showed promising biological activity in terms of their antioxidant properties (Li et al., 2009).

The phlorotannins from *E. cava*, *E. kurome*, and *E. bicyclis* had significant radical scavenging activities against the superoxide anion (IC<sub>50</sub>: 6.5–8.4 µM) and DPPH (IC<sub>50</sub>: 12–26 µM), which were more effective as compared with ascorbic acid and α-tocopherol (Shibata et al., 2008). In another study, diphlorethohydroxycarmalol was isolated from brown algae, *Ishige okamurae* and antioxidant activity was evaluated as IC<sub>50</sub> value of DPPH to be 3.41 µM and 4.92 µM (Heo et al., 2008). The scavenging activity of the fraction against superoxide anion radicals was estimated to be 1.0 mg/mL (IC<sub>50</sub>), which was approximately five times stronger than that of catechin (Nakai et al., 2006). The IC<sub>50</sub> values of 974-A, 974-B, phlorofucofuroeckol-A, and dieckol were significantly smaller than those of phlorofucofuroeckol-B, phloroglucinol, α-tocopherol, and ascorbic acid (Yotsu-Yamashita et al., 2013).

### Anti-inflammatory Activity

Chronic inflammation is one of the main reasons behind majority of diseases. Several kind of anti-inflammatory drugs are available in the market to treat the inflammatory diseases, but those drugs are often associated with some side effects. Therefore, much attention has been paid to natural marine-based anti-inflammatory compounds (Cheung et al., 2016; Balboa et al., 2012; Shin et al., 2006). Marine-based compounds are unique and have proven to be potentially useful to treat inflammatory diseases. Yang et al. (2016) proposed that the phlorotannin-rich *E. cava* can be used to treat sepsis. Eom et al. (2017) investigated anti-inflammatory effects of eckol on *Propionibacterium acnes* induced human skin keratinocytes (HaCaT) cells. The functions of eckol in production of nitric oxide, matrix metalloproteinase 2 and 9, NO synthase, cyclooxygenase-2 and necrosis factor-α were also studied. Eckol significantly inhibited the expression or formation of proinflammatory mediators and cytokines in HaCaT cells (Eom et al., 2017). In most of the studies, LPS (lipopolysaccharide) was used to induce the inflammation in RAW 264.7 cells. Kang et al. (2015) reported that diphlorethohydroxycarmalol reduced interleukin 6 (IL-6), DPHC (12.5 and 100 µM) and suppressed the phosphorylation and nuclear translocation of NF-kappa (NF κB). In another study, Wijesinghe et al. (2013) reported that phlorotannin-rich fermented *E. cava* by-product extract dose-dependently inhibited nitric oxide production, prostaglandin-E2 production and suppressed inducible nitric oxide synthase and cyclooxygenase-2 expressions in LPS stimulated RAW 264.7 cells. Sugiura et al. (2013) reported the inhibitory effect of histamine release from rat basophile leukaemia (RBL) 2H3 cells. The anti-inflammatory activity potential of the methanolic extract and its fractions from *Eisenia bicyclis* was in the order of dichloromethane > methanol > ethyl acetate > n-butanol (Jung et al., 2013). 6,6'-Bieckol, and phlorofucofuroeckol A also suppressed LPS-induced iNOS, COX2 and PEG2 production and inflammatory cytokine expression in macrophages (Yang et al., 2012; Kim et al., 2009, 2011a).

### Antiproliferative Activity

Phlorotannin derived from *Sargassum muticum* showed antiproliferative effect against HT-29 cells (Montero et al., 2016). Compounds from *E. cava* have shown cytotoxic action against HeLa, HT1080, A549, and HT-29 cells (Li et al., 2011b). In another study, the antiproliferative effect of phlorotannins derived from *Laminaria japonica* was evaluated against hepatocellular carcinoma cells (BEL-7402) and murine P388 leukemic cells (Yang et al., 2010).

### Antitumor Activity

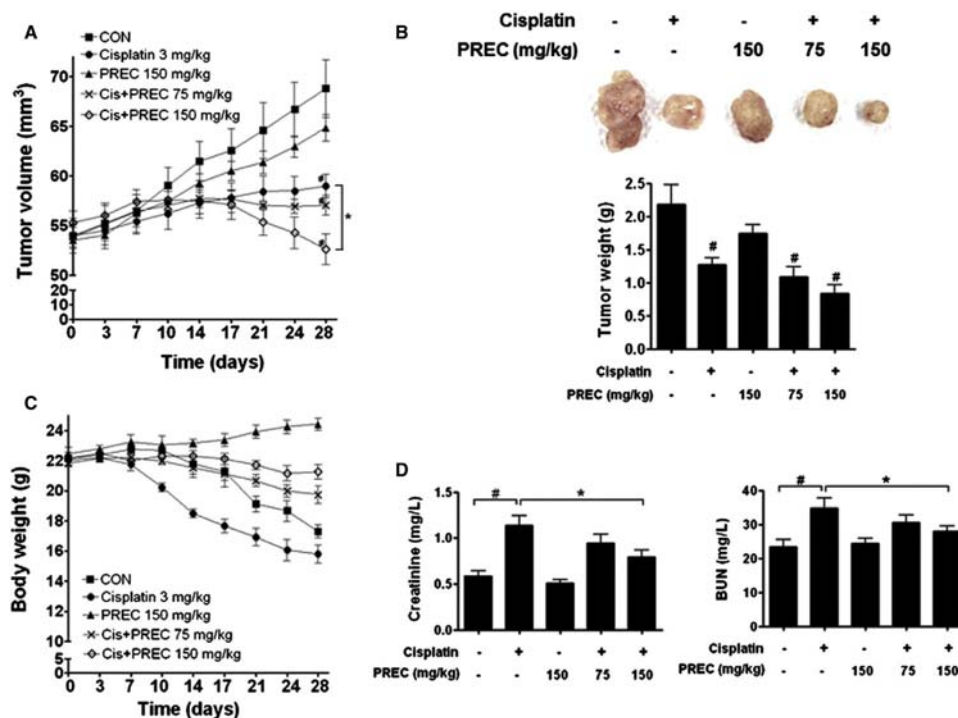
Antitumor effect of phlorotannin-rich extract with cisplatin were checked and results (Fig. 5) suggest that the combination improved the properties of cisplatin due to the synergetic effect (Yang et al., 2015).

Dieckol was isolated from *E. stolonifera* and its anticancer activity was checked against human Hep3B hepatocellular carcinoma cells. The compound has excellent ability to control cancer cell proliferation in a dose-dependent manner. In addition to this, important genes were expressed such as caspases-3, 7, 8 and 9 (Yoon et al., 2013). In another study, phlorotannin eckol suppressed stemness and malignancies in glioma stem-like cells (Hyun et al., 2011).

### Antidiabetics Activity

Diabetes mellitus is a chronic disease and mainly classified as type 1 and type 2. Type 2 diabetes incidence in people has increased considerably throughout the world due to the consumption of high-fat and carbohydrate foods. Even though, several chemically-derived antidiabetic drugs are available to treat diabetes, the problem persists with adverse side effects. Ultimately, there is a need to find effective molecules with less or none side effects. Polyphenol-rich molecules from marine seaweeds show promising effects in treating diabetes mellitus in terms of digestive enzymes, hepatic glucose metabolizing enzymes, lipid peroxidation and lowering the glucose plasma levels (Murugan et al., 2015).

Lee and Jeon (2015) conducted a study on intake of dieckol-rich extract and measured the glycemic parameters, serum biochemistry and hematology. About 80 pre-diabetic male and female adults were randomized and separated into two groups, namely, placebo control and dieckol-rich extract (1500 mg/day). The results showed that dieckol rich extract group decreased in insulin and C-peptide levels in 12 weeks, however there is no significant difference between the groups (Lee and Jeon, 2015). Antidiabetic



**Figure 5** Phlorotannins improve the antitumor activity of cisplatin. SKOV3 xenograft treated three times per week for 4 weeks (A), tumor weight after the experiment has been conducted (B), mouse body weight changes (C), Plasma creatinine and BUN levels were measured using a calorimeter testing kit. The figure was adapted with permission from (Yang et al., 2015).

effect of phlorotannins can follow diverse mechanisms, such as to improve insulin resistance or inhibition of hepatic glucose by stimulating GK (Hepatic glucokinase) activity (Lee and Jeon, 2013). Dieckol isolated from brown seaweed significantly attenuated type II diabetes which was investigated in C57BL/KsJ-db/db mouse model. Dieckol was administered through intraperitoneal route at 10 mg/kg and 20 mg/kg dose for 14 days. The results show that reduced thiobarbituric acid reactive substances (TBARS), as well as increased activities of antioxidant enzymes including SOD, catalase (CAT) and GSH-px in liver tissues were observed in the dieckol administered group (Kang et al., 2013d). Even though the phlorotannins from *E. kurome* showed some amount of toxicity, their consumption might be useful in ameliorating diabetes-related complications (Xu et al., 2012).

Extracts from *Palmaria*, *Ascophyllum* and *Alaria* inhibited  $\alpha$ -amylase activity to some extent, but *Ascophyllum* extracts were very effective with an  $IC_{50}$  of approximately 0.1  $\mu$ g/mL gallic acid equivalent (GAE). The *Ascophyllum* extracts inhibited  $\alpha$ -glucosidase, another key enzyme involved in starch digestion and blood glucose regulation, at low levels, e.g.  $IC_{50}$  of approximately 20  $\mu$ g/mL GAE (Nwosu et al., 2011). It is evidenced that diphlorethohydroxycarmalol (DPHC) has the ability to reduce the effects of  $\alpha$ -glucosidase and  $\alpha$ -amylase. The  $IC_{50}$  values of DPHC against  $\alpha$ -glucosidase and  $\alpha$ -amylase were 0.16 and 0.53 mM, respectively. In addition to this, increased postprandial blood glucose levels were significantly suppressed in the DPHC-administered group than those in the streptozotocin-induced diabetic or normal mice. Moreover, the area under curve (AUC) was significantly reduced via DPHC administration (2022 versus 2210 mmol min/L) in the diabetic mice as well as it delayed the absorption of dietary carbohydrates (Toume et al., 2004; Lee et al., 2012). High glucose (30 mM) treatment induced the death of rat insulinoma cells, but treatment with 10 or 50  $\mu$ g/mL 6,6'-bieckol significantly inhibited the high glucose-induced glucotoxicity. In addition to this, usage of 6,6'-bieckol significantly reduced the level of TBARS, generation of intracellular ROS and NO level, all of which were increased at high glucose concentrations (Park et al., 2015).

### Radio-Protective Effects

Drug-induced toxicity such as ototoxicity from platinum-based compounds and aminoglycosides, often leads to hearing loss and hair loss. This may be caused because of the extensive formation of reactive species. Several small molecules have been used to suppress the reactive oxygen species to prevent further damages (Shin et al., 2014). Some of the compounds from marine resources have also been used such as dieckol and dioxinodehydroeckol (DHE) from *E. cava*. Chang et al. (2016) investigated drug-induced ototoxicity with dieckol in mice model; mice pre-treated with gentamicin were later exposed to dieckol for 48 h. Partial protection of gentamicin induced hair loss was observed after the treatment of dieckol (Chang et al., 2016). DHE has been explored for protective effect against UVB-induced damages in HaCaT cells. The cells were exposed to 20 mJ cm<sup>-2</sup> of UVB irradiation which is the minimal

erythema dose (MED) for individuals to be able to tan. Furthermore, the expression levels of Bax/Bcl-2 and caspase-3, -8, -9, which are the genes associated with apoptosis were investigated when cells were either treated with DHE doses after UVB irradiation or exposed to UVB only. Clear UVB-protective effects were observed in DHE-treated cells (Ryu et al., 2015). In another study, DPHC was used to protect from UVB-induced DNA damages in HaCaT cells (Piao et al., 2015).

Doxorubicin-induced hepatotoxicity was protected with several brown seaweed extracts (Jung et al., 2014b). In another study, Kang et al. (2013c) used zebrafish model to check the protective effect against ethanol-induced damages. Phloroglucinol, eckol and dieckol were used, and dieckol showed highest protective effect against ethanol-induced cell apoptosis. Furthermore, the dieckol-treated group scavenged intracellular ROS and prevented lipid peroxidation and ethanol-induced cell death in the zebrafish embryo (Kang et al., 2013c). From the same group, reports suggest that most of the phlorotannins possess noticeable free radical scavenging activity, which can be potentially useful in diseases concerned with oxidative stress (Kang et al., 2013b). Eckstolonol from *E. cava* showed the protective effective against UVB-induced ROS in human keratinocytes (HaCaTs). Cell viability was decreased by UVB radiation and restored by the treatment with different eckstolonol concentrations (0, 5, 50, 100, and 200  $\mu$ M). Furthermore, eckstolonol reduced UVB-induced ROS, lipid peroxidation, damaged DNA levels, and cell death (Jang et al., 2012). In another study, Heo et al. (2010) showed the UVB-protective effect of DPHC via damaged DNA tail length and morphological changes in fibroblast. Dieckol also showed protective effect from the UVB-induced damages (Heo et al., 2009), and phloroglucinol and eckol showed strong protective effects against gamma irradiated damages (Moon et al., 2008; Kang et al., 2010).

### Antiadipogenic Activity

Obesity is one of the most common health problems, which leads to several diseases such as type 2 diabetics, cardiovascular diseases, and hypertension. Increase in the number of mature adipocytes is the main cause of obesity (Kim et al., 2013a). Some of the phlorotannins have shown inhibitory activity towards adipogenesis and obesity. Kang et al. (2015) investigated the phlorotannin from *E. cava* for inhibitory effect of adipogenesis. The researchers also examined the adipogenic activities by measuring glycerol release level and adipogenic-related gene expression in differentiating 3T3-L1 preadipocytes. The phlorotannin increased glycerol secretion and reduced glucose consumption level in cells. In addition, effects on PPAR $\gamma$ , C/EBP $\alpha$  and differentiation-dependent factor 1/sterol regulatory element-binding protein 1c, as well as downstream genes such as fatty acid binding protein-4, fatty acid transport protein-1, fatty acid synthase, leptin and acyl-CoA synthetase 1 were also studied (Kong et al., 2015). In another study by Karadeniz et al. (2015), it was reported that phlorotannin suppressed adipogenesis in preadipocytes. Triphlorethol-A, eckol and dieckol were checked against the antiadipogenesis activity. Phlorotannin (20  $\mu$ M) reduced lipid accumulation and suppressed the adipogenic differentiation markers (Karadeniz et al., 2015). In another study, dieckol from *E. cava* suppressed lipid accumulation in animal model. Mice were used in the study with normal diet, high-fat diet and dieckol-treated groups. Dieckol-supplemented groups showed significant decreases in body weight gain (36%) when compared with the high-fat diet group. In addition, dieckol downregulated the adipogenic factors and decreased the triacylglycerol content in MT3-L1 cells (Choi et al., 2015).

Phloroglucinol, eckol, dieckol, dioxinodehydroeckol, and phlorofucofuroeckol A from *E. stolonifera* were isolated and checked for their abilities to inhibit adipogenesis over a range of concentrations (12.5–100.0  $\mu$ M). Phloroglucinol, eckol, and phlorofucofuroeckol A significantly and concentration-dependently inhibited lipid accumulation in 3T3-L1 cells without affecting cell viability. All the five compounds reduced the expression levels of several adipocyte marker genes, including PPAR $\gamma$  and CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ) (Jung et al., 2014a). In another study, dieckol showed adipogenesis inhibition and down-regulated the expression of PPAR $\gamma$ , CCAAT/enhancer-binding proteins (C/EBP $\alpha$ ), sterol regulatory element-binding protein 1 (SREBP1) and fatty acid binding protein 4 (FABP4) in a dose-dependent manner. The specific mechanism mediating the effects of dieckol was confirmed by AMP-activated protein kinase (AMPK) activation (Ko et al., 2013).

### Other Activities

Several other biological activities of phlorotannins have been reported earlier in the literature. These include sleep induction (Cho et al., 2012, 2014b), arousal inhibitory effect (Cho et al., 2014a), SARS-CoV 3CL inhibition (viral replication) (Park et al., 2013), antiviral activity (Kwon et al., 2013), anti-Alzheimer disease (Kang et al., 2013a), inhibitory effect against melanin synthesis (Kang et al., 2012c), neuroprotection (Kang et al., 2012b), hepatoprotection (Kang et al., 2012a; Kim et al., 2011b), osteogenesis (Ali and Hasan, 2012), neuraminidase inhibitory activity (Ryu et al., 2011), and inhibition of high glucose-induced oxidative stress (Lee et al., 2010). Other reported effects include antioxidant (Kim et al., 2010), anti-arthritis (Ryu et al., 2009), hepatocellular carcinoma inhibition (Yoon et al., 2008), inhibition of glycosidase (Shibata et al., 2002, 2003), antiplasma inhibitor (Fukuyama et al., 1989a, 1989b, 1990; Nakayama et al., 1989), carbolytic enzyme inhibition activity (Kellogg et al., 2014), antiallergic activity (Ahn et al., 2015; Sugiura et al., 2006, 2007; Le et al., 2009) and anti-HIV activity (Karadeniz et al., 2014; Ahn et al., 2004; Artan et al., 2008; Vo and Kim, 2010).

## Conclusion

To conclude, the information provided in this chapter point to phlorotannins as excellent materials to treat various diseases at a lower concentration due to their exceptional biological activity. Recently, considerable attention has been paid on natural materials for the development of cosmeceuticals, pharmaceuticals and nutraceuticals. Currently, few companies around the world have been utilizing the extract of phlorotannin for the development of cosmeceutical and nutraceutical products. However, the utilization of seaweed in many countries is not yet well-explored.

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<https://en.wikipedia.org/wiki/Phlorotannin>.

# Gamma-Aminobutyric Acid

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## Introduction

Gamma-aminobutyric acid (GABA) is a non-proteinogenic amino acid composed of four carbon atoms, which is widely present in bacteria, plants and vertebrates (Diana et al., 2014). GABA has multiple health-promoting properties including acceleration of the synthesis of proteins in the brain, increase of the secretion of growth hormones (Takahara et al., 1977; Tujioka et al., 2007, 2009; Powers et al., 2008; Suwanmanon and Hsieh, 2014), as well as anti-hypertensive, diuretic, anti-diabetic, anticancer, antihypercholesterolemic, and tranquilizer effects (Adeghate and Ponery, 2002; Huang et al., 2013; Roohinejad et al., 2009, 2010; Shimada et al., 2009; Suwanmanon and Hsieh, 2014). Some other studies have revealed the role of GABA as stress and anxiety reducer (Enna and McCarson, 2006; Lydiard, 2003). GABA is biosynthesized from glutamic acid by the irreversible decarboxylation of L-glutamic acid or its salts (Fig. 1). It is involved in the Krebs cycle metabolism when synthesized in plants and bacteria, and as neural signal transmitter in mammalian cells. Some physiological and metabolic processes of GABA were previously reviewed (Shelp et al., 1999). Due to its biological functions, GABA is largely employed in certain countries as a food additive or supplement (e.g. GABA is a GABA-rich chocolate product that has been exclusively commercialized in Japan by Glico Company).

The enzyme catalyzing the biosynthesis of GABA is glutamic acid decarboxylase (GAD; EC 4.1.1.15) (Fig. 1), which has been biochemically characterized (Nomura et al., 1999) and identified in numerous organisms including bacteria (e.g. lactic acid bacteria (LAB) (Bertoldi et al., 1999), *Aspergillus* (Kato et al., 2002), etc.), plants (e.g. tea (Zhao et al., 2011), cowpea (Johnson et al., 1997), etc.), mammalian animal brain (Nathan et al., 1994), and insects (Anthony et al., 1993). In this chapter, the main production processes of GABA will be discussed.

## GABA Production

Multiple GABA synthetic pathways have been proposed during the last decade. Most of them involve lactic acid bacteria (LAB)-producing strains. However, the synthetic pathways of GABA by other microorganisms (e.g., yeasts, other bacterial strains) as well as its production by plants have also been described (Diana et al., 2014). Current development of novel culturing techniques and genetic tools has led to optimize GABA production using microorganisms. These techniques include cell immobilization and co-culturing technologies as well as enhancement of growth conditions by the formulation of modified culture media (Kim et al., 2009a; Li and Cao, 2010; Seok et al., 2008).

## Chemical Synthesis of GABA

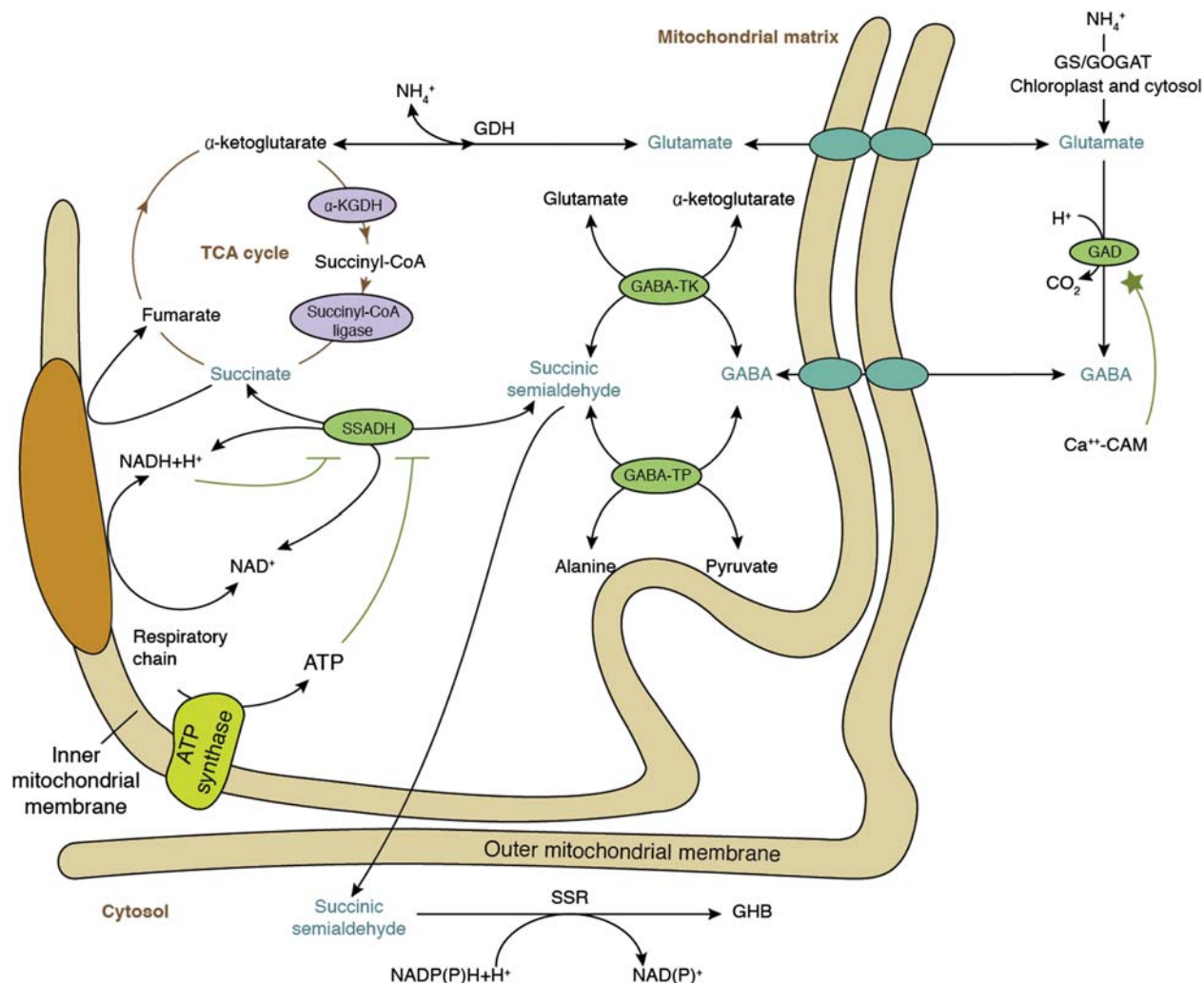
Recent efforts have been made for amplifying GABA production using various chemical processes from different bio-based or non-chemical precursors. Pure GABA is isolated as a white microcrystalline powder that melts at about 203.7 °C. Usually, about three or four chemical steps are required to obtain the target amino acid in good yields and sufficient purity. To date, many attempts have been concentrated to reduce the number of steps. Unfortunately, in the literature no real improvement of the process has been reported using organometallic complexes-catalyzed reactions. For example, a Pd/Cu methoxy carbonylation of *N*-propargylphthalimide derivative or a ruthenium complex catalyzed cross-metathesis between 2-propen-1-amine and acrylic acid can afford an unsaturated GABA precursor that needs further noble metal catalyzed reduction of their unsaturated bond in pressurized hydrogen atmosphere to lead to the target amino butyric acid (Ego et al., 1986; Robinson et al., 2014).

Typical chemical synthesis pathway involves the condensation of a 4-halobutyric acid ester on a source of nitrogen such as potassium phthalimide or ammonia aqueous solution. Besides, some results in a recent Chinese patent described the total GABA synthesis after 3 steps using the  $\gamma$ -butyrolactone (GBL) as the starting material (Fig. 2) (Zhiyong et al., 2013).

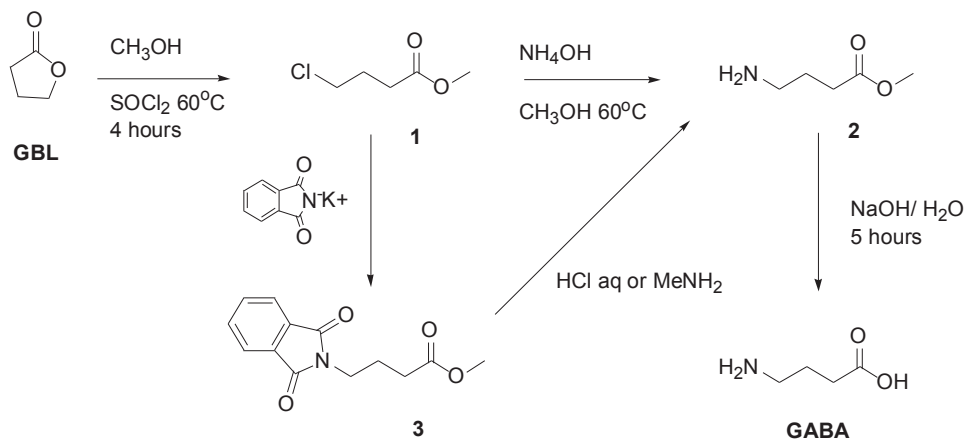
To access the GABA, GBL is first exposed to thionyl chloride (SOCl<sub>2</sub>) in methanol, amination of the resulted alkyl halide 3 then leads to the amino ester 2, which is generally saponified in presence of sodium hydroxide at room temperature. However, a pH adjustment is necessary to obtain the desired GABA under its dipolar ion, also called zwitterionic form. Interestingly, Talbot et al. (1958) have already reported a shorter pathway leading to GABA synthesis. They claimed that direct condensation of potassium phthalimide on GBL was possible without using a catalyst and gave the carboxylic acid derivative of compound 3. Thus, simple acidic treatment in hot hydrochloric acid of the *N*-protected amino acid afforded water-soluble GABA hydrochloride in solution after removal by filtration of the hydrophobic phthalates.

Instead of the 4-chlorobutyrate derivatives,  $\gamma$ -halobutyronitrile (compound 4 in Fig. 3) could react by the same manner with potassium phthalimide to produce a cyano derivative (compound 6) (Dewitt, 1943). When this compound is stirred in dilute sulfuric acid solution, simultaneously, the phthalimide protecting group is cleaved releasing the free primary amino group, and the cyano function is hydrolyzed into carboxylic acid group. Thus, GABA could be rapidly produced in good yields (Fig. 3).

On the above synthetic scheme, Nordcliffe et al. (2011) developed a strategy to produce GABA in mixture with traces of pyrrolidone, a five-member nitrogen rich heterocycle, starting this time from an alkyl azide derivative (compound 5 in Fig. 3).



**Figure 1** Metabolism and role of GABA. GS/GOGAT, glutamine-synthetase/glutamate-synthase cycle; GDH, glutamate dehydrogenase; GAD, glutamate decarboxylase (GAD); CaM, Ca<sup>2+</sup>-calmodulin complex; GABA-TK,  $\alpha$ -ketoglutarate-dependent GABA transaminase; GABA-TP, pyruvate-dependent GABA transaminase; SSADH, succinic semialdehyde dehydrogenase; TCA, tricarboxylic acid cycle;  $\alpha$ -KGDH,  $\alpha$ -ketoglutarate dehydrogenase; GHB,  $\gamma$ -hydroxybutyric acid; SSR, semialdehyde reductase. Reprinted with permission from Poojary, M.M., Dellarosa, N., Roohinejad, S., Koubaa, M., Tylewicz, T., Gómez-Galindo, F., Saraiva, J.A., Dalla Rosa, M., Barba, F.J., 2017. Influence of innovative processing on  $\gamma$ -aminobutyric acid (GABA) contents in plant food materials. *Compr. Rev. Food Sci. Food Saf.* 16, 895–905.



**Figure 2** Chemical synthesis of GABA from  $\gamma$ -butyrolactone (GBL).



The compound **5** is transformed into its 4-aminobutyronitrile counterparts through a reaction using sodium thiophosphate after 3 h reaction at 90 °C. GABA could then be obtained by further hydrolysis of the nitrile function.

Using shorter molecule; 2-bromopropanoic acid (compound **7** in Fig. 4), the halogen needs to be replaced by cyanide in order to extend the carbon chain (He et al., 2014; Nudelman et al., 2008). Obviously, the hazardous character of the cyanide could limit the industrial exploitation of this process. Only two steps are required and the second one is the reduction of the cyano moiety into primary amine group on platinum oxide in H<sub>2</sub> pressurized atmosphere.

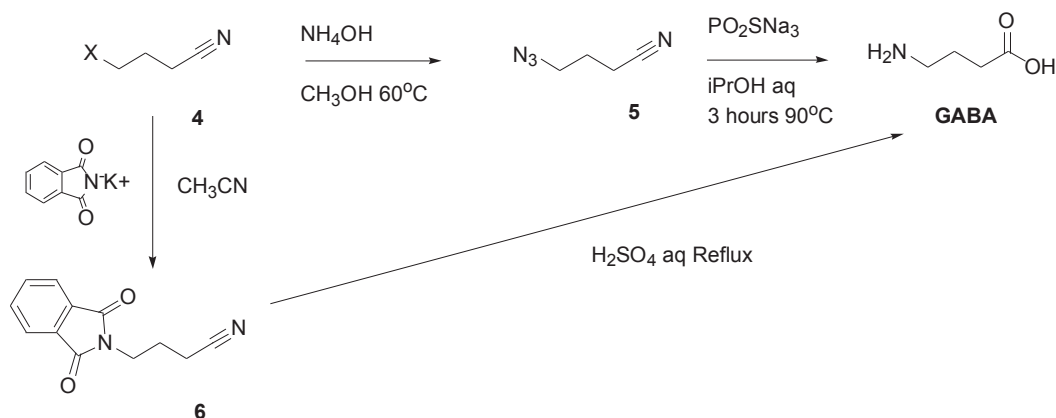
On the other hand, another patent reported direct oxidation of 4-aminobutanol into GABA in presence of sodium periodate (NaIO<sub>4</sub>) and sodium dichromate (Na<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>). Both are strong oxidative agents active in sulfuric acid aqueous solution (Alsters et al., 2001).

As another starting material, ornithine or 2,5-diaminopentanoic acid could be oxidized by osmium (VIII) in association with diperiodatocuprate (III) or diperiodato argentate (III) in alkaline aqueous solution (Abbar et al., 2010; Malode et al., 2010). In this case, the amino acid is subjected to successive oxidative deamination and decarboxylation leading to the production of GABA. Apparently, it seems to be difficult to transfer this reaction on an industrial scale due to the formation of by-products. On the other hand, some other works reported the ring opening reaction of a lactam or other types of heterocycles to get the desired GABA. Bon et al. (1994) started from a *N*-protected butyrolactame or more exactly the *N*-tosylpyrrolidone attacked by amino group of tertibutylamine in presence of 1.3 equivalents of aluminium chloride (AlCl<sub>3</sub>), a Lewis acid. Then, the ring was opened from its amide function and a disubstituted  $\gamma$ -aminobutylamide was formed prior to its protecting groups cleavage in hydrobromic acid phenolic aqueous solution. Compared to recent Chinese articles and patents (Wang et al., 2010; Zhan, 2005; Zhang et al., 2015), the method appeared to be less economical than the direct opening of pyrrolidone ring in refluxing water in alkaline condition (e.g., KOH, Ca(OH)<sub>2</sub>, NaOH, etc) or catalyzed by potassium fluoride (KF) supported on alumina (Al<sub>2</sub>O<sub>3</sub>). Here the cost of GABA production should be strongly correlated to the price of the lactam on the market.

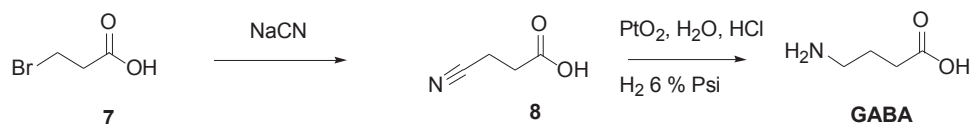
Starting from the commercial 4-hydroxyproline, Hausler (1987) reported the GABA synthesis, but, using a too long synthetic route that need to be improved to become industrially exploitable. Another nitrogen containing heterocycle called glutanamide (compound **10** in Fig. 5) could be used as a promoter to synthesize GABA. According to a patent proposed by Hua and Cai (1995), glutaric anhydride (compound **9** in Fig. 5) is allowed to stir in ammonia aqueous solution forming its useful corresponding imide (compound **10**) derivative, which is oxidized in presence of sodium hypochlorite (NaOCl) in sodium hydroxide aqueous solution. Unfortunately, from a carbon economy point of view, this process is not the greener reaction because of loss of one atom of carbon for each molecule of GABA formed during the opening of the ring.

### Microbial Production of GABA

For the GABA production, chemical synthesis is more and more rejected due to the corrosive reactants used and the health-related issues caused by the presence of toxic chemicals. Therefore, more “greener” methods are currently employed. Nevertheless, recovering GABA from naturally producing microorganisms remains difficult due to the low amounts of GABA accumulated in biological tissues.

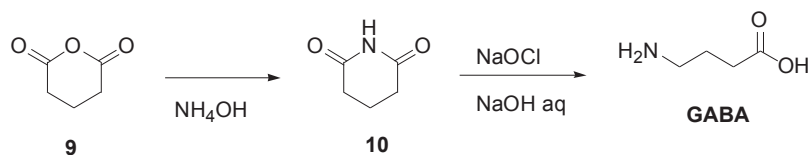


**Figure 3** Chemical synthesis of GABA from  $\gamma$ -halobutyronitrile.



**Figure 4** Chemical synthesis of GABA from 2-bromopropanoic acid.





**Figure 5** Chemical synthesis of GABA from glutaric anhydride.

Numerous works have reported the presence of GAD enzyme in LAB cells and their ability to produce high concentrations of GABA. For instance, concentrations from 15 to 63 mg/kg have been reported when culturing LAB (e.g., *Lactobacillus brevis* PM17, *Lactobacillus plantarum* C48, *Lactobacillus paracasei* PF6, *Lactobacillus delbrueckii* sbsp. bulgaricus PR1 and *Lactococcus lactis* PU1) in different media (Siragusa et al., 2007). *L. brevis* strains have been widely investigated for the production of GABA. Park and Oh (2005) and Park and Oh (2007) investigated the production of GABA from the *L. brevis* strains OPY-1 and OPK-3, and found respectively 0.825 g/L and 2.023 g/L. Other strains of *L. brevis*; NCL912 and GABA057 produced respectively 35.66 g/L and 23.40 g/L (Li et al., 2010). In addition, fermentation of black raspberry juice (Kim et al., 2009b) and sea tangle solution (Baejin et al., 2010) using *L. brevis* strains has been reported to produce 27.6 mg/mL and 2.465 mg/L, respectively. Higher concentrations were obtained using other strains of *L. brevis* such as that reported by Yokoyama et al. (2002) producing 1.049 g/L, while, the highest one reported was 76.36 g/L (Huang et al., 2007a, 2007b), which shows the interesting production way of GABA that could be scaled-up. These bacteria were isolated from different sources including kimchi (Cho et al., 2007; Lu et al., 2008; Seok et al., 2008), koumiss (Sun et al., 2009), cheese starter (Nomura et al., 1998), and red seaweed beverage (Ratanaburee et al., 2011). Obviously, obtaining high concentrations of GABA in fermented food is highly correlated to the concentration of glutamic acid and the activity of GAD enzyme.

In addition to LAB, other microorganisms such as filamentous fungi (e.g., *Aspergillus nidulans*, *Aspergillus niger* and *Neurospora crassa*, etc.) have been also reported as producers of GABA (Kubicek et al., 1979; Schmit and Brody, 1975). More recently, production of GABA using *Monascus purpureus* by fermenting rice and nutrient media (Jannoey et al., 2010; Su et al., 2003), as well as using *Rhizopus microsporus* strains by fermenting soybeans (Aoki et al., 2003) has been demonstrated. Some other authors have investigated the isolation of GABA producer microorganisms from marine environment, such as *Pichia* (Guo et al., 2009), *Saccharomyces* (Masuda et al., 2008), and some pseudomonas species (Mountfort and Pybus, 1992).

### Production of GABA by Plants

Producing GABA by plants remains less interesting than that of microorganisms due to the low concentrations accumulated and the unclear mechanisms of production. Many research works have reported that GABA could be accumulated after exposure to stress factors or infection by some fungal species (Solomon and Oliver, 2001). For instance, GABA level was reported to increase 20 to 40 times within 5 min following either a cold shock or mechanical manipulation (Wallace et al., 1984).

Accumulation of GABA was also observed during the developmental stages of plants, their differentiation, and their reproduction (Gallego et al., 1995; Molina-Rueda et al., 2015; Yang, 2003; Roohinejad et al., 2009; Nikmaram et al., 2017). Also, it plays a crucial role in the regulation of pH, where increased levels of  $\text{H}^+$  and  $\text{Ca}^{2+}$  have been reported as GAD enzyme activators (Poojary et al., 2017). Another factor influencing GABA accumulation in plants is culture conditions, which was previously observed during germination of fava beans (Li et al., 2010) and brown rice (Roohinejad et al., 2011; Banchuen et al., 2010; Oh, 2003).

### GABA Extraction and Purification

GABA was mainly extracted and purified from fermented broth cultures, which allows the recovery of higher quantities than that naturally accumulated. For instance, high concentration of GABA ( $997 \pm 51$  mM) was first obtained by fermentation using *L. brevis* NCL912. Sequential recovery steps of GABA including centrifugation, filtration, decoloration, desalination, ion-exchange chromatography (IEC), and crystallization were performed. Using this methodology, the authors were successful to reach approx. 50% recovery yield. The purity of the extract was  $98.66 \pm 2.36\%$ , determined using thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) (Li et al., 2011).

In another work, GABA was produced by fermentation of defatted rice bran extract using *L. brevis* VTTC-B-454 (Tuan et al., 2017). Rice bran, used as substrate in this study, contains high concentrations of nutrient compounds, including glutamic acid that could be converted into GABA using LAB. After fermentation, GABA was purified using cation exchange resin. Results showed that a GABA recovery yield of 84.2% with a purity of 44% was obtained using an eluent with a pH of 6.5 and a concentration of 0.8 M NaCl. The authors concluded about the feasibility of using cation exchange resin for GABA purification from fermentation broth.

In another recent work, GABA was produced by fermentation using *Enterococcus raffinosus* TCCC11660 strain, and then separated and purified by flocculation and chromatographic methodologies (Gao et al., 2013). GABA concentration in the fermentation broth was 31.2 g/L. A flocculation step was first performed using chitosan and sodium alginate in order to remove the impurities. Following this pre-treatment, ultrafiltration followed by two discontinuous diafiltration steps was used in order to remove macromolecules. The resulting permeate was then loaded into DA201-CII resin for further decoloration. GABA was finally purified

using ion exchange chromatography with Amberlite 200C resin. GABA crystals with high purity (99.1%) were then obtained by precipitation using ethanol. The authors concluded that their approach is promising for possible scaling-up of GABA extraction and purification from fermentation broth.

When GABA content is relatively low in human diet, its intake via GABA-enriched foods is required (Oh et al., 2003). Numerous GABA-enriched products are currently commercialized including fermented dairy products (Hayakawa et al., 2004), soy sauces (Yamakoshi et al., 2007), cheeses (Siragusa et al., 2007), and many others, which were very well reviewed recently (Diana et al., 2014). Some of the health-related benefits of GABA-enriched foods have been reported in the literature, which were either tested on human or animals. For instance, antihypertensive effects and other benefits have been demonstrated either on animals or humans following the administration of GABA enriched foods such as fermented milk, soymilk drink, soy sauce, green tea, purple sweet potato fermented milk, cheese, fermented vinegar, etc (Hayakawa et al., 2004; Inoue et al., 2003; Joye et al., 2011; Liu et al., 2011; Shizuka et al., 2004).

## Conclusion

Several GABA synthetic pathways have been reported during the last decade. GABA has been synthesized from different materials such as  $\gamma$ -butyrolactone (GBL),  $\gamma$ -halobutyronitrile, 2-bromopropanoic acid, and glutaric anhydride. However, chemical synthesis of GABA has been rejected due to the corrosive reactants used and the health-related issues caused by the presence of toxic chemicals. Thus, more “greener” methods such as GABA production using microorganisms or in plants have been suggested. GABA produced by these methods needs to be extracted and purified to allow recovery of higher quantities than that naturally accumulated. However, numerous health-promoting GABA-enriched foods have been produced and commercialized, especially fermented products, which are used to supplement the low GABA content in human diets. Although these achievements in GABA production and food use have been demonstrated, further research works need to be conducted to better understand the mechanisms involved in enhancing the accumulation of GABA in plants or its production by microorganisms.

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## Phenolic Acids

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### Introduction

Phenolic acids belong to a highly diversified group of phytochemicals, phenolics which are found in all foods of plant origin in the human diet. Phenolics are secondary metabolites composed of an aromatic ring bearing one or more hydroxyl substituents together with a number of other side groups (Shahidi and Yeo, 2016; Shahidi and Nazck, 2004). Phenolic compounds most widely occurring in plants include phenolic acids, flavonoids, coumarins, stilbenes, tannins, lignans and lignins (Nazck and Shahidi, 2006).

Phenolics are synthesized as first line of defense chemical compounds against biotic (infections, wounding) and abiotic (nutrient deficiencies or excess, cold and visible light) stresses of plants (Beckman, 2000; Beggs et al., 1987; Christie et al., 1991; Graham, 1991; Hahlbrock and Scheel, 1989). In addition, phenolics contribute to different attributes of foods such as bitterness, astringency, color, flavor, odour and stability against lipid oxidation (Shahidi and Nazck, 2004). Phenolic acids possess a myriad of health benefits such as anti-inflammatory, antibacterial, antiproliferative, anticarcinogenic and antioxidative activities (Ambriz-Pérez et al., 2016; Cheng et al., 2007). *In vivo* and epidemiological studies suggest that phenolic compounds through their antioxidant properties may exert health benefits thus ameliorating chronic diseases associated with oxidative damage (Arts and Hollman, 2005; Kennedy, 2014; Kris-Etherton et al., 2002; Slavin, 2003). Dietary phenolics exert their health benefits by various biological activities such as free radical scavenging, metal chelation, reducing potential, chain breaking, and modulation of enzymatic activity as well as alteration of signal transduction pathways (Cheng et al., 2007; Rice-Evans et al., 1996; Reiners et al., 1999).

In general, the phenolic profile of a plant food is species specific (Mekeehen et al., 1999). However, the level of individual phenolic compounds available in a given species of plant material depends on a number of factors such as cultivar, environmental conditions, cultural, and postharvest practices, as well as processing and storage conditions (Abdel-Aal and Hucl, 1999; Chethan and Malleshi, 2007; Chandrasekara and Shahidi, 2011a; Kumari et al., 2017; Levakova and Lacko-bartošová, 2017; Li and Zhang, 2001; Rao and Muralikrishna, 2002; Siebenhandl et al., 2007; Yang et al., 2001; Yu et al., 2002; Zhou et al., 2004).

This review discusses phenolic acids and their derivatives for potential *in vitro* and *in vivo* bioactivities. Food sources of phenolic acids are reviewed first, followed by discussion of the extraction methods of different forms of phenolic acids from food materials. *In vitro* antioxidant activities and structural significance of compounds of interest are discussed. Finally, *in vivo* studies on the bioactivities of phenolic acids are presented.

### Chemistry and Overview of Phenolic Acids

Two classes of phenolic acids based on C1–C6 and C3–C6 backbones are hydroxybenzoic acids and hydroxycinnamic acids, which are ubiquitously found in plant materials at varying levels (Shahidi and Nazck, 2004). Hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, vanillic, syringic, and protocatechuic acids (Fig. 1). The hydroxycinnamic acids commonly found in foods and beverages are *p*-coumaric, caffeic, ferulic, sinapic and cinnamic acids (Fig. 2).

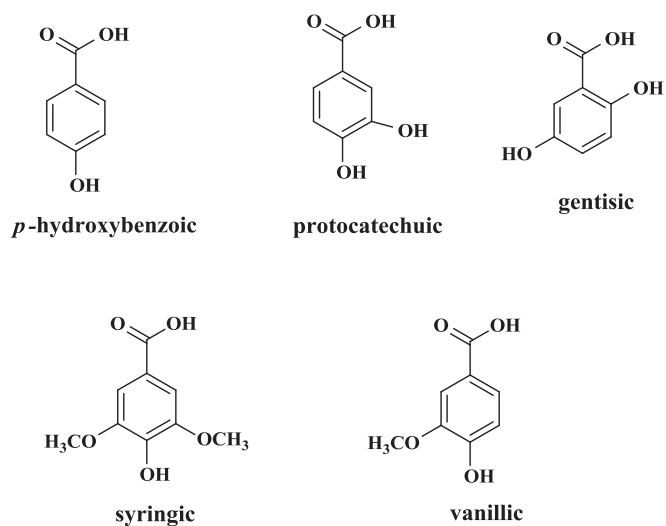
Phenolic compounds are derived from a limited pool of biosynthetic precursors such as pyruvate, acetate, a few amino acids, acetyl CoA and malonyl CoA via the action of pentose phosphate, shikimate, and phenylpropanoid metabolism pathways (Robards et al., 1999; Randhir et al., 2004). Two main amino acids involved in the synthesis of phenolics in plants include phenylalanine and to a lesser extent tyrosine (Shahidi, 2002; Herrmann, 1989).

Phenolics which are extractable into aqueous or aqueous - organic solvent mixtures are generally referred to as soluble phenolics and these includes phenolic compounds existing in the free, non-conjugated form as well as phenolic compounds conjugated to soluble carbohydrates by ester (esterified) and ether (etherified) bonds (Fig. 3; Shahidi and Yeo, 2016; Shahidi and Nazck, 2004). Furthermore, the left over residue after extraction of the soluble phenolics can be used to obtain insoluble bound phenolic compounds, especially hydroxycinnamic acids which are mainly esterified to the sugar residues of polysaccharides providing cross-linking between cell wall polymers (Ishii and Hiroi, 1990). They also form ether bonds and C–C linkages with lignins (Grabber et al., 2000). Some studies have shown that conjugated and insoluble bound phenolics may be released at the variable alkaline and acidic gastro-intestinal conditions and under colonic fermentation and may impart health benefits even at the local sites such as intestinal epithelium and beyond after absorption (Andreassen et al., 2001a; Chandrasekara and Shahidi, 2012).

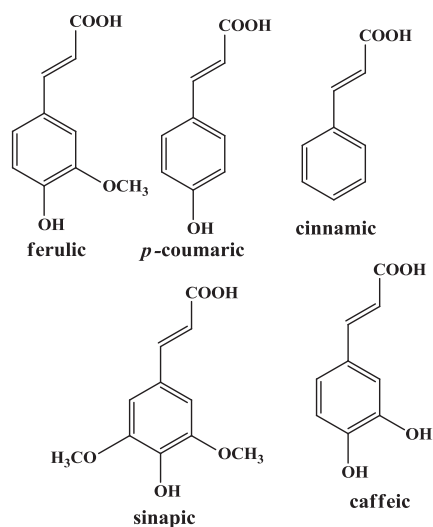
Cereals contain mainly free and conjugated forms of phenolic acids, which include derivatives of hydroxybenzoic and hydroxycinnamic acids (Chandrasekara and Shahidi, 2011a). The occurrence of phenolic acids in different food groups is presented in Table 1.

### Extraction and Analysis of Phenolic Acids

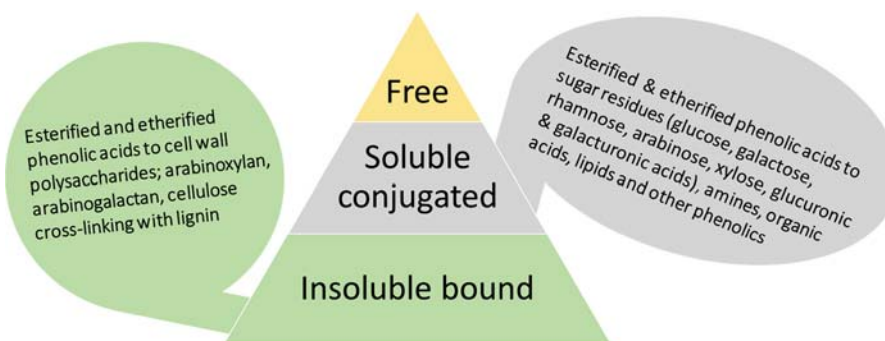
The identification of phenolic acids depends on the ability to isolate them from plant tissues. In general, the structural diversity of the phenolic compounds makes the analysis difficult and complicated. Several phenolic compounds are easily hydrolyzed, oxidized



**Figure 1** Hydroxybenzoic acids.



**Figure 2** Hydroxycinnamic acids.



**Figure 3** Schematic presentation of phenolic acids occurrence in cereal grains.



**Table 1** Representative Food Sources of Phenolic acids

Food source	Phenolic acids and derivatives	References
<b>Cereals</b>		
Barley	ferulic, <i>p</i> -coumaric, caffeic, chlorogenic acids, 8- <i>O</i> -4 diFA, 5-5 diFA, 8-5 diFA (benzofuran) 8-5 diFA	Yan et al. (2001); Hernanz et al. (2001)
Corn	ferulic, <i>p</i> -coumaric, 8-5 diFA, 8-8 diFA (cyclic), 8- <i>O</i> -4 diFA,	Bunzel et al. (2008)
Rice	ferulic, <i>p</i> -coumaric, caffeic acids, 5-5 diFA, 5-8 diFA (benzofuran form), 8-5 diFA, 8-8 diFA, 8- <i>O</i> -4 diFA, 4- <i>O</i> -5 diFA, 6'- <i>O</i> -( <i>E</i> )-feruloylsucrose,	Bunzel et al. (2000, 2002); Zhou et al. (2004); Tian et al. (2004)
Rye	ferulic, caffeic, <i>p</i> -coumaric, sinapic acids, 8- <i>O</i> -4 diFA	Weidner et al. (1999); Zielinski et al. (2001); Andreassen et al. (2001a); Amarowicz and Weidner (2001)
Wheat	ferulic, caffeic, <i>p</i> -coumaric, sinapic acids, campestanil, sitostanyl (steryl ferulates), 8-8 diFA (cyclic), 5-8 diFA, 5-5 diFA, 8- <i>O</i> -4 diFA, 8-5 diFA (benzofuran)	Sosulski et al. (1982); Hakala et al. (2002); Parker et al. (2005); Liyana-Pathirana and Shahidi (2005, 2006); Liyana-Pathirana et al. (2006)
Millet	ferulic, caffeic, <i>p</i> -coumaric, sinapic Gallic, <i>p</i> -hydroxybenzoic, protocatechuic, syringic, gentisic, vanillic acids	Rao and Muralikrishna, 2001, 2002, 2004; Chethan and Malleshi, 2007; Shobana et al., 2009; Vishwanath et al., 2009; Chandrasekara and Shahidi, 2011b
<b>Vegetables</b>		
Celery	caffeic, <i>p</i> -coumaric, ferulic, chlorogenic acids	Viña and Chaves (2007)
Lettuce	caffeoyl tartaric, chlorogenic, dicaffeoyl tartaric, isochlorogenic acids	Cantos et al. (2001)
Tomato	chlorogenic, caffeic, <i>p</i> -coumaric, ferulic acids	Martinez-Valverde et al. (2002)
<b>Oilseeds</b>		
Flax	<i>p</i> -coumaric, ferulic, caffeic acids	Kazimierz et al. (1984)
Mustard	<i>p</i> -coumaric, ferulic, caffeic, sinapic ( <i>trans</i> and <i>cis</i> ) acids	Kazimierz et al. (1984)
Rapeseed	ferulic, sinapic ( <i>trans</i> and <i>cis</i> ) acids, sinapine	Nazck and Shahidi (2006), Kazimierz et al. (1984), Larsen et al. (1983)
Safflower	<i>p</i> -coumaric, ferulic, caffeic acids	Kazimierz et al. (1984)
Sunflower	<i>p</i> -coumaric, ferulic, caffeic, chlorogenic acids	Kazimierz et al. (1984)
<b>Legumes</b>		
Beans (including soybeans)	ferulic, <i>p</i> -coumaric, sinapic, caffeic, chlorogenic; gallic, protocatechuic, vanillic, syringic, acids	Xu and Chang (2008); Garcia et al. (1998); Dabrowski and Sosulski (1984); Espinosa-Alonso et al. (2006); Diaz-Batalla et al. (2006); Luthria and Pastor-Corrales (2006); Ranilla et al. (2007)
Peas	caffeic, <i>p</i> -coumaric ( <i>trans</i> and <i>cis</i> ), ferulic, cinnamic, <i>trans p</i> -coumaroylmalic, <i>trans</i> feruloyl-malic acids	Cai et al. (2003); Duenas et al. (2004)
<b>Fruits</b>		
Apple	chlorogenic acids	Duda-Chodak et al., 2011; McGhie et al. (2005); Guyot et al. (1998)
Mangoes	Chlorogenic, gallic, vanillic, protocatechuic acids	Palafox-Carlos et al. (2012)
Grapes	<i>trans</i> -caftaric acid, <i>p</i> -coumaric ( <i>trans</i> and <i>cis</i> ) acids, gallic, protocatechuic, vanillic and syringic	Baderschneider and Winterhalter, 2001; Lu and Foo (1999)
Blackberries	gallic, caffeic, ferulic, <i>p</i> -coumaric, and ellagic	Hager et al. (2008)
Pears	chlorogenic, <i>p</i> -coumaroylquinic, <i>p</i> -coumaroylmalic, and dicaffeoylquinic acids	Simirgiotis et al. (2016); Schieber et al. (2001)

and subjected to isomerization (Jeandet et al., 1997), thus precautionary steps should be taken during sample handling to assure the integrity of the analyte (Montedoro et al., 1992a,b).

Sample preparation vary from a simple to a very complicated process depending on the nature and particle size of the food material, chemical nature of phenolic compounds, storage time and conditions and presence of substances that interfere with the isolation process (Nazck and Shahidi, 2006). Cereals, legumes, oilseeds, nuts, dried fruits and vegetables are ground to generate a powder of a defined particle size followed by defatting with an organic solvent such as hexane (Chandrasekara et al., 2016; Chandrasekara and Shahidi, 2011a,c; Liyana-Pathirana, and Shahidi, 2006; Madhujith and Shahidi, 2006a,b). Phenolic extracts obtained from the plant materials are a mixture of different classes of compounds, which are soluble in the solvent used (Nazck and Shahidi, 2006). Methods of extraction vary depending on the location of phenolic compounds as well as their occurrence as free, conjugated or bound compounds.

Solid-liquid extraction of free and soluble esters of phenolic acids is done with water as well as absolute and aqueous organic solvents. Different solvents are used such as hot water, methanol, ethanol, acetone, methanol-ammonia-water and ethyl acetate (Table 2). Reported extraction times vary with the technique, which include soxhlet extraction (Sun et al., 2001), vortexing followed by centrifugation (Montedoro et al., 1992a,b), ultrasound-assisted extraction (Chandrasekara and Shahidi, 2010), mechanical

stirring by homogenization (Chandrasekara et al., 2016; Krygier et al., 1982a,b), continuous rotary extraction (Guillen et al., 1996a,b), pressurized liquid extraction (Bonoli et al., 2004) and microwave-assisted extraction (Beejmohun et al., 2007; Pomponio et al., 2002) (Table 2). Ultrasound extraction is considered as an alternative method to classical extraction methods of phenolic compounds due to its high efficiency, low energy and water consumption (Wang et al., 2008). Ultrasound waves contribute to the disruption of the cell walls and reduction of the particle size. Furthermore, they enhance the mass transfer of the cell contents into the solvent through collapse of the bubbles produced by cavitation (Paniwnyk et al., 2001). Phenolic compounds released could vary with the extraction techniques used (Chandrasekara et al., 2016) (Table 3).

Insoluble residue left is separated by centrifugation or filtration. The extraction of phenolic compounds is influenced by ratio of solvent-to-sample (Nazck and Shahidi, 2006). Bound phenolic compounds are released by alkali, acid or enzymatic treatment (Table 4). Many studies have reported the use of alkaline hydrolysis which can be a rapid hydrolysis (from 1 to 4–6 h) or long hydrolysis, when the digestion time is more than 16 h (de Camargo et al., 2016; Sosulski et al., 1982; Maillard and Berset, 1995; Sun et al., 2001; Hernanz et al., 2001; Bonoli et al., 2004). A number of investigators have allowed alkaline hydrolysis reactions to occur at room temperature (Tian et al., 2004). Some studies were carried out in the dark and under inert atmosphere such as argon or nitrogen to minimize the isomerization of compounds such phenolic acids and other compounds (Kazimierz et al., 1984). Few studies have used acid hydrolysis for the recovery of bound phenolics (Bonoli et al., 2004).

Enzymatic reactions have been used to release bound phenolic acids. Enzymes such as amylases, pectinases and cellulases have been employed for the degradation of carbohydrate linkages (Yan et al., 2001; Zhou et al., 2004). Upon enzymatic hydrolysis, the phenolic acids are released by cleavage of an acetal or hemiacetal bond between carbohydrate moieties and the hydroxyl groups on the aromatic rings (Robbins, 2003).

**Table 2** Representative examples of sample preparation to determine free phenolic acids of cereals, legumes and oilseeds

<i>Food</i>	<i>Solvents</i>	<i>Extraction method</i>	<i>References</i>
Millet	80% Acetone	Sonication for 25 min at 60° C under reflux conditions	Chandrasekara and Shahidi, 2010
Barley	Hot water	Mix with hot water and heated in a boiling water bath for 1 h	Yan et al., 2001
Barley	80% MeOH	Under Reflux conditions in a water bath at 60 °C for 40 min	Madhujith and Shahidi, 2007
Wheat	80% EtOH	Continuous stirring at 4 °C for 16 h	Liyana-Pathirana et al., 2006
Wheat	Absolute EtOH	Soxhlet extractor under N gas for 3 h	Yu et al., 2001
Wheat bran	64% EtOH	Sonication for 25 min at 60 °C	Wang et al., 2008
Oats, corn, wheat, rice	80% chilled EtOH	Blending for 10 min	Adom and Liu, 2002
Oats	Absolute MeOH	Stirring for 2 h	Emmons et al., 1999
Oats, wheat, rye, barley buckwheat, corn	Methanol/10% acetic acid (85:15)	Homogenized and then ultrasonicated for 30 min	Mattila and Kumpulainen, 2002
Beach pea	70% acetone containing 1% conc HCl	Polytron homogenization for 1 min at 10000 rpm	Shahidi et al., 2001
Soybean, black soybean, adzuki bean mung bean	80% MeOH	Continuous shaking at 60 °C for 2 h	Lin and Lai, 2006
Cowpea	70% acetone	Continuous stirring at 25 °C for 24 h	Siddhuraju and Becker, 2007
Black bean seed coat	Chilled 80% acetone	Homogenized for 3 min using chilled warring blender then homogenized for 3 min with polytron homogenizer	Dong et al., 2007
Peas, chickpeas, soybeans	50% acetone	Shaken at 300 rpm at RT on an orbital shaker for 3 h then extracted for another 12 h in the dark	Xu and Chang, 2008
Rapeseed/canola hulls	70% acetone	Blending for 2 min at maximal speed at RT	Amarowicz and Weidner, 2001
Rapeseed	70% MeOH/70% acetone (1:1)	Homogenized by Polytron at RT	Krygier et al., 1982a,b
Flaxseed	70% MeOH supplemented with 1 M NaOH	Microwave assisted extraction for 3 min	Beejmohun et al., 2007
Canola	10% (w/w) ammonia in MeOH or 10% ammonia in MeOH containing 5% water After 15 min with hexane	Blending for 2 min at 15000 rpm in a Waring blender at RT	Naczck and Shahidi, 1989

RT, room temperature; EtOH, ethanol; MeOH, methanol.

**Table 3** Examples of sample preparation to determine bound phenolic acids of cereals, and legumes

Food	Method	Description	References
Barley	Acid hydrolysis	With 0.2 N H <sub>2</sub> SO <sub>4</sub> heated for 1 h in a boiling water bath. Reaction terminated by cooling in an ice-water bath for 10 min	Yan et al., 2001
Barley, rice	$\alpha$ -amylase hydrolysis	Samples were first acid hydrolyzed and then added 2% (w/v) $\alpha$ -amylase prepared in 2.5 M aqueous sodium acetate. Incubated for 1 h at 30 °C.	Zhou et al., 2004; Yan et al., 2001
Barley	Cellulase hydrolysis	First samples were hydrolyzed with acids and then with $\alpha$ -amylase. Then samples were added 2% (w/v) cellulase prepared in 0.1 M aqueous sodium acetate. Incubated for 10 h at 30 °C.	Yan et al., 2001
Oat, wheat, rye, barley, buckwheat, corn	Alkaline hydrolysis and acid hydrolysis	10 M NaOH stirred 16 h at 20 °C then conc. HCl 85° C for 30 min.	Mattila and Kumpulainen, 2002
Finger millet	Alkaline hydrolysis	Fat and free phenolics free dried samples were added 1 M NaOH containing 0.5% sodium borohydride under N gas	Rao and Muralikrishna, 2002
Barley	Alkaline hydrolysis	2 M NaOH at RT, shaken under N gas for two periods at 4th h and 20th h	Bonoli et al., 2004
Barley	Soft –acid hydrolysis	96% EtOH and 25% HCl at 65 °C for 30 min	Bonoli et al., 2004
Lentils	Acid hydrolysis	1 N HCl heated for 10 min in a steam bath	Sosulski and Dabrowski, 1984; Duenas et al., 2004

RT, room temperature; EtOH, ethanol.

After the extraction of phenolic compounds, the determination of their content may be carried out by spectrophotometric analysis (SA), high performance liquid chromatography (HPLC), or gas chromatography (GC). GC and HPLC techniques are widely used for both separation and quantification of phenolic compounds (Nazck and Shahidi, 2006).

HPLC is frequently used for the analysis of phenolic compounds. Examples for different procedures and detectors used for the analysis of food materials are given in Table 4. HPLC coupled with mass spectrometry, <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance spectrometry or infrared spectroscopy are used to identify structural characters of phenolic compounds (Beninger and Hosfield, 2003; Gujer et al., 1986; Kruger et al., 2003; Wu and Prior, 2005).

### Mechanism of Action *In Vitro* and *In Vivo*

Protective activities of phenolic compounds are due to three main different mechanisms, namely hydrogen atom transfer (HAT), electro transfer-proton transfer (ETPT) and sequential proton loss-electron transfer (SPLET) (Ajitha et al., 2012; Wright et al., 2001; Fiorucci et al., 2007; Litwinienko and Ingold, 2007; Tishchenko et al., 2008; Leopoldini et al., 2011). In the process of free radical scavenging, phenolic acids produce phenolic radicals and they can be stabilized by establishing intra-molecular hydrogen bonds and extending delocalization and conjugation of the electrons enhanced by resonance stabilization (Chen et al., 2015). Phenolic acids are absorbed when they are in free form and bound phenolic acids can be released in the body. Ferulic acid can be absorbed from rat stomach in its free form and is likely to be metabolized into conjugated ferulic acid in the liver (Zhao

**Table 4** Examples of HPLC procedures for determination of phenolic acids in cereals and legumes

Food	Phenolics	Stationary phase	Mobile phase and method	Detection	References
Millet	Phenolics	Supelcosil LC-18 (250 mm × 4.6 mm, i.d. 5 µm)	Gradient; methanol, acetonitrile, formic acid	Diode array (254–280 nm)	Chandrasekara and Shahidi, 2011b
Rice	Phenolic acids	Cosmosil 5C18 (250 mm × 4.6 mm, i.d. 5 µm)	Gradient; acetonitrile, H <sub>2</sub> O, TFA	Diode array (280–325 nm)	Tian et al., 2004
Rice	Phenolic acids	Alltima C18 (150 mm × 4.6 mm, i.d. 5 µm)	Gradient; acetic acid, H <sub>2</sub> O, MeOH, acetonitrile	UV–vis detector	Zhou et al., 2004
Barley	Phenolic acids	Zorbax SB-C18 (150 mm × 4.6 mm, id. 5 µm)	Gradient; acetic acid, H <sub>2</sub> O, acetonitrile	Diode array (280–325 nm)	Quinde-Axtell and Baik, 2006
Oat, rye, wheat, barley, buckwheat, millet, rice, corn	Phenolic acids	Inertsil ODS-3 (150 mm × 4 mm, i.d. 3 µm)	Gradient & Isocratic; H <sub>3</sub> PO <sub>4</sub> , acetonitrile	Diode array (254–329 nm)	Mattila and Kumpulainen, 2002

TFA, Trifluoroacetic acid; EtOH, ethanol; MeOH, methanol.

et al., 2004). In addition, excretion of ferulic acid in its sulphate and glucuronide forms in human subjects has been observed, thus suggesting its absorption and metabolism in the body (Virgili et al., 2000). Bound phenolic acids, especially hydroxycinnamates such as ferulic and *p*-coumaric acids in cereal grains are linked via an ester bond to the arabinoxylans in the plant cell wall (Boz, 2015). It has been reported that gastrointestinal esterase from intestinal mucosa and microflora (both human and rat) can release ferulic and diferulic acids from cereal bran (Andreasen et al., 2001b).

### ***In Vitro* Antioxidant Activities of Hydroxycinnamates**

Ferulic acid has been shown to be an effective antioxidant in several *in vitro* assays. It is also approved in some countries as an additive to prevent oxidation in foods (Graf, 1992; Itagaki et al., 2009). Ferulic acid has demonstrated *in vitro* free radical scavenging properties against hydroxyl radical, peroxynitrite and LDL oxidation (Yu et al., 1999; Kanski et al., 2002; Kikuzaki et al., 2002; Ogiwara et al., 2002). Kampa et al. (2004) showed time and dose-dependent inhibitory effects of caffeic acid, syringic acid, sinapic acid, protocatechuic acid, ferulic acid and 3,4-dihydroxy-phenylacetic acid (PAA) on growth of T47D human breast cancer cells. However, the antioxidative activity of these phenolic acids in T47D cells does not coincide with their inhibitory effect on tumoral proliferation suggesting a direct effect on anti-proliferation.

Caffeic acid and some of its derivatives, namely caffeic acid phenethyl ester (CAPE), rosmarinic acid, chlorogenic acid, and caffeic acid have shown potent antioxidant properties (Sudina et al., 1993; Kikuzaki et al., 2002; Gulcin, 2006). Caffeic acid has the ability of scavenging several reactive species, such as DPPH (Silva et al., 2000; Kikuzaki et al., 2002; Gulcin, 2006), peroxyl (Castelluccio et al., 1995), hydroxyl (Kono et al., 1997), superoxide radical anion (Sudina et al., 1993; Gulcin, 2006), peroxynitrite (Pannala et al., 1998), and singlet oxygen (Foley et al., 1999). Furthermore, caffeic acid demonstrated antimutagenic activity against nitrosamines (Kono et al., 1997; Nardini et al., 2001; Tapiero et al., 2002) and anti-inflammatory activity against 5- and 12-lipoxygenase (Koshihara et al., 1984; Sudina et al., 1993). Among several hydroxycinnamates, chlorogenic, and caffeic acids reported higher antioxidant activity than ferulic and *p*-coumaric acids in peroxidation of lipid systems mediated by metmyoglobin (Castelluccio et al., 1995).

A higher radical scavenging activity of chlorogenic and caffeic acids compared to *p*-coumaric can be explained by the arrangement of substituents in the molecule to favour the reactions with free radicals. In ferulic acid, the hydroxyl group at the third position of the benzene ring is methoxylated, which decreases the scavenging activity, compared to caffeic acid in which hydroxylation occurs in place of methoxylation. However, ferulic acid is more effective than *p*-coumaric acid due to its electron-donating methoxy group, which facilitates increased stabilization of the resulting phenoxyl radical following hydrogen donation by the hydroxyl group (Castelluccio et al., 1995).

Hydroxyl groups contribute to the antioxidant activity of phenolic acids and the number available for activity determines their potency as an effective antioxidant. Caffeic acid contains two hydroxyl groups whereas rosmarinic acid contains four hydroxyl groups and show high antioxidative efficiency (Chen and Ho, 1997). However, esterification of caffeic acid by a sugar moiety decreases its antioxidant activity (Cuvelier et al., 1992). This has been demonstrated using DPPH radical scavenging activity, and oxidation inhibition in an oil-in-water emulsion system (Chen and Ho, 1997).

Caffeic acid inhibit LDL oxidation (Nardini et al., 1995; Meyer et al., 1998; Wang and Goodman, 1999; Cartron et al., 2001). At a concentration of 7.5  $\mu$ M, caffeic acid showed 97.5% inhibition of copper-catalyzed human LDL oxidation (Meyer et al., 1998). Cheng et al. (2007) showed that caffeic acid and chlorogenic acid showed equally high antioxidant activities by inhibiting AAPH and copper-catalyzed human LDL peroxidation. Furthermore, they found that each molecule of caffeic and chlorogenic acid may trap about two peroxyl radicals, thus more active as antioxidants than other hydroxycinnamates such as sinapic, ferulic and *p*-coumaric acids.

Chlorogenic acid and caffeic acid have been shown to scavenge nitrogen sesquioxide (Kono et al., 1995a), organic free radicals, hypochlorous (Kono et al., 1995b), superoxide, hydroxyl radicals and peroxynitrite (Kono et al., 1997) as well as peroxyl radicals (Laranjinha et al., 1994; Kono et al., 1997) effectively *in vitro*; DNA is sensitive to peroxynitrite-mediated oxidative damage. Grace et al. (1998) showed that chlorogenic acid inhibited the formation of single strand breaks in supercoiled pBR322 DNA by acting as a peroxynitrite scavenger.

Caffeic acid had showed greater activity than ferulic acid in inhibiting the formation of N-nitroso compounds in simulated gastric fluid (Kuenzig et al., 1984). Bakalbassis et al. (2001) showed that the calculated heat of formation value in radical formation could be used to describe antioxidant activity of caffeic, ferulic, *p*-coumaric and sinapic acids. In accordance with experimental data, caffeic acid showed lower difference in the heat of formation of its phenoxyl radical, which could be raised from the less energy demanding hydrogen atom abstraction (Bakalbassis et al., 2001). This explained the high antioxidant activity of caffeic acid in DPPH radical scavenging system among other tested hydroxycinnamates such as sinapic, ferulic and *p*-coumaric acids (Bakalbassis et al., 2001).

### ***In Vivo* Bioactivities of Phenolic Acids and Derivatives**

Oxidative stress is known to involve in a number of non-communicable chronic diseases and neurodegenerative disorders. Antioxidants scavenge free radicals and reduce the incidence of oxidative stress-provoked damage, in addition to maintain cellular redox

balance (Chauhan and Chauhan, 2006; Di et al., 2012). Itagaki et al. (2009) demonstrated protective effects of ferulic acid on ischaemia-reperfusion (IR) associated intestinal injury using a rat model. The amount of lipid peroxide after IR was significantly reduced by treatment with ferulic acid (Itagaki et al., 2009). Elevated vascular permeability is one of the indicators of IR injury (Pompermayer et al., 2007) and ferulic acid prevented the elevation of vascular permeability following IR injury in the intestine. This could be due to the possible chain breaking activity ferulic acid that plays a protective role in oxidative injury.

Neuroprotective efficacy of vanillic acid against  $\beta$ -amyloid<sub>1-42</sub> peptide-induced oxidative stress, neuroinflammation and cognitive impairment was demonstrated using an *in vivo* Alzheimer's disease mouse model (Amin et al., 2017).  $\beta$ -amyloid peptide is known as a major component of senile plaques formed during the pathogenesis of Alzheimer's disease (Behl, 1999). The results showed that vanillic acid treatment (30 mg/kg intraperitoneally for 3 weeks) reversed cognitive deficits in A $\beta$ <sub>1-42</sub> treated mice. The investigators further showed that the antioxidant activity of vanillic acid treatment was associated with an increased expression of HO-1, which is mediated by the activation of Akt/GSK-3 $\beta$ /Nrf2 signaling pathway. In a previous study Yan et al. (2001) demonstrated chemopreventive activity of ferulic acid *in vivo* against Alzheimer's disease by resisting  $\beta$ -amyloid peptide-induced oxidative stress in a rat model. Results of this study further demonstrated that long-term administration of ferulic acid induces resistance to  $\beta$ -amyloid peptide toxicity in the brain, suggesting potency of ferulic acid as a protective agent against Alzheimer's disease.

Ferulic acid treatment has shown beneficial effects by attenuating oxidative stress in diabetic subjects (Balasubashini et al., 2004). A significant decrease in the levels of thiobarbituric acid reactive substances (TBARS), hydroperoxides and free fatty acids was demonstrated in the liver along with an increase in glutathione and antioxidant enzymes. The antioxidant enzymes include glutathione peroxidase, superoxide dismutase and catalase. Furthermore, Jung et al. (2007) reported the hypoglycemic effects of ferulic acid in a type 2 diabetic mice model. Ferulic acid oral administration for 17 days decreased blood glucose levels and increased plasma insulin levels. In addition, ferulic acid had significantly elevated hepatic glycogen synthesis and glucokinase activity of mice compared to those in the control group. Plasma total cholesterol and LDL cholesterol concentrations were significantly decreased by ferulic acid administration, suggesting the overall beneficial effects of ferulic acid for treatment of type 2 diabetes (Jung et al., 2007).

The topical application of caffeic and ferulic acids exerted a significant protection to the human skin against ultraviolet radiation-induced erythema (Saija et al., 2000). Ferulic acid showed a better protection to the skin than caffeic acid and these results are in line with the findings of an *in vitro* assay, which demonstrated better percutaneous absorption of ferulic acid than that of caffeic acid (Saija et al., 2000).

The effect of ferulic acid on blood pressure was investigated in spontaneously hypertensive rats (Suzuki et al., 2002). The oral administration of ferulic acid (1 to 100 mg/kg) significantly decreased systolic blood pressure in a dose-dependent manner. A 60 days treatment with syringic acid prevented diabetic cataract of rat lenses by inhibiting aldose reductase activity (AR) and gene expression (Wei et al., 2012). Syringic acid forms a complex with AR that may reduce the occurrence and progression of diabetic cataracts caused by glycometabolism dysfunction. Furthermore, investigation of the mechanism of syringic acid action showed that it downregulated mRNA expression of AR and inhibited AR activity in a noncompetitive and dose dependent manner (Wei et al., 2012).

Sinapic acid demonstrated anti-inflammatory potential using serotonin- and carrageenan-induced paw edema of mice (Yun et al., 2008). Suppression of the expressions of iNOS, cyclooxygenase-2, tumor necrosis factor-R and interleukin-1 $\beta$  via nuclear transcription factor kappa-B inactivation were responsible for the anti-inflammatory effects of sinapic acid. Nuclear transcription factor kappa-B plays an important role in the pathogenesis of inflammation.

Oral administration of *p*-coumaric acid (317 mg/day) to adult rats for 30 days significantly inhibited LDL oxidation and reduced LDL cholesterol levels by 33% in serum (Zang et al., 2000). The levels of high-density lipoprotein (HDL) cholesterol were not affected by the respective treatment of *p*-coumaric acid. Further, it was showed that *p*-coumaric acid significantly reduced the levels of 8-epiprostaglandin F<sub>2 $\alpha$</sub>  (8-EPI), by 60% in the serum. The 8-EPI, is a product of lipoprotein peroxidation and is reported to be a potent vasoconstrictor in rats and rabbits. This lipoprotein by-product can be used as a marker of oxidative stress in atherosclerosis and carcinogenesis (Morrow et al., 1990a,b). Chiang et al. (2014) showed that caffeic acid derivatives inhibit the growth of colon cancer through the involvement of phosphatidylinositol 3-kinases (PI3-K)/Akt, and AMP-activated protein kinase (AMPK) signaling pathways.

## Conclusions

Major plant food groups, in addition to the basic macro and micro nutrients provide phenolic compounds, among other bioactive phytochemicals that may provide beneficial health effects. Ferulic acid is the predominant phenolic acid reported continuously in all cereals. Generally, the major phenolic acids detected in legumes include ferulic acid, protocatechuic acid and *p*-coumaric acid. Phenolic acids in oilseeds include hydroxylated derivatives of benzoic and cinnamic acids. Sample preparation and extraction of phenolic compounds of food matrices have been done using different methodologies though a standardized procedure is not reported. Chromatographic and spectrometric methods are employed for the determination and quantification of phenolic acids in foods and beverages. Phenolic acids are effective antioxidant compounds that can attenuate chronic diseases associated with oxidative stress.



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# Phospholipids

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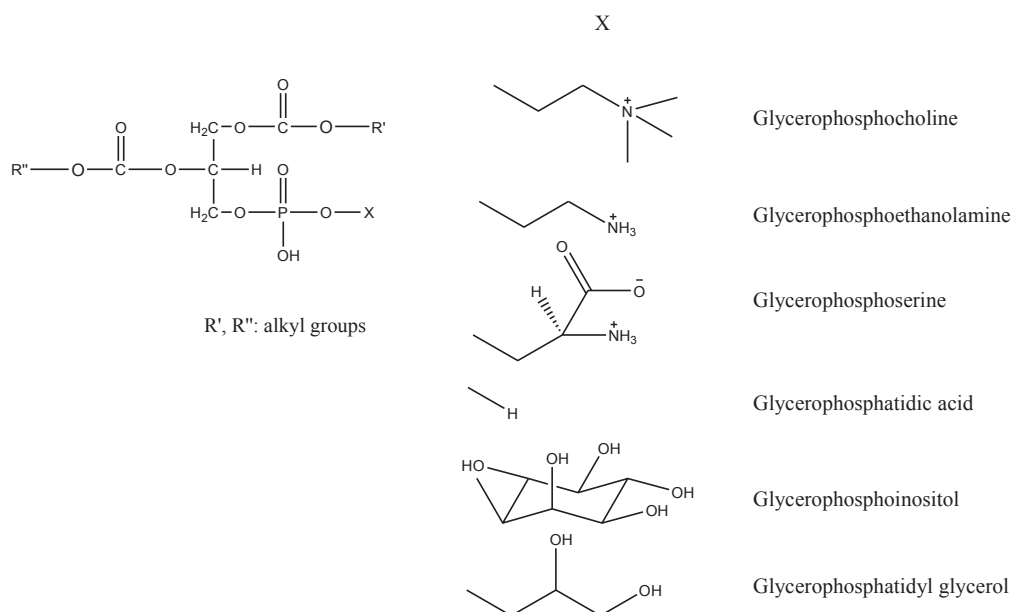
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## Structure and Occurrence

Phospholipids (PLs) are important components for human body, especially in cell membrane formation. They are amphiphilic lipids consisting of a glycerol backbone (glycerophospholipids, GPLs) or an amino-alcohol sphingosine backbone (sphingophospholipids, SPM), which is esterified with one or two fatty acids (FAs), a phosphate group and a hydrophilic residue such as choline, ethanolamine, inositol, etc (Fig. 1). The normal dietary intake of PLs is 2–8 g per day, which just represents a small part of PLs in the intestinal lumen while the rest is derived mostly from bile (Cohn et al., 2010; Iqbal and Hussain, 2009). Foods with a high PLs content are soybeans, egg yolk, milk, or marine organisms like fish, roe or krill. Lately, the PLs from marine organisms have captured increasing interest due to being abundant in health-beneficial omega-3 polyunsaturated fatty acids (n3-PUFAs) (Burri et al., 2012). PLs are supposed to be highly effective in delivering their FA residues for incorporation into the membranes and altering the FA composition of membrane PLs within a certain cell type (Küllenberg et al., 2012). Therefore, dietary supplementation of PLs carrying n-3 PUFAs is believed to contribute to a higher health benefit.

## Extraction

Extraction of PLs from food materials is the first step for their further analysis and utilization. For analysis purposes, the modified extraction procedures based on a mixture of chloroform/MeOH introduced by Folch et al. (1957) and Bligh and Dyer (1959) are most commonly used. In recent years, a low toxic extraction procedure employing methyl *tert*-butyl ether (MTBE) instead of chloroform, which was introduced by Matyash et al. (2008) is widely applied. Meanwhile, the direct extraction of lipids from food materials using single and mixed solvents is also used in some cases (Cajka and Fiehn, 2014). However, the aforementioned extraction methods are not applicable for food industrial use due to the high toxicity of chloroform and methanol. Thus, low or non-toxic organic solvents of relatively high polarity such as ethanol, acetone and ethyl acetate are more often used for edible PLs extraction. For example, a two-step solvent extraction using acetone and ethyl acetate as well as a supercritical CO<sub>2</sub> in combination with approximately 20% ethanol were used to extract PLs enriched oils from Antarctic krill (Sampalis, 2012; Bruheim et al., 2015). Single SC–CO<sub>2</sub> can not efficiently extract PLs from food materials, but can be used to remove neutral lipids from materials for extracting PLs with high purity by a subsequent extraction (Ali-Nehari and Chun, 2012).



**Figure 1** Structure of glycerophospholipids.

## Analysis

Recently, high performance liquid chromatography-mass spectrometry (HPLC–MS) has been used extensively for the quantification of phospholipids (PLs) according to both class and molecular species due to its greater specificity, selectivity, and, in many cases, better sensitivity than HPLC-ultraviolet detector (UV), HPLC-evaporative light scattering detector (ELSD), HPLC-charged aerosol detector (CAD) and  $^{31}\text{P}$  nuclear magnetic resonance (NMR) (Fong et al., 2013). Hydrophilic interaction chromatography-triple quadrupole tandem mass spectrometry (HILIC-QqQ-MS/MS) method is usually used to quantify PLs by class (Peterson and Cummings, 2006). This is because that HILIC can separate PLs by class, while QqQ-MS/MS can specifically detect a class of PLs by precursor-ion scanning (PIS) or neutral loss scanning (NLS) due to the loss of the head group (Peterson and Cummings, 2006; Nováková and Vlčková, 2009). However, there are no special requirements for the types of chromatographic column and MS for the quantification of PLs according to molecular species (lipidomics analysis). Reversed-phase HPLC, normal-phase HPLC, HILIC, and even direct-infusion without HPLC coupled to QqQ-MS/MS, quadrupole time-of-flight (Q-TOF)-MS/MS and Orbitrap MS are now widely used for lipidomics study. It is not feasible to get reference standards for each target PLs molecular species in food materials due to their unavailability or prohibitive price. Therefore, it is acceptable to quantify the individual molecular species using one corresponding standard for each lipid class through single-point internal standard method or standard curve method (Wang and Zhou, 2017).

In the intestinal lumen, dietary PLs are cleaved into 1-lysophospholipid and free fatty acids (FFAs) by activated pancreatic phospholipase  $A_2$  (Iqbal and Hussain, 2009). The resulting FFA is mainly re-synthesized into triacylglyceride (TAG) whereas 1-lysophospholipid is mainly re-synthesized into PLs before they are transferred into the lymph and the blood (Cohn et al., 2008). This means the fate of FAs at the *sn*-1/*sn*-2 position of oral PLs *in vivo* is different, which suggests that analysis of FA distribution in PLs is necessary for further revealing the health-beneficial effects of PLs-containing FFAs. Initially, an empirical rule that the *sn*-2 of GPL is the preferred position for the more unsaturated FFAs was used to determine the distribution of the two FFAs in the glycerol backbone of PLs (Napolitano et al., 1992). Then a phospholipase  $A_2$  hydrolysis and gas chromatography (GC) analysis procedure was used to determine the stereospecific distribution of the FFAs in the glycerol backbone of GPLs (Wang et al., 1997). However, this method still can not reveal the distribution of the two FFAs in the glycerol backbone of each individual PL.

## Health Benefits

PLs are essential in living organisms since they are the major component of double-layer cell membranes, containing hydrophobic fatty acyl chains on the inside and hydrophilic phosphate head groups on the membrane surface. In addition to their structural function, PLs are degraded by phospholipases to release FFAs, which are a source of cellular energy.  $\beta$ -oxidation of FFAs produces acetyl-CoA, nicotinamide adenine dinucleotide (NADH), and flavin adenine dinucleotide ( $\text{FADH}_2$ ), that are further metabolized via tricarboxylic acid (TCA) cycle and oxidative phosphorylation, to obtain energy molecule known as adenosine triphosphate (ATP). Another function of PLs is to regulate cell metabolism. PLs constitute a source of signaling molecules such as diacylglycerol (DAG), inositol phosphate, etc (Espinosa-Salinas et al., 2011). The detail of some major PLs is included.

Phosphatidylcholine (PC), the most abundant PL in mammalian cells, is de novo biosynthesized majorly via cytidine diphosphate (CDP)-choline pathway by a rate-limiting step, CTP:phosphocholine cytidyltransferase, and two other enzymes using choline and cytidine triphosphate (CTP) substrates. Only in the liver that PC is alternatively synthesized from the conversion of phosphatidylethanolamine (PE) via PE N-methyltransferase. PC plays a role in lipoprotein secretion. It is required for assembly and secretion of not only chylomicron and very low-density lipoprotein (VLDL) from the intestinal cells and the liver, respectively to the circulation (Van Der Veen et al., 2017), but also high-density lipoprotein (HDL) and low-density lipoprotein (LDL) via reverse PC transport to supply PC to the liver (Vance, 2008). PC also influences lipid droplet formation in the cells. Inhibition of PC biosynthesis during conditions that promote TAG storage increases the size of the lipid droplets presumably due to the ratio of the surface area (or the amount of phospholipid) to the volume (TAG) of the droplets. In addition, PC controls de novo lipogenesis via regulation of sterol regulatory element-binding proteins (SREBPs). Experimental studies have demonstrated SREBP-1 accumulation and increased lipogenic gene expression when PC synthesis was attenuated (Van Der Veen et al., 2017). In response to cell stimulation, PC also generates signaling molecules including diacylglycerol (DAG) via hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to an activation of protein kinase C (PKC) isoforms that have been implicated in the regulation of cell division (Cui and Houweling, 2002).

PE, the second most abundant phospholipid in mammalian cells, is synthesized via two major pathways: 1) CDP-ethanolamine pathway from ethanolamine and CTP substrates in endoplasmic reticulum, and 2) phosphatidylserine decarboxylase reaction via the conversion of phosphatidylserine (PS) in mitochondria (Patel and Witt, 2017). Based on charge-carrying structure of PE, it can bind to membrane proteins and assist their folding, introducing not only the change in physical structure, but also their functions. PE, which is normally enriched in the inner leaflet of the cell membrane, transiently accumulates on the external leaflet as an important factor regulating a late-stage cell division. The fusion of Golgi membranes that occurs after cell division also requires PE to increase the head group spacing between lipids on the membrane, allowing conformational changes during membrane fusion (Calzada et al., 2016). Regarding lipoprotein secretion, in addition to PC, PE also affects VLDL metabolism by stimulating VLDL removal from the circulation. In mitochondria, an increase in PE content, as well as a decrease in the PC/PE ratio, stimulates



mitochondrial respiration and activities of proteins of the electron transport chain to increase energy production (Van Der Veen et al., 2017).

PS is a minor membrane PL. In mammalian cells, it is synthesized by PS synthase-1 and PS synthase-2 from PC, and PE substrates, respectively. Since PC, PE, and PS are synthesized using one molecule or another as a substrate, perturbation of one PL contributes to cellular homeostasis of the others. PS synthase-1 mRNA is distributed through most murine tissues with highest expression in the liver, heart, brain, kidney and testis, while PS synthase-2 is only highly expressed in the testis. PS is mostly enriched in the inner leaflet of the plasma membrane and acts as an enzyme cofactor. One of the best-known roles of PS is as a specific activator of the classical PKC isoforms. PS induces the insertion of one of the protein domains to cell membrane, allowing the kinase to bind to DAG, thereby stimulating the enzyme activity. Another example is on the selective binding of heat-shock proteins (Hsps) on the target cell surface, which induces the formation of an ion conductance channel in the membrane and decreases cell viability. Exposure of PS on the cell surface also increases during the early-stage apoptosis. It makes apoptotic cells recognizable and subsequently removed by phagocytes (Vance and Steenbergen, 2005).

Phosphatidylinositol (PI) is also a minor constituent of plasma membrane. It is synthesized in the endoplasmic reticulum from CDP-DAG and myo-inositol substrates by a PI synthase (Balla, 2013). PI is a precursor of important signaling molecules, phosphoinositides, which are the products of PI phosphorylation at D3-, D4- or D5-positions of the inositol ring. The most abundant phosphoinositides are phosphatidylinositol 4,5-bisphosphate and its main precursor, phosphatidylinositol 4-phosphate. Phosphatidylinositol 4,5-bisphosphate is known as a substrate of phospholipase C, and class I phosphatidylinositol 3-kinases, which are enzymes producing major secondary messengers. In canonical mammalian pathway, phospholipase C produces two secondary messengers: 1) DAG and 2) inositol 3,4,5-trisphosphate, which contribute to the regulation of a wide-range of cellular functions such as cell homeostasis, fertilization, motility, sensory transduction, as well as the switch between proliferation and differentiation. In metazoan cells, phosphatidylinositol 4,5-bisphosphate is metabolized to phosphatidylinositol 3,4,5-trisphosphate by class I phosphatidylinositol 3-kinases. This major signaling pathway also involves regulation of various biological processes including cell directional movements, embryogenesis, immune response, neuronal patterning, and wound healing (Delage et al., 2013).

Although de novo synthesis of PLs occurs in the cells, experimental studies have demonstrated positive impacts of dietary PLs in different illnesses and symptoms, such as inflammation, cancer and coronary heart disease. This raises the possibility of using synthetic or naturally occurring PL isolates as nutraceuticals or functional foods. Beneficial effects of PLs can be partially explained by the effectiveness of PL in delivering their fatty acid residues and incorporating them into cell membranes that is involved in different diseases such as immune or cancer cells. Alteration of membrane composition is assumed to influence characteristics of cellular membrane including activity of membrane proteins, and biosynthesis of lipid secondary messengers (Küllenberg et al., 2012). The following examples demonstrate biological activities of dietary PLs. A PC-rich krill oil and soybean PC were shown to be effective in reducing inflammatory arthritis in patients and in chronic murine model, respectively (Deutsch, 2007; Erős et al., 2009). In addition, PLs improved anti-inflammatory and analgesic activity of nonsteroidal anti-inflammatory drugs in acute and chronic arthritis models by enhancing transport and bioavailability of the medicine (Lichtenberger et al., 2001, 2009). In a murine cancer model, PC showed a significant reduction of cancer cells by promoting apoptosis, and synergistically acted with menaquinone-4 (vitamin K2) to suppress nodule formation (precursors of hepatic cancer) and preneoplastic liver lesions (Sakakima et al., 2007). In case of patients with coronary artery disease, supplementation with krill oil changed blood lipid profile by reducing total cholesterol, LDL and TAG levels, while increasing HDL. The significant increase of blood HDL after PL supplementation could be attributed to the fact that PLs can be partially absorbed by the intestine and are preferentially incorporated into HDL (Küllenberg et al., 2012).

## **Oxidative/Antioxidative Properties**

In addition to health benefits, PLs, especially lecithins, are widely used as natural emulsifiers with gaining interest as they are natural antioxidants to control lipid oxidation. For example, sunflower lecithin was shown to possess better oxidative stability in oil-in-water emulsions than the system stabilized by synthetic food-grade surfactants, such as Tween 20 (Liang et al., 2017). In soybean oil, PC and other phospholipids components in the oil enhanced synergistic effect in inhibiting oxidation (Jiang et al., 2016). The antioxidant activity of the PLs has been attributed to their properties in free radical scavenging, metal ion chelation, the formation of specific structures that can prevent pro-oxidants interacting with lipids, as well as synergism between PL and other compositions in the food system (McClements and Decker, 2017). However, the presence of unsaturated fatty acids in dietary PLs, such as meats and dried milk products, makes them susceptible to lipid oxidation. Bulk oil, a heterogeneous system containing not only TAG, but also 200–800 ppm water and a variety of amphiphilic minor components such as monoacylglycerols (MAGs), DAGs, free FAs, PLs, phytosterols and oxidation products, is an example in which PLs act either as antioxidants or prooxidants depending on critical micelle concentration (Cui and Decker, 2016).



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## Phytochemicals and Hormonal Effects

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### Phytochemicals: A Brief Review

Naturally occurring chemical compounds in plants are called Phytochemicals. Several studies have shown the therapeutic potentials (Raul et al., 2016). They are widely distributed in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi, herbs and spices and in plant-based beverages such as wine and tea.

### Classifications

Phytochemicals can be classified into groups based on their chemical structure: alkaloids, terpenoids, sulfur-containing phytochemicals, carotenoids and polyphenols.

### Polyphenols

Polyphenols form a large part of the phytochemicals. They can be classified as flavonoids and non-flavonoids considering their chemical structures. Flavonoids are a large group of compounds found in many fruits, vegetables and legumes.

Flavonoids can be further divided into six subfamilies on the basis of differences in their molecular backbone structure: flavonols, flavones, flavanols, flavanones, anthocyanidins and isoflavonoids. Examples of flavonols, are quercetin (Lee et al., 2010; Hossion et al., 2011; Liu et al., 2010; Al-Saif et al., 2014), galangin (Cushnie and Lamb, 2006; Eumkeb et al., 2010; Pepeljnjak and Kosalec, 2004), kaempferol and myricetin (Ansari et al., 2015). Flavones are only found in citrus varieties as un-conjugated polymethyl-flavone (Hollman and Arts, 2000). Anthocyanins are a large group of compounds that are responsible for the colors of many flowers, vegetables, fruits and berries (Pazmiño-Durán et al., 2001). Flavanols are a large group of flavonoids, which include catechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate. These polyphenols are the most important constituents of tea leaves and appear to have great health benefits in humans (Daglia et al., 2014). Flavanones are mostly found in citrus fruits such as oranges, grapefruit and lemons. Isoflavones are mainly found in leguminous plants, especially soybean.

The non-flavonoid polyphenol groups include: phenolic acids, stilbenes, coumarins and tannins. Phenolic acids are plant metabolites widely spread throughout the plant kingdom. They are mainly contained in chokeberry, blueberry, dark plum and cherry among fruits, while among beverages the best sources of phenolic acids are coffee as well as green and black teas. Gallic acid is found in almost every plant part: bark, wood, leaf, fruit, root and seed, and in many common foodstuffs such as blueberry, blackberry, strawberry, plums, grapes, wine and tea among others (Daglia et al., 2014). Ferulic acid occurs in the seeds and leaves both in free form and covalently linked to lignin and other biopolymers. It is found in wheat and some vegetables and sweet corn (Balasubashini et al., 2003).

### Sources of Phytochemicals

There are various sources where phytochemicals can be obtained, including plant foods, medicinal plants and nutraceuticals.

### Plant Foods

Examples of phytochemicals derived from plant foods are; Ellagic acid conjugates are the major components of raspberry fruits, with other phenols, such as flavonoids and phenolic acids, occurring at significantly lower concentrations (Mazur et al., 2014). Epigallocatechin gallate is the ester of gallic acid and epigallocatechin. This polyphenolic compound is mostly present in green tea (Khan et al., 2006; Wang et al., 2012a,b). Quercetin, a compound present in many plants including apple, apricot, broccoli, spices, brussels sprout, cauliflower, grape, honey, kale, lettuce, lemon juice, citrus peel, onion, strawberry, wine, juice, wolfberry, black tea infusions, and tomato (Le et al., 2007; Hertog et al., 1992; Lin et al., 2007; Pellegrini et al., 2010; Petrus et al., 2011; Adefegha and Oboh, 2012; Ademosun et al., 2015a).

Genistein, an isoflavonoid compound, is found in some dietary plants such as alfalfa, soybean, fava bean, kudzu, psoralea, cowpea, yellow pea, green lentil, and lupine (Kaufman et al., 1997). Pterostilbene is a phenolic phytoalexin found in almond, rabbit, eye blueberry, and deerberry (Rimando et al., 2004; Xie and Bolling, 2014). Resveratrol is a stilbenoid and a type of natural phenolic compound, which is abundant in many fruits including blueberry, bilberry, cranberry, deerberry, lingonberry, partridge

berry, cocoa, grape, peanut, plum, red wine, and tomato (Sanders et al., 2000; Burns et al., 2000; Rimando et al., 2004; Ragab et al., 2006; Hurst et al., 2008; Sebastia et al., 2012). Ellagic acid, a natural phenol antioxidant, occurs in some fruits such as blackberry, green leafy vegetables, cloudberry, cranberry, strawberry, raspberry, wolfberry, grape seed, grape pulp, grape skin, pecan, and walnut (Daniel et al., 1989; Koponen et al., 2007; Bobinaite et al., 2012; Lee et al., 2012; Oboh et al., 2015a; Pantelic et al., 2016; Donno et al., 2016; Nwanna et al., 2016). Lycopene, a noncyclic carotenoid found in tomatoes (*Solanum lycopersicum*, Solanaceae), has received considerable scientific interest in recent years (Rao et al., 2006; Ademoseun et al., 2013).

## Medicinal Plants

Medicinal plants include various types of plants used as herbs and some of these plants have medicinal properties. These plants are considered as rich sources of phytochemicals, which can be used in drug development. Examples include; Gallic acid, which is found in almost every plant parts including: bark, wood, leaf, fruit, root and seed, and in many common food such as blueberry, blackberry, strawberry, plums, grapes, wine and tea among others (Oboh et al., 2016a; Daglia et al., 2014). Curcumin is a combination of three molecules: diferuloylmethane (1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione), p-dihydroxycinnamoylmethane (1,7-bis-(4-hydroxyphenyl)-hepta-1,6-diene-3,5-dione), and phydroxycinnamoylferuloylmethane (1-(4-hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione). This polyphenol is present in turmeric rhizomes (Ademiluyi et al., 2012; Kim et al., 2013; Akinyemi et al., 2017a).

Benzyl isothiocyanate is the myrosinase-induced hydrolysis product of benzyl glucosinolate (glucotropaeolin), which occurs in some cruciferous plants (for example, cabbage, horseradish root, and garden cress) and papaya seed (Bennett et al., 2004; Agneta et al., 2014; Nakamura et al., 2007; Williams et al., 2009). Phenethyl isothiocyanate, an isothiocyanate compound, is widely distributed as gluconasturtiin in some cruciferous plants, including broccoli, cabbage, cauliflower, kale, horseradish, kohlrabi, black radish, and watercress (Bennett et al., 2004; Gupta et al., 2014; Ares et al., 2014; Loebers et al., 2014; Ediage et al., 2011; Yi et al., 2015). Sulforaphane is an isothiocyanate compound found as glucoraphanin in cruciferous plants including broccoli sprout, mature broccoli, brussels sprouts, cabbage, cauliflower, kohlrabi, kale, radish, arugula, black radish, turnip, and rucola (Zhang et al., 1992; Bennett et al., 2004; West et al., 2004; Nakagawa et al., 2006; Higdon et al., 2007; Sivakumar et al., 2007; Ediage et al., 2011; Sasaki et al., 2012; Aires et al., 2013; Yi et al., 2015; Ku et al., 2016; Oboh et al., 2017). Indole-3-carbinol, a natural indole carbinol compound, is produced by the breakdown of glucobrassicin. It is present in cruciferous vegetables like broccoli, brussels sprouts, cauliflower, cabbage, horseradish, kale, and watercress (McNaughton and Marks, 2003; Higdon et al., 2007; Pellegrini et al., 2010; Loebers et al., 2014). 3,3'-Diindolylmethane, a dimer of I3C, is widely distributed as glucobrassicin in cruciferous vegetables such as broccoli, brussels sprouts, cauliflower, cabbage, horseradish, kale, and watercress (Kushad et al., 1999; Suzuki et al., 2006). Diallyl disulphide is a sulphur containing organic phytochemical. It is mostly found in garlic (Lee et al., 2003; Wang et al., 2015). Oleoanolic acid is a member of triterpenoids found in apple, grape, olive fruit and leaf, rosemary, sage, and virgin olive oil (Pensec et al., 2014).

## Nutraceuticals

Nutraceuticals are isolated food bioactive constituents that provides medical or health benefits, including the prevention and/or treatment of a disease. In other words, it provides medical or health benefits and helps in the prevention and treatment of diseases (Zeisel, 1999). The major active nutraceutical ingredients in plants are flavonoids. As is typical for phenolic compounds, they can act as potent antioxidants and metal chelators. Flavonoids such as quercetin have been reported to possess anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic properties (Pal et al., 2009; Oboh et al., 2016b).

## Metabolism and Bioavailability of Phytochemicals

Phytochemicals have preventive and therapeutic potential for diseases. Most of these compounds have low levels of solubility, stability, and bioavailability in the body. For instance, resveratrol found in grapes, epigallocatechin gallate found in green tea, quercetin found in red onions and curcumin found in turmeric, Ferulic acid found in wheat, which are known for the prevention and treatment of many pathologies.

## Resveratrol

Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a polyphenol abundant in the skin of red grapes and other fruits such as berries. Resveratrol possess two structural isomers: *cis*- and *trans*-resveratrol. Under UV exposure, *trans*-resveratrol is converted into *cis*-resveratrol (Orallo, 2006). Nanoencapsulation protects *trans*-resveratrol against light-exposure degradation and hence increases its stability (Sanna et al., 2012).

## Curcumin

A study reported that curcumin has a poor aqueous solubility, has low bioavailability and is quickly metabolized by hepatic enzymes in humans and research animals (Akinyemi et al., 2015; Bansal et al., 2011). The bioavailability of curcumin is low. After oral administration of 1 and 2 g/kg body weight of curcumin to rats, the peak blood concentrations detected after 48min were 1.4 and 3.7  $\mu$ M, respectively (Wahlstrom and Blennow, 1978). Nanoencapsulation increases curcumin bioactivities (Mohanty and Sahoo, 2010; Yallapu et al., 2010; Anand et al., 2010; Yekollu et al., 2011; Kim et al., 2011; Doggui et al., 2012; Wang et al., 2012a,b). Nanocurcumin significantly enhanced the inhibitory effect of curcumin on cancer cell viability, which is accompanied by an increase in curcumin uptake by cancer cells (Mohanty and Sahoo, 2010; Yallapu et al., 2010; Kim et al., 2011; Nair et al., 2012).

## Quercetin

Quercetin is a hydrophobic compound, and its solubility in an aqueous solution varies from 0.00215 g/L at 25 °C to 0.665 g/L at 140 °C (Srinivas et al., 2010). It was reported that quercetin has low aqueous solubility and bioavailability and is quickly metabolized in the body, which may reduce its efficacy as an application in preventing or treating diseases (Kumari et al., 2012; Oboh et al., 2016b). Quercetin has a high solubility in organic solvents such as ethanol, dimethyl sulfoxide (DMSO) and dimethyl formamide. The solubility of quercetin is approximately 2 g/L in ethanol and 30 g/L in DMSO at 25 °C (Srinivas et al., 2010). Quercetin in human bodies is rapidly metabolized by enzymes in the liver as well as the other organs or tissues (Hong and Mitchell, 2004), found that at least 21 metabolites of quercetin are detected in human urine after ingestion of quercetin glycosides from onions.

## Therapeutic Potentials of Phytochemicals

Phytochemicals are considered as potential agents against a wide range of diseases, such as neurodegenerative diseases, diabetes, cancer and cardiovascular disorders (Soobrattee et al., 2005; Oboh et al., 2016b). These phytochemicals are widely distributed in the plant kingdom and they are an integral part of the diets, with significant amount present in vegetables, fruits and beverages (Luximon-Ramma et al., 2005).

### Anticancer Effect

Free radicals are considered as important factors in the origin of cancer. Phytochemicals can exert anticancer activity, partially based on their ability to quench ROS and thereby protecting insulin (Roy et al., 2003). Phytochemicals may also interfere with intracellular signaling pathways, such as those, which regulate proliferation, induction of apoptosis and response to oxidative stress (Loo, 2003). Curcumin (Plummer, 1999), Gingerol (Bode et al., 2001) and citrus peel phenolic rich-extracts (Ademosun et al., 2015a,b) have been shown to possess anticancer effects.

### Antidiabetic Effect

Diabetes is the most common endocrine disorder characterized by the hyperglycemia, which causes overproduction of free radicals, thereby result in oxidative stress (Arango et al., 2000). This stress is an imbalance between the levels of prooxidants and antioxidants in the biological systems, leading to cellular injury (Villa-Caballero et al., 2000). Studies have shown that phenolics such as ferulic acid reduced blood glucose level (Villa-Caballero et al., 2000; Balasubashini et al., 2004; Noumura et al., 2003; Ohnishi et al., 2004). Quercetin and its derivative-rutin have also been reported to inhibit key enzymes linked to type-II diabetes in vitro (Oboh et al., 2015b).

### Neuroprotective Effect

An example of neurodegenerative disease is Alzheimer's disease (AD). This disease is an age associated dementing disorder, and many other neurodegenerative disorders, are characterized by free radical-mediated oxidative stress in the brain (Butterfield et al., 2002). Studies have shown that phytochemicals has been reported to act as a potent scavenger of ROS and RNS, thereby reducing the chance of free radical attack on proteins and hence preventing their oxidative modifications (Joshi et al., 2006; Oboh et al., 2016c). Curcumin has been reported to offer neuroprotective effect and was recently reported to synergize the anticholinesterase activity of donepezil-a major drug for AD (Akinyemi et al., 2017a). Proposed mechanism behind the neuroprotective properties of curcumin includes anticholinesterase, anti-inflammatory and antioxidant properties (Akinyemi et al., 2017a,b), as well as suppression of the acetylcholinesterase gene expression (Akinyemi et al., 2017b). Furthermore, caffeine and caffeic acid

(predominant phytochemicals in coffee and other beverages) have been reported extensively to offer neuroprotective properties in various experimental models essential via anticholinesterase, antioxidant and cytoprotective properties (Obboh et al., 2016a,b,c; Deshmukh et al., 2016; Akomolafe et al., 2017a, b; Winerdal et al., 2017).

## Sexual and Reproductive Hormones

Over the years, the use of plant-derived chemicals otherwise known as phytochemicals have been shown to influence human health most especially hormonal related diseases (Slavin et al., 1997; Manson et al., 2003). This is owing to the fact that hormonal imbalances i.e. too high or too low, or inability of the body to respond to hormones in the appropriate ways, which could be an indication of endocrine system disorder, is a hallmark of many human diseases such as infertility, diabetes, depression, among others (Verthelyi, 2001). In simple term, a hormone is a substance that affect physiological activity when get to the target site/cell differentiation from the producer cell via the bloodstream (Nelson et al., 2008). Basically, all animals/human hormones can be categorized into three: lipid-derived, amino acid-derived, and peptide hormones. Lipid-derived hormones differ from others in that is that they can diffuse across plasma membranes, whereas the amino acid-derived and peptide hormones cannot. Primarily, lipid hormones in humans belongs to the class of steroid hormones such as sex hormones e.g., estradiol (Adlercreutz, 1990; Behl, 2002). Sex hormones otherwise known as gonadocorticoids and gonadal steroid hormones establish their effects by interacting with vertebrate androgen or estrogen receptors, thereby mediating most of their biological activities via both genomic and nongenomic mechanisms (Handa et al., 1994). Earlier report has suggested that sex hormones also act via the thymus gland, and influence immune system (Ahmed et al., 1985). Sex hormones are of three types; androgens, oestrogen and progesterones.

## Sex Hormones

Androgen: a sex/steroid hormone that controls the growth and development of male characteristics, including the activity of the primary male sex organs (Lindzey et al., 1994; Debes and Tindall, 2004). Although, androgens are regarded as dominant male sex hormones, its presence has also been found in female vertebrates in varying degrees, and increase during puberty (Carlson, 2012). Example of androgens includes progesterone, dehydroepiandrosterone, androstenedione (A4), androstenediol (A5), androsterone, dihydrotestosterone (DHT). Androgens have been reported to be used in hormone replacement therapy to treat hypogonadism, while androgen deprivation therapy is used to treat prostate cancer (Kaufman and Graydon, 2004; Rhoden and Morgentaler, 2004; Sharifi et al., 2005).

Estrogen or oestrogen is the primary female sex hormone that is responsible for the development and regulation of the female reproductive system and sex characteristics. It is synthesized in all vertebrates and some insects, therefore, suggesting their ancient evolutionary history. The three major naturally occurring forms of estrogen in women are estrone (E1), estradiol (E2), and estriol (E3), estetrol (E4) is produced only during pregnancy (Burger, 2002). While estrogen is known as female hormones, it has also been found significantly amount in male vertebrates, carrying out important physiological roles (Lombardi et al., 2001). Among these, estriol is the most abundant, but also the weakest, while estradiol is the strongest with a potency of approximately 80 times that of estriol. However, all of them are synthesized from androgens, specifically testosterone and androstenedione, by the activity of the enzyme aromatase (Nelson and Bulun, 2001). Their medical uses in the past include the management of prostate cancer in men (Oh, 2002), breast cancer in women (Miller et al., 2002; Lumachi et al., 2011), induction of growth attenuation in tall girls (Gunther and Diekema, 2006). It has also been used in the treatments of the cognitive behavioral disorder and also women suffering from bulimia nervosa: an eating disorder characterized by binge eating followed by purging, which has been linked to a hormonal imbalance in the brain (Slade, 1982).

## Reproductive Hormones

Reproductive hormones play pivotal roles in the reproductive systems of both male and female puberty development, as well as their growth and sexual character. Although the synthesis of both hormones is stimulated by the hypothalamic gonadotropin releasing hormone (GnRH) and they are produced in the same cells, the expression and circulating levels of each hormone are distinct, in accordance with their different roles in reproductive function. In the female ovulatory cycle, FSH is responsible for inducing follicular growth, while also increasing estrogen production, while LH, apart from enhancing cholesterol availability for steroidogenesis, also signals ovulation following its peak mid-cycle. The reproductive hormones, including testosterone (T), and free testosterone (free T), follicle-stimulating hormone (FSH), luteinizing hormone (LH), sex hormone-binding globulin (SHBG), oestrogen (E2) are present in the broad range of tissues (Kjeldsen and Bonefeld-Jørgensen, 2013).



## Progesterone and Its Actions

Progesterone belongs to the class of steroid hormone, and is essential for reproductive functions in males and females of several species (Spark and Willis, 2012). Though, it is synthesized by the placenta and ovaries in the females, adrenal glands and testes in males, but it is involved in the regulation of other tissues, including the brain, breast, and bone (Singh and Su, 2013). Progesterone causes its effects by ligand binding to nuclear progesterone receptors (PRs), which subsequently binds to gene regulatory regions, including progesterone response elements (PREs) to initiate transcription of progesterone-responsive genes, and also through non-classical signaling pathways. Studies have shown that progesterone has other biological effects other than being a reproductive hormone. For example, it has been reported to possess neuroprotective potentials (Singh and Su, 2013), reduce the formation of lipid peroxidation, thus possess antioxidant activity (Roof et al., 1997), and reduced the expression of pro-inflammatory genes (Pettus et al., 2005), cardiovascular and venous systems and breast cancer in the climacteric and postmenopausal women (Schindler et al., 2003).

Follicle-stimulating hormone (FSH) is a glycoprotein hormone that plays a major role in the development and maturation of ovarian follicles before the release of an egg from one follicle at ovulation, increases oestradiol production, and the secretion of gonadal hormones (Kim et al., 2013). In men, FSH acts on the testes' Sertoli cells to stimulate production of sperm otherwise known as spermatogenesis (De Kretser et al., 1998). Although, it plays a major sex-related role, but it's usually not regarded as sex hormone. FSH is synthesized and secreted by the gonadotropic cells of the anterior pituitary gland (Nett et al., 2002) and regulates the development, growth, pubertal maturation, and reproductive processes of the body (Lincoln, 1998). FSH and luteinizing hormone (LH) are structurally related, and also work together in the reproductive system (Smitz et al., 2016).

Luteinizing hormone (LH) is another gonadotropic hormone, produced by gonadotropic cells of the anterior pituitary gland (Soldani et al., 1995). LH triggers ovulation and development of the corpus luteum, and also involved in the stimulation of Leydig cell production of testosterone (Lin et al., 2001; Acosta and Miyamoto, 2004). LH acts synergistically with FSH, which is frequently used in assisted reproductive technology (ART), in the stimulation of follicular growth and ovulation (Raju et al., 2013).

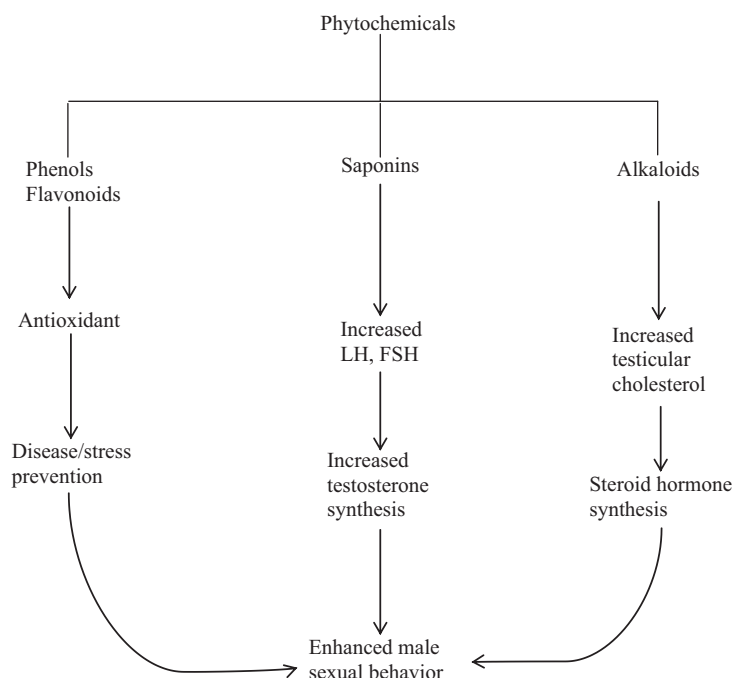
## Enhancement of Reproductive Hormones by Phytochemicals

Men develop and experience premature coronary artery disease earlier than women, increasing the risk of cardiovascular mortality by more than twofold. This led to the belief that testosterone exerts a detrimental influence upon the cardiovascular system; however, evidence has emerged over recent years to suggest that a number of the cellular mechanisms associated with the atherosclerotic process are beneficially modulated by testosterone. Indeed, it is well established that total and biologically available testosterone in men decreases with age and the age-associated decline may be related to the increased prevalence of cardiovascular disease (CVD) and comorbidities. Lack or reduced level of testosterone has been reported in population studies to be associated with an increase in mortality, and this has been shown to be accounted for mainly by CVD (Khaw et al., 2007; Vikan et al., 2009; Araujo et al., 2011). Moreover, accumulating evidence suggests that testosterone deficiency is an independent cardiovascular risk factor and many recent reviews have focused on the link between hypogonadism, erectile dysfunction, type-2 diabetes mellitus and CVD (Makhrida et al., 2005; Shabsigh et al., 2008; Yassin et al., 2008; Corona et al., 2009). Testosterone is converted to 17 $\beta$  oestradiol (E2) by the enzymatic activity of aromatase in adipose tissue. Thus, higher expression of aromatase in adipocyte reduces bioavailability of testosterone. Falling testosterone promotes increasing adipocyte number and fat deposition, which gradually leads to a further lowering effect on testosterone levels.

The relevance of androgen in the normal functioning of the entire male reproductive system and male sexual activity has been documented (Aversa and Fabbri, 2001; Yakubu et al., 2005). Neurons in the hypothalamus secrete gonadotropin-releasing hormone into the median eminence. This in turn stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the general circulation. The LH binds to receptors in the Leydig cells of the testes to synthesize pregnenolone, and via a two-step reaction produce dehydroepiandrosterone before finally synthesizing and releasing testosterone. Testosterone then acts on androgen receptors to stimulate the components of sexual behavior (Yakubu et al., 2005; Aversa and Fabbri, 2001; Subramoniam et al., 2007). Testosterone is the main synchronizer of sexual activity, acting both centrally and peripherally to regulate libido, the synthesis and release of enzymes like nitric oxide synthase (NOS) and phosphodiesterase-5 (PDE5), which are crucial for the erectile process (Vignozzi et al., 2005). It can enhance erection by attenuating the alpha-adrenergic vasoconstrictor activity in vascular smooth muscles of the corpus carvenosum (Heaton and Varrin, 1994). Furthermore, testosterone and FSH can act on the receptors in the Sertoli cells to stimulate spermatogenesis in the seminiferous tubule.

Invariably, most of the plants with acclaimed aphrodisiac activities act centrally by altering the concentrations of specific neurotransmitters or elevating testosterone concentration as reported in previous studies (Vignozzi et al., 2005; Yakubu et al., 2005; Aversa and Fabbri, 2001; Yakubu and Jimoh, 2015). Hence, the enhanced sexual stimulating activities of several plant extracts/phytochemicals has been considered as an important factor that contributed to the entire restoration of sexual activity in sexually impaired male rats (see Fig. 1) (Chauhan et al., 2013). Experimentally induced male infertility with ethanol reported a significant decrease in the androgenic hormones (testosterone, LH, FSH and inhibin B) (Akomolafe et al., 2017b) however, in ethanol-induced rats pretreated with aqueous extract of African Walnut leaf (*Tetracarpidium conophorum* (Mull.Arg.) Hutch & Dalziel) showed a significant reversal in the decreased bioavailability of these androgenic hormones. These decreased androgenic hormones induced by ethanol in this study was equally accompanied by reduced testicular weight, sperm count viability and abnormalities (Akomolafe





**Figure 1** Proposed mechanism of action of plant phytochemicals on sexual hormones.

et al., 2017b). Quercitrin, luteolin and quercetin have previously been reported by same author to be the predominant phenolic phytochemicals present in African Walnut leaf and was associated with its androgenic properties including modulation of the androgenic hormones (Akomolafe et al., 2015, 2017b,c). In another study, Akomolafe et al. (2017d) examined the effect of co-administration of caffeine and caffeic acid on androgenic properties in normal rats. They observed that there was significant increase in reproductive hormones (testosterone, FSH and LH) and decreased oxidative stress in the testes and epididymides of the treated rats as typified by elevation in antioxidant molecules. In addition, photomicrographic examination of sperm cells revealed no significant alterations. The authors hypothesized that these combination exhibited additive/synergistic effects on the reported biochemical markers and thus, suggested that the combination of these phytochemicals could improve male reproductive function.

Interestingly, studies has shown that the enhanced stimulation of testosterone has been reported to be accompanied by corresponding elevations of gonadotropins which involve a possible impact of plant phytochemicals on the pituitary gland resulting in the release of both LH and FSH. Findings from previous studies revealed elevations in both levels of testosterone and gonadotropins following the administration of some plants extract to sexually compromise male rats (Akomolafe et al., 2017d).

## Conclusion

Phytochemicals are non-nutritive components of plants that studies have shown to exhibit various therapeutic properties, especially in the management of several diseases including cancer, diabetes, cardiovascular disease and erectile dysfunction. The therapeutic properties of phytochemicals are often as a result of interaction with the various biological systems including the endocrine and hormonal systems. However, the complex interactions between sex hormones have made comprehensive studies on pathophysiology roles hormones and interactions of therapeutic phytochemicals challenging. Nevertheless, with the few available studies, more in-depth studies are still encouraged.

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# Tocopherols and Tocotrienols: Sources, Analytical Methods, and Effects in Food and Biological Systems

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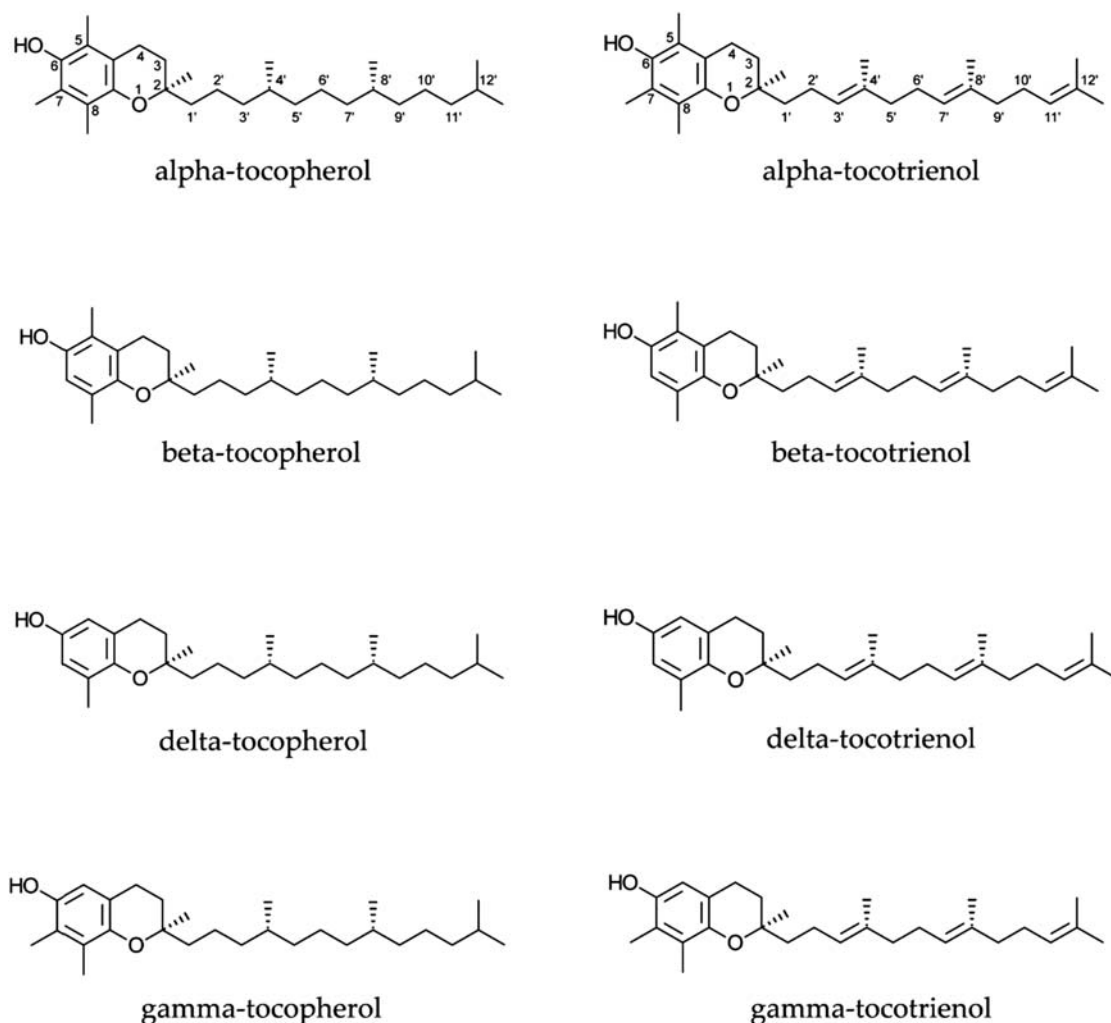
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## Introduction

Tocopherols and tocotrienols, collectively known as tocopherols, are lipid-soluble phenolic compounds. Tocopherols (Fig. 1) exist as four homologues (alpha, beta, gamma, and delta) and they differ from each other according to the location and number of methyl groups in their chemical structures, while the side chain in tocotrienols is saturated, there are three double bonds at positions 3', 7', and 11' in the side chain of tocotrienols. Tocopherols have three chiral centres, thus explaining the existence of eight stereoisomers for each of them. In contrast, the absence of chiral centres in the side chain of tocotrienols explains the existence of only two stereoisomers for each one. In addition to the difference between each homologue, different isomers may also have distinct activity and/stability (Shahidi and de Camargo, 2016). Tocopherols and tocotrienols are monophenols, therefore, like other phenolic compounds, they act as antioxidants in the human body and in animal tissues. Their antioxidant properties in food systems have also been well substantiated. Fat depots, lipoproteins (e.g. low-density lipoprotein cholesterol) and lipid-rich regions of cells such as mitochondrial membranes may contain tocopherols. Besides the antioxidant activity, which protects both lipid and protein fractions from oxidation (Estévez, 2011; Nagy et al., 2016), tocopherols may also act through regulation of gene expression, signal transduction and modulation of cell functions (Zingg, 2007). As for the health benefits, a myriad of evidences on the potential protective effects of tocopherols and tocotrienols against cardiovascular diseases, certain types of cancer, and metabolic disorders, neurodegeneration, and oxidative stress has been reported. Additionally, immune regulation and anti-inflammatory properties have also been highlighted (Meganathan and Fu, 2016; Nesaretnam and Meganathan, 2011; Reiter et al., 2007; Shahidi and de Camargo, 2016). Plant foods containing high concentrations of unsaturated fatty acids are good sources of tocopherols and fewer sources of tocotrienols are currently known. The concentration of tocopherols in animal food products is influenced by their dietary intake (Mercier et al., 1998; Ventanas et al., 2007). Oilseeds (Syväoja et al., 1986), nuts (Kornsteiner et al., 2006) and other common edible oils (e.g. olive, palm, rice bran, wheat, and corn) are among the major sources of tocopherols, whereas their contribution in most fruits and vegetables is negligible, possibly due to their low lipid contents (Shahidi and de Camargo, 2016). Furthermore, seeds and other plant processing by-products are emerging as novel sources of these bioactives (da Silva and Jorge, 2014). In terms of food chemistry, the presence and/or concentration of tocopherols may be influenced by different feedstocks (Kornsteiner et al., 2006; Syväoja et al., 1986), cultivars, crop year (Lavedrine et al., 1997; Shin et al., 2009), climate and stress conditions, life cycle (Holländer-Czytko et al., 2005; Munné-Bosch, 2005), soil quality (Egesel et al., 2008), storage and processing conditions (Raiola et al., 2015).

## Natural Sources of Tocopherols and Tocotrienols

Among all homologues, alpha-tocopherol is for sure the most studied form. As can be noted in Table 1, wheat germ oil presents a high concentration of alpha-tocopherol, which is also the major homologue in common edible oils (e.g. barley, cottonseed, olive, palm, safflower, and sunflower) and emerging specialty seed oils (e.g. grape, lemon, orange, papaya, and tangerine) (Table 2). Cashew nut oil is the only feedstock presenting beta-tocopherol as the predominant form (Gómez-Caravaca et al., 2010). Likewise, among the examples given in the latter contribution, only one feedstock (passion fruit seed oil) showed delta-tocopherol as the main one. Beta-tocopherol in linseed and rapeseed was detected only in trace amounts (Schwartz et al., 2008), whereas the same has been reported for the presence of delta-tocopherol in cottonseed (Schwartz et al., 2008). Nuts are well recognized sources of tocopherols (Alasalvar and Pelvan, 2011). Several tree nut oils have been examined (Miraliakbari and Shahidi, 2008), including almonds, Brazil nuts, hazelnuts, pecans, pine nuts, pistachios and walnuts. According to the above-mentioned study, alpha- and gamma-tocopherols were the predominant homologues present in these samples. The same trend has been found for peanuts (de Camargo et al., 2012; Shin et al., 2009). However, among the reported nuts, macadamia seems to be an exception, for which the contribution of tocopherols has been found to be minor or non-existent (Elisia et al., 2013; Kornsteiner et al., 2006; Wall, 2010). It has been reported that alpha-tocopherol is found mostly in the chloroplasts of plant cells, whereas beta-, gamma-, and delta-tocopherols are commonly found outside these organelles. Tocotrienols are usually absent in the green parts of the plants, but may be found in the bran and germ fractions of certain seeds and cereals (Shahidi and Ambigaipalan, 2015). Data about the presence and contribution of tocotrienols is still scarce in emerging dietary sources. However, all forms of tocotrienols have been detected in barley, palm, rice bran, and wheat germ (Shahidi and de Camargo, 2016). The presence of alpha-tocotrienol in almond has been reported (Piironen et al., 1986; Zhu et al., 2015). According to Zhu et al. (2015), some samples presented similar or higher concentrations compared to that of gamma- and beta-tocopherols, thus suggesting that the presence of alpha-tocotrienols should be monitored in this oilseed. Additionally, as mentioned earlier, macadamia also appears to be an exception amongst other nuts. Kaijser et al. (2000) evaluated several macadamia samples from different cultivars and locations. Their study



**Figure 1** Chemical structures of tocopherols and tocotrienols (Shahidi and de Camargo, 2016).

demonstrated that the content of alpha-tocotrienols may be up to 44 and 11 times higher than that of alpha- and delta-tocopherols, respectively. The presence of both alpha- and gamma-tocotrienols in grapeseeds was reported by Choi and Lee (2009). Regardless of the fraction (hexane-soluble fraction versus methanol-soluble fraction), tocotrienols were the most prominent homologues compared with that of alpha- and gamma-tocopherols. In fact, the methanol-soluble fraction showed about 54-fold higher recovery of gamma-tocotrienol compared to the recovery of gamma-tocopherol. Furthermore, gamma-tocopherol was not detected in the hexane-soluble fraction. In the nineties, the presence of tocotrienols in tocotrienol-free vegetable oils was proposed as a means to indicate their authenticity (Dionisi et al., 1995). The advance in the breeding technology has, however, brought new challenges for such assumption. In contrast to common sunflower cultivars, at which alpha-tocopherol is the dominant tocol, the development of a new cultivar with higher contents of gamma-tocopherol has been reported (García-Moreno et al., 2012). Therefore, the use of the dominant tocol to indicate oil adulteration requires reevaluation (Shahidi and de Camargo, 2016).

### Identification and Quantification of Tocopherols and Tocotrienols

Tocols are lipophilic phenolic compounds, therefore, the American Oil Chemists' Society (AOCS) has published an official method (AOCS, 2003) for determination of tocopherols and tocotrienols in vegetable oils and fats by high-performance liquid chromatography (HPLC). Extraction of tocopherols is influenced by the solvent system employed (Table 3). Hexane is the most commonly used solvent system, but ethanol (Bueno-Borges et al., 2017; Ng et al., 2004) and solvent mixtures such as heptane–diethyl ether (Schwartz et al., 2008), and ethyl acetate–hexane (Shin et al., 2009) have also been used for their recovery. Furthermore, the lipid fraction extracted with chloroform–methanol rendered higher contents of tocopherols than that of hexane (Miraliakbari and Shahidi, 2008). The contribution of each tocopherol has been found to be quite different in ethanolic oil-rich miscella (71% gamma-

**Table 1** Tocopherols (mg/100 g of oil) in common and specialty oils

Feedstock	Alfa-Toc <sup>a</sup>	Beta-Toc	Gamma-Toc	Delta-Toc
Almonds <sup>b,c</sup>	nd-34.9	nd	1.26–1.77	nd
Barley <sup>d</sup>	14.2–20.1	0.60–1.90	3.50–15.1	0.90–4.60
Brazil nuts <sup>b,c</sup>	nd-2.2	nd	13.8–16.8	nd
Camelina <sup>d</sup>	2.81–3.80	0.09	72.0–74.2	1.50–2.04
Cashew <sup>b,e</sup>	nd-7.84	134	30.0	0.3–0.63
Coconut <sup>d</sup>	0.20–1.82	tr-0.25	tr-0.12	nd-0.39
Corn <sup>d</sup>	18.0–25.7	0.95–1.10	44.0–75.2	2.20–3.25
Cottonseed <sup>d</sup>	30.5–57.3	0.04–0.30	10.5–31.7	tr
Grape <sup>d</sup>	11.8–18.8	nr-0.01	2.22–60.1	nr
Guariroba <sup>d</sup>	1.19	nd	nd	0.78
Guava <sup>d</sup>	45.8	nr	93.1	nr
Hazelnut <sup>b,c</sup>	15.7–42.1	nd	9.7–13.6	nd-to 0.3
Jatoba <sup>d</sup>	88.6	nr	nr	16.9
Jerivá <sup>d</sup>	1.10	0.10	nd	0.78
Lemon <sup>d</sup>	10.2	0.22	0.13	1.9
Linseed <sup>d</sup>	0.54–1.20	nd-tr	52.0–57.3	0.75–0.95
Macadamia <sup>f</sup>	0.08–0.11	nr	nr	0.35–0.48
Macaúba <sup>d</sup>	1.44	0.08	nd	0.79
Melon <sup>d</sup>	20.5	nr	250	nr
Olive <sup>d</sup>	11.9–17.0	nd-0.27	0.89–1.34	nd-tr
Orange <sup>d</sup>	30	nd	nd	1.86
Palm <sup>d</sup>	6.05–42.0	nd-0.42	tr-0.02	tr-0.02
Papaya <sup>d</sup>	5.18	0.21	0.18	1.89
Passionfruit <sup>d</sup>	nd	5.40	16.7	27.9
Peanut <sup>b,g,h</sup>	8.86–30.4	nd-0.38	3.50–19.2	0.85–3.10
Pecan <sup>b,c</sup>	nd-1.82	nd	44.0–4.73	nd-0.7
Pine <sup>b,c</sup>	2.2–16.6	2.26–3.18	23.0–24.7	nd-0.7
Pinha <sup>d</sup>	1.20	0.33	12.3	<b>0.02</b>
Pistachios <sup>b,c</sup>	nd-32.8	nd	3.06–4.78	nd-2.3
Pumpkin <sup>d</sup>	7.30	nr	294.5	nr
Rapeseed <sup>d</sup>	18.9–24.0	nd-tr	37–51	0.98–1.90
Rice bran <sup>d</sup>	0.73–15.9	0.19–2.5	0.26–8.00	0.03–2.70
Safflower <sup>d</sup>	36.7–47.7	nd-1.20	tr-2.56	tr-0.65
Sesame <sup>d</sup>	0.24–36.0	0.28–0.80	16.0–57.0	0.17–13.0
Soybean <sup>d</sup>	9.53–12.0	1.00–1.31	61.0–69.9	23.9–26.0
Sunflower <sup>d</sup>	32.7–59.0	tr-2.40	1.40–4.50	0.27–0.50
Soursop <sup>d</sup>	22.1	nr	7.10	nr
Tamarind <sup>d</sup>	1.24	0.70	3.82	0.02
Tangerine <sup>d</sup>	11.6	0.22	nd	1.92
Tomato <sup>d</sup>	nd	nr	32.9	nr
Walnut <sup>b,c</sup>	nd-3.80	nd	34.9–37.6	1.98–5.40
Wheat germ <sup>d</sup>	151–192	31.2–65.0	tr-52.3	nd-0.55

<sup>a</sup>Toc is tocopherol.<sup>b</sup>Adapted from Kornsteiner et al. (2006).<sup>c</sup>Adapted from Miraliakbari and Shahidi (2008).<sup>d</sup>Adapted from Shahidi and de Camargo (2016).<sup>e</sup>Adapted from Gómez-Caravaca et al. (2010).<sup>f</sup>Adapted from Kaijser et al. (2000).<sup>g</sup>Adapted from Syväoja et al. (1986).<sup>h</sup>Adapted from Carpenter (1979).

25% delta-, 3% alpha-, and 1% beta-tocopherol) compared to the oil procured by hexane extraction (82% gamma-, 11% alpha-, 6% delta-, 1% beta-tocopherol) (Bueno-Borges et al., 2017). Tocotrienols are also recovered in hexane, but the literature has demonstrated that methanol may be a better option in recovering tocotrienol-rich fractions (Choi and Lee, 2009). The AOCS (2003) official methods recommends the use of normal-phase liquid chromatography, but the use of reverse phase chromatography has also been reported (Choi and Lee, 2009). The literature (Moreau et al., 2007; Ryyänen et al., 2004) has demonstrated that tocopherols elute before tocotrienols during chromatographic separation. The technique seems to be very simple, but requires the use of authentic standards for identification and quantification of all tocol homologues. Furthermore, due to co-elution, gamma- and

**Table 2** Tocotrienols (mg/100 g of oil) in common and specialty oils

Feedstock	Alfa-T3 <sup>a</sup>	Beta-T3	Gamma-T3	Delta-T3
Barley <sup>b</sup>	46.5–76.1	nd–12.4	8.50–18.6	0.50–2.6
Coconut <sup>b</sup>	1.09–3.00	nd–0.17	0.33–0.64	nd–0.10
Corn <sup>b</sup>	0.94–1.50	nd	1.30–2.00	nd–0.26
Macadamia <sup>c,d</sup>	1.25–4.84	nr	0.87–3.43	0.30–1.77
Olive <sup>b</sup>	nd–tr	nd	nd	nd–tr
Palm <sup>b</sup>	5.70–26.0	nr–0.82	11.3–36.0	3.33–8.00
Rice bran <sup>b</sup>	0.84–13.8	tr–2.6	1.74–23.1	0.14–2.53
Sesame <sup>b</sup>	tr	nd	0.34	nr
Sunflower <sup>b</sup>	0.11	nd	tr	tr
Wheat germ <sup>b</sup>	2.5–3.6	nd–8.2	nd–1.85	nd–0.24

<sup>a</sup>T3 is tocotrienol.<sup>b</sup>Adapted from Shahidi and de Camargo (2016).<sup>c</sup>Adapted from Kaijser et al. (2000).<sup>d</sup>Adapted from Wall (2010).**Table 3** Chromatographic methods for identification and quantification of tocopherols and tocotrienols

Extraction solvent/dissolution	Analytical method	Column	Mobile phase
n-hexane <sup>a</sup>	HPLC-FD	Microsorb 100 Si	hexane/isopropanol (99.5:0.5, v/v)
n-hexane <sup>b</sup>	HPLC-FD-MS	Diol	hexane/tetrahydrofuran (980:20, v/v) and isopropanol
heptane–diethyl ether (50:50, v/v) <sup>c</sup>	HPLC-FD-MS	Varian Inertsil 5 Si	n-heptane/1,4-dioxane (97:3.0, v/v)
n-hexane <sup>d</sup>	HPLC-FD	LiChrosorb Si60	di-isopropyl ether
n-hexane <sup>e</sup>	HPLC-FD	LiChrosorb Si60	3% dioxane in n-hexane
n-hexane <sup>f</sup>	HPLC-DAD	μPorasil	1.5% isopropanol in hexane
ethanol <sup>g</sup>	HPLC-PDA	Develosil RP Aqueous	methanol
ethanol <sup>h</sup>	HPLC-FD	Lichrospher Si60	hexane/isopropanol (99:1, v/v)
petroleum ether <sup>i</sup>	HPLC-FD	Silica	hexane/isopropanol (99.5:0.5, v/v)

HPLC, high-performance liquid chromatography; FD, fluorescence detection; MS, mass spectrometry; DAD, diode array detection; PDA, photodiode array detection.

<sup>a</sup>da Silva and Jorge (2013, 2014), Luzia and Jorge (2013), Malacrida and Jorge (2012), Malacrida et al. (2011, 2012).<sup>b</sup>Moreau et al. (2007).<sup>c</sup>Schwartz et al. (2008).<sup>d</sup>Syväoja et al. (1986).<sup>e</sup>Desai et al. (1988).<sup>f</sup>Carpenter (1979).<sup>g</sup>Ng et al. (2004).<sup>h</sup>Bueno-Borges, et al. (2017).<sup>i</sup>Coimbra and Jorge (2012).

beta-tocopherols have often been reported (Delgado-Zamarreño et al., 2016; Kornsteiner et al., 2006) as their sum, which jeopardizes the prediction of their actual benefits in food and/or biological systems. Co-elution of gamma- and beta-tocopherols may be explained by the presence of three methyl groups in their chromanol ring structures. The influence of the number of methyl groups in the chromanol rings of tocopherols is higher than that of the steric effects of methyl groups. Although some research groups (Moreau et al., 2007; Schwartz et al., 2008; Tang et al., 2015) have used hyphenated techniques such as LC-MS<sup>n</sup>, data procured using such a technique is still scarce. Both atmospheric pressure chemical ionization (APCI) (Moreau et al., 2007; Schwartz et al., 2008) and electrospray ionization (ESI) have been used (Bustamante-Rangel et al., 2007). Likewise, both positive (Moreau et al., 2007) and negative ionization modes (Bustamante-Rangel et al., 2007) have been employed. Ionization of non-polar substances may be hindered because of the lack of protonation and/or deprotonation sites (Bustamante-Rangel et al., 2007; Rentel et al., 1998). Therefore, the use of metal salts in the mobile phase has been practiced to overcome such a problem. Silver–tocopherol adducts have been found to enhance the ionization of tocopherols (Rentel et al., 1998), but addition of acid to the mobile phase, which is more common, has also been found to be a good option (Mottier et al., 2002). Finally, due to different reporting methods, comparing literature results might be challenging. Due to the lipophilic nature of such compounds, it is recommended to publish the final results on the basis of their fat and/or oil fraction (e.g. mg/100 g of oil).

## Properties and Stability of Tocopherols and Tocotrienols in Food Systems

The antioxidant properties of tocopherols and tocotrienols are well substantiated. This property stems in part from their ability to donate their phenolic hydrogen to free radicals (Kamal-Eldin and Appelqvist, 1996). Both the number and position of methyl groups of tocols affect their capacity to donate a hydrogen atom and, in turn, their antioxidant effectiveness (Kamal-Eldin and Budiarto, 2015). Tocols may protect lipids from oxidation by either physically quenching or by reacting with ROS (Kamal-Eldin and Appelqvist, 1996; Shahidi and Ambigaipalan, 2015), but prooxidant effects of tocopherols at high concentrations have also been reported (Kamal-Eldin and Appelqvist, 1996). However, the contents of tocopherols in natural systems seem to be close to their optimal values. In food manufacturing, the recommended levels of total alpha-tocopherol (naturally occurring or added) should be between 50 and 500 ppm (Kiokias et al., 2008). The prooxidant effect of alpha-tocopherol is inversely proportional to the temperature of the system. Under low-to-mild temperatures, and at optimum concentrations to achieve their antioxidant potency, the order of activity of tocopherols is alpha- > beta- > gamma- > delta-tocopherol. However, at higher temperatures their potency follows a different trend. Additionally, under different conditions (60 °C or 160 °C), gamma- and delta-tocotrienols were more active in preventing coconut fat oxidation than their corresponding tocopherols (Wagner et al., 2001). Furthermore, gamma-tocotrienol was also a better antioxidant than alpha- and delta-tocopherol in stripped corn oil (Dolde and Wang, 2011). Regardless of the chemical nature (tocopherols or tocotrienols), their activity is influenced by light exposure, presence of trace metal ions, substrate, medium pH, presence of synergists, and/or regenerating substances such as ascorbic acid, chitosan, glutathione, dehydrozingerone, flavonoids, carotenoids, melanoidins, and phospholipids (Georgantelis et al., 2007; Hudson and Mahgoub, 1981; Kamal-Eldin and Appelqvist, 1996; Kancheva et al., 2014; Pekkarinen et al., 1999; Shahidi and Ambigaipalan, 2015; Zou and Akoh, 2015b). In addition, the efficacy of tocols may differ according to the parameter evaluated (e.g. hydroperoxides, aldehydes, etc) (Huang et al., 1994). Because of its amphiphilic nature, the synergistic effect of phospholipids has been attributed to their ability to form microemulsions in organic solvent systems. Tocopherols are then solubilized in these microemulsions and their polar hydroxyl groups are located near the membrane surface where they can easily scavenge aqueous peroxy radicals. Furthermore, the hydrocarbon chain length in the phospholipid has been found to be positively correlated to its ability to enhance the antioxidant activity of alpha-tocopherol (Koga and Terao, 1995). The loss of tocopherols during the refining of vegetable oils may exceed 30% (Gogolewski et al., 2000). During the industrial processing and production of refined-bleached-deodorized soybean oil, delta- and beta-tocopherols were the most affected homologues (Bueno-Borges et al., 2017). Tocopherols and tocotrienols are procured by distillation of the residue generated and collected in the deodorizer-distillate during the refining of several vegetable oils. These tocols, recovered in such a process, may be used to enhance the oxidative stability of sensitive oils such as those from the marine origin (Shahidi and de Camargo, 2016). Despite their wide range of health benefits, marine oils contain a high percentage of polyunsaturated fatty acids, which makes them very sensitive to oxidative reactions, thus potentially jeopardizing their quality and biological function. As an alternative to the use of tocopherols and tocotrienol as food additives, the literature (Wang and Shahidi, 2017) has demonstrated that vegetable oils such as wheat germ oil, which contain high concentrations of tocols, are also able to improve the oxidative stability of marine oils (Wang and Shahidi, 2017). The application and proven efficiency of tocols in food emulsions (e.g. salad dressings, infant formulas, and meat) are also numerous (Georgantelis et al., 2007; Let et al., 2007; Zou and Akoh, 2015a) and, as mentioned for fats and bulk oils, the literature (Zou and Akoh, 2015a) supports the higher potency of tocotrienols compared to that of tocopherols. Furthermore, tocopherols and tocotrienols (alpha and gamma) were found to be more effective in inhibiting fatty acid oxidation than anthocyanins. Nitrosating agents react with primary and secondary amines producing potentially carcinogenic *N*-nitrosamines (NAs) (Tricker and Preussmann, 1991). Malondialdehyde, a well-accepted biomarker for lipid oxidation in muscle food, influences the generation of *N*-nitrosamines (NAs) (Kurechi et al., 1980) and the degree of unsaturation of the feedstock plays a crucial role in the level of NAs formation in cured meats (Gray et al., 1984). Several studies support the use of alpha-tocopherol to prevent/decrease the formation of NAs in cured meat products (Fiddler et al., 1978; Gray et al., 1984; Wang et al., 2015). During the storage of meat products, oxymyoglobin is oxidized to metmyoglobin, which is easily noted by a brownish colour development of the product (Pegg and Shahidi, 1997). Furthermore, metmyoglobin is also able to initiate lipid oxidation (Melo et al., 2016). Endogenous (from the diet) or added alpha-tocopherol has been used to reduce myoglobin oxidation (Djenane et al., 2002; Morrissey et al., 1998). The higher the degree of unsaturation of the lipid fraction, the lower the stability of tocols (Corsini et al., 2009). Finally, tocopherols incorporated in active packaging may be released to the surface of different food products, thus acting as antioxidants (Chen et al., 2012).

## Tocols as Antioxidants and Anti-inflammatory Compounds

Several studies have supported the potential benefits of tocopherols and tocotrienols under physiological conditions. Regardless of the chronic ailment, different homologues may have a different bioavailability (Saito et al., 2010). Gene mutations, health status (e.g. metabolic syndrome, inflammation, and oxidative stress) also affect the uptake of tocopherols and tocotrienols, in turn, their concentration in plasma and other tissues. In addition, the presence and action of tocols also differs among several tissues (Jansen et al., 2016). Fenton reaction is often used to generate hydroxyl radical, a highly reactive oxygen species. In this system, Fe(II) and H<sub>2</sub>O<sub>2</sub> are present, therefore, it has been hypothesized that the protective effects of several plant food phenolics may include both the chelation of iron and/or the scavenging of hydroxyl radicals. The scavenging activity of alpha-tocopherol towards hydroxyl radicals generated via Fenton reaction has been reported by Inami et al. (2012). Phenolic extracts from different plant sources are known to



exhibit chelating capacity (Ayoub et al., 2016). Likewise, both alpha-tocopherol and tocotrienol-rich fractions showed chelating effect towards ferrous ion, with the latter one presenting the highest efficiency. The oxidation of low-density lipoprotein cholesterol (LDL-c) has long been used as a biomarker to predict potential development of cardiovascular diseases. Pearce et al. (1994) have ranked antioxidant effect of tocopherols using the copper-induced LDL-c oxidation model *in vitro*. The protective effect was found to be in the order of gamma-tocotrienol > gamma-tocopherol > alpha-tocotrienol > gamma-tocopherol. Additionally, gamma-tocotrienol ( $IC_{50} = 1.8 \mu M$ ) showed around twice as much oxidative prevention than gamma-tocopherol ( $IC_{50} = 3.9 \mu M$ ). Likewise, peroxy and hydroxyl radical-induced DNA damage has also been accepted as an early stage indicator of cancer development. Besides that, toxins and NAs are found to induce DNA fragmentation (strand breaks, oxidized pyrimidines and oxidized purines) (Delgado et al., 2008; Shahidi and de Camargo, 2016). Both alpha-tocopherol and gamma-tocotrienol have been reported to inhibit DNA damage (Abid-Essefi et al., 2003; Pathak et al., 2016). Other factors involved in antitumour action of tocopherols (e.g. cell cycle arrest, antiangiogenesis, anti-metastasis, nuclear factor- $\kappa B$  suppression, and telomerase inhibition) have also been reported (Eitsuka et al., 2016). Furthermore, tocopherols and tocotrienols are known to perform apoptotic and antiproliferative functions in cancer cell lines (Campbell et al., 2006; Wada et al., 2005; Yu et al., 1999), with the advantage of being safe towards normal cells (Shahidi and de Camargo, 2016). Nonetheless, in human breast cancer cells, tocopherols and tocotrienols also play a synergistic effect in combination with tamoxifen (Guthrie et al., 1997). Tocopherols and tocotrienols exhibit significant anti-inflammatory activity *in vitro* and *in vivo*, and their molecular mechanisms of action include, at least in part, modulation of inflammatory mediators responsible for orchestrating several stages of inflammation (Ahsan et al., 2014; Jiang, 2014; Jiang et al., 2001; Mathur et al., 2015; Reiter et al., 2007; Singh and Jialal, 2004; Yang et al., 2010). According to the literature (Jiang et al., 2000), gamma-tocopherol treated RAW 264.7 macrophages and human epithelial cells show reduction in prostaglandin E2 (PGE2) synthesis. The same study further reported that 2,7,8-trimethyl-2-(b-carboxyethyl)-6-hydroxychroman, the main metabolite of gamma-tocopherol, also showed anti-inflammatory effect in the same cell lines. According to these authors, the mechanism of action was related to inhibition of cyclooxygenase-2 (COX-2) enzymatic activity. In another study (Jiang and Ames, 2003), the treatment with gamma-tocopherol (at 33 or 100 mg/kg) or with its main metabolite (2 mg/pouch) *in vivo* was likewise effective in reducing the synthesis of PGE2 at carrageenan-induced inflammation sites in rats. In addition, gamma-tocopherol showed inhibitory activity on leukotriene B4 (LTB4) and tumour necrosis factor-alpha (TNF- $\alpha$ ) synthesis. Yam et al. (2009) examined the *in vitro* anti-inflammatory activity of tocotrienols and found that tocotrienols (gamma-, alpha- and delta-tocotrienol) or a tocotrienol-enriched fraction had a significant anti-inflammatory effect on lipopolysaccharides (LPS)-stimulated macrophages. Among their findings, delta-tocotrienol was found to be most effective in inhibiting the production of interleukin 6 (IL-6), nitric oxide and PGE2 as well as gene expression of COX-2. The synthesis and release of inflammatory mediators by activated cells is controlled by different intracellular signaling pathways, which involves the participation of protein kinases, such as mitogen-activated protein kinases (MAPK) as well as transcription factors (Arthur and Ley, 2013; Li and Verma, 2002). Several studies on tocopherols and tocotrienols have demonstrated that the anti-inflammatory activity of these compounds stems from their ability in inhibiting inflammatory intracellular pathways (Azzzi et al., 2004; Cook-Mills and McCarty, 2010; Rimbach et al., 2002). Ekstrand-Hammarström et al. (2007) have shown that treatment with alpha-tocopherol reduced interleukin 8 (IL-8) production in human alveolar type II and bronchial epithelial cells stimulated with TNF- $\alpha$ . The intracellular mechanism of action of alpha-tocopherol was found to be related to decreased phosphorylation of p38 MAPK and extracellular signal-regulated kinases (ERK) 1/2 as well as of nuclear factor kappa B (NF- $\kappa B$ ). A study by Shibata et al. (2010) suggested that gamma-tocotrienol displays anti-inflammatory activity by suppressing the expression of COX-2, interleukin 1 beta (IL-1 $\beta$ ), IL-6 and monocyte chemoattractant protein-1 (MCP-1) genes in HaCaT cell cultures exposed to UVB irradiation. Furthermore, treatment with gamma-tocotrienol decreased phosphorylation of c-Jun amino-terminal kinases (JNK), p38 and ERK. A tocotrienol-rich fraction (15.4% alpha-tocotrienol, 28.2% gamma-tocotrienol, 6.4% delta-tocotrienol, 15.3% alpha-tocopherol, 12.8% plant squalene and 5.1% phytosterol), led to increased anti-inflammatory activity as compared to those of alpha-tocopherol and alpha-tocopheryl acetate in a mouse peritoneal macrophage culture. Inhibition of the activation of the transcription NF- $\kappa B$  was found to be the main intracellular modulatory mechanism of the enriched fraction (Ng and Ko, 2012). Besides activating the synthesis and release of inflammatory mediators, the transcription factor NF- $\kappa B$  also regulates the expression of adhesion molecules on endothelial cells (Tak and Firestein, 2001). Adhesion molecules play a crucial role in leukocyte migration into the inflammatory focus. Therefore, modulation of cell adhesion during inflammatory processes constitute a promising pharmacological approach for development of novel anti-inflammatory compounds (Griffith and Luster, 2013; Mackay, 2008; Peres et al., 2016). Muid et al. (2016) investigated the activity of all four homologues of tocotrienols in activating endothelial cells. These authors demonstrated that delta-tocotrienol was the most potent inhibitor of intercellular adhesion molecule type 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) as well as the transcription factor NF- $\kappa B$ . Zapolska-Downar et al. (2000) investigated the effects of alpha-tocopherol on the expression of adhesion molecules in human vascular endothelial cells and found a significant down regulation of VCAM-1. Such pharmacological activity was therefore associated with the antiatherogenic activity of alpha-tocopherol. In another study (Yoshikawa et al., 1998), cocubation of neutrophils with alpha-tocopherol and pretreatment of human umbilical vein endothelial cells reduced platelet-activating factor (PAF)-induced CD11b/CD18 expression and IL-1 $\beta$ -induced positive regulation of ICAM-1 and VCAM-1, respectively. Preclinical and clinical studies have confirmed the efficiency of tocopherols and tocotrienols, alone or by using tocopherol-enriched fractions, in preventing the development of inflammatory diseases (Reiter et al., 2007; Jiang, 2014). A tocotrienol-rich fraction from palm oil (23.5% alpha-tocotrienol, 43.2% gamma-tocotrienol, 9.8% delta-tocotrienol, and 23.5% alpha-tocopherol) attenuated the progression of atherosclerosis in mice by modulating the action of peroxisome proliferator-activated receptors (Li et al., 2010). The therapeutic effect of delta-tocotrienol by attenuating the development of collagen-induced arthritis in rats by reducing edema, joint damage, and T-cell proliferation



has also been demonstrated (Haleagrahara et al., 2014). According to Kim et al. (2016), gamma-tocotrienol was able to attenuate the activation of NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome and delayed the progression of type 2 diabetes. A preclinical trial with gamma-tocopherol demonstrated that its administration was effective in attenuating moderate colitis in mice (Jiang et al., 2013). In addition, Wagner et al. (2008) showed that acute administration of gamma-tocopherol over a 4-day period led to extensive protection of ovalbumin-sensitized Brown Norway rats challenged with allergen via the airways. Among the parameters analyzed, a reduction in the recruitment of inflammatory cells and in the production of inflammatory mediators (e. g. PGE2 and leukotrienes) was reported. Later on, the same research team (Hernandez et al., 2013) conducted pre-clinical and clinical trials and demonstrated that gamma-tocopherol-enriched supplementation was effective in reducing airway neutrophil recruitment following challenge with inhaled LPS. Furthermore, Devaraj et al. (2008) carried out a clinical study in patients with metabolic syndrome and showed that alpha-tocopherol supplementation, alone or in combination with gamma-tocopherol, not only reduced biomarkers related to oxidative stress (e.g. lipid peroxidation), but also reduced inflammation markers, such as TNF- $\alpha$ . Therefore, supplementation with the combination of alpha- and gamma-tocopherol seemed to have a greater protective effect compared to the use of isolated homologues. As mentioned before, a wealth of evidences have demonstrated the antioxidant properties of tocopherols and tocotrienols. However, in several chronic ailments the oxidative stress comes along with inflammatory responses. Therefore, this contribution demonstrates that tocols may counteract both oxidative and inflammatory responses.

## Conclusions

In this contribution, sources, analytical methods, and effects of tocols in food and biological systems were discussed. While alpha-tocopherol is the most studied form, fewer sources of tocotrienols, especially for emerging dietary sources (e.g. specialty oils), are currently known. The dominant tocol as an indicator of product adulteration has been proposed by some authors, however, advances in the breeding technology has brought new challenges which require reevaluation. In terms of analytical methods, co-elution of gamma- and beta-tocopherols may be a challenge in some cases, therefore, special attention should be paid to this potential problem. In addition, regardless of the application, some studies have supported the superior activity of tocotrienols compared to tocopherols. Therefore, production of high-tocotrienol cultivars and prospection of already available sources of tocotrienols and further application as functional food ingredients and nutraceuticals are promising areas of interest.

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## Resistant Starch

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### Glossary

**Gastrointestinal** Part of digestion system comprising stomach and intestines.

**Glycemic index** The potential of food product to raise postprandial blood glucose level.

**In vitro** A biological process made to occur in an artificial environment such as laboratory vessel or other controlled experimental environment instead of within a living organism or natural setting.

**In vivo** A biological process made to occur in the living body of a plant or animal.

**Resistant starch** Part of starch that is not digested in the upper gastrointestinal tract and reaching the colon either unchanged or slightly changed.

**Retrogradation** Loss of the crystalline structure of amylose and amylopectin molecules when native starch is heated and dissolved in water.

### Introduction

Starch is the main storage carbohydrate in plants and mainly functions as a reserve for supplying about 50% of the nutrient and energy requirements in the human diet. Moreover, starch quantity and quality is a major concern in terms of blood glucose level and homeostasis in human body (Ahuja et al., 2013). Structurally starch has two major constituents of amylose and amylopectin. Amylose is a linear glucan polymer formed through  $\alpha$ -1,4 glycosidic bonds, whereas amylopectin is a highly-branched glucan polymer consisting of  $\alpha$ -1,6 glycosidic linkages. Native starch involves 15%–30% amylose, depending on its botanical origin, maturity level, growing condition and the method of analysis while the remaining part is amylopectin (Hasjim et al., 2009). The amylose/amylopectin ratio may be considered as one of the most significant factors that affect starch properties.

Starch digestion is a multi-step process and it is initiated by the oral salivary  $\alpha$ -amylases, then pancreatic  $\alpha$ -amylase and finally intestinal brush border glucoamylases, maltase-glucoamylase, and sucrase-isomaltase that convert the resultant products into molecules suitable for absorption into the vascular system (Nichols et al., 2003; Ahuja et al., 2013). Starches are classified according to their rates of digestion such as; rapidly digestible starch (RDS); slowly digestible starch (SDS) and resistant starch (RS). RDS is digested in the small intestine and results in a high glycemic response while SDS is digested at a slower rate, having a relatively lower glycemic response in blood. Glycemic response or glycemic index is defined as the potential of food product to raise postprandial blood glucose level. It is expressed as a percentage of area under the blood glucose response curve of a test food, divided by the area under the curve after eating a similar amount of control/reference food (usually white bread) (Ludwig and Eckel, 2002). RS, on the other hand, is not digested in the upper gastrointestinal tract. RS is resistant to enzymatic hydrolysis within 120 min of consumption; therefore, it does not contribute to postprandial hyperglycemia. Instead microorganisms available in the large intestine ferment the RS into short chain fatty acids (mainly butyrate, propionate and acetate) and gases ( $\text{CO}_2$ ,  $\text{CH}_4$  and  $\text{H}_2$ ) (Englyst et al., 1992). Short chain fatty acids in the large intestine are significant in terms of colonic health in addition to their reducing role on certain diseases such as irritable bowel syndrome, inflammatory bowel disease, cardiovascular diseases and cancer (Roe-diger, 1980; Wong et al., 2006). Current interest in RS is not only related to its health effects, but also its formation mechanisms and functional properties in cereal products such as heat or processing stability.

### Structure and Types of RS

The part of starch which is not digested in the small intestine and reaches the large intestine in the intact form is called RS. Therefore, RS is the part of starch that acts like a dietary fiber within the gastrointestinal tract. The resistance against enzymatic digestion in the small intestine (Englyst et al., 1992) has been recently linked to numerous positive physiological benefits on the health such as; prebiotic effect on colon microflora, improvement of cholesterol metabolism and reducing the risks of ulcerative colitis and colon cancer (Shi et al., 2013; Ozturk et al., 2009). RS is defined under 5 sub-classes: (1) physically inaccessible (RS1), (2) native B- and C-type polymorphic starch granules or ungelatinized starch (RS2), (3) retrograded or crystalline starch (RS3), (4) starch chemically modified (RS4) and (5) amylose-lipid complexes (RS5) (Shi et al., 2013; Hasjim et al., 2013). All different sub-classes of RS are generally assumed to have similar metabolic fate by being fermented in the large intestine. However, differences in site, magnitude and diversity of biochemical and physiological actions through the large intestine might be possible.



RS1 is not accessible to digestive enzymes physically because it is bound within the fibrous cell walls, in other words, it is captured in the food matrix such as whole or coarsely ground grains or seeds (Sajilata et al., 2006; Champ et al., 2003). The thick cell walls and the protein matrix in cereal and in particularly legumes, such as beans or lentils grains, prevent water penetration into the starch. Because water cannot penetrate the starch granule, there is lack of necessary moisture that is required to gelatinize and swell; therefore, it is not readily susceptible to enzymatic hydrolysis. RS2 is defined as the characteristic of B-type starches such as those present in uncooked potato starch, green banana starch and high-amylose maize starch that are very resistant to enzymatic hydrolysis. In the native (raw) form, RS2 is digested at very low levels in the small intestine but becomes totally digested when freshly cooked due to the gelatinization of starch and loss of the B- and C-type crystalline structures. The compact structure of RS2 is a limiting factor to accessibility by digestive enzymes, including some of the amylases. Since cooking is highly effective, raw banana starch is known as the most significant example of RS2 (Sajilata et al., 2006). RS3 is also slowly and partially digested but has reversible characteristics, and digestibility may be enhanced by reheating. Amylose retrogradation is a causative factor of RS3 generation. Amylose molecules tend to form double helices around refrigeration temperatures (4 °C) because of their linear structures. In addition, retrograded amylose has higher gelatinization temperatures, and they cannot be dissociated by cooking. Among all RS types, RS3 has been the most commonly studied particularly because of its advantages such as thermal stability, high gelatinization temperature, very low water holding properties, as well as improved texture, appearance and organoleptic properties (Sajilata et al., 2006). Some previous works had aimed to increase the amount of RS3 in food samples using different methods (such as heat treatment, partial acid hydrolysis, enzymatic modifications together with acidic modification and heat treatment, extrusion or chemical methods) (Shi et al., 2013; Ozturk et al., 2009; Dundar and Gocmen, 2013; Van Hung et al., 2016; Liao and Hung, 2015; Reddy et al., 2013; Demirkesen-Bicak et al., 2018; Faraj et al., 2004; Hasjim and Jane, 2009). Although the formation of RS is generally related to retrogradation of amylose, retrograded amylopectin has also been stated as a contributor to RS content (Thompson, 2000). Amylopectin retrogrades slower than amylose and crystalline amylose is more resistant to heat in comparison to similar amylopectin structures. Therefore, temperature and branched structure of amylopectin may be considered as limiting factors for RS formation in these amylopectin moieties. The preventive effect of amylopectin structure has been overcome using enzymatic treatment with pullulanase or isoamylase enzymes. They are able to cleave  $\alpha$ -1,6 glycosidic bonds in amylopectin molecule to increase the number of linear glucans so that re-association to form RS3 becomes easier (Thompson, 2000). Some of very recent studies on enzymatic treatment with pullulanase (Shi et al., 2013; Reddy et al., 2013; Demirkesen-Bicak et al., 2018) focused not only on the different conditions for enzymatic treatment of starch with pullulanase but also the use of different starch sources.

RS4 is basically the chemically modified starch and consists of starches in which chemicals have been used to etherize, esterify or crossly-bond the molecules in order to decrease their digestibility. They can also be classified into four sub-categories according to their solubility characteristics in water or methods used for analysis (Nugent, 2005). Chemical modification methods that include conversion, substitution or cross-linking may be used to block the enzyme access and form different linkages such as  $\alpha$ (1  $\rightarrow$  4) and  $\alpha$ (1  $\rightarrow$  6) linkages to prevent starch digestion (Kim et al., 2008; Sajilata et al., 2006). Cross-linking raises the gelatinization temperature and it permits the starch granule to stay intact at high temperatures. RS4 type can also be formed by dextrinization during which new linkages that cannot be degraded by mammalian digestive enzymes are formed between starch molecules. RS5 has been quite recently introduced as high-amylose starch that are preheated, debranched with isoamylase and complexed with palmitic acid to form an amylose-lipid complex that is resistant to enzymatic digestion (Hasjim et al., 2010). According to this finding, lipids form complexes with the hydrocarbon portions found in the helical cavity of amylose, dominated by unbranched (linear molecule with  $\alpha$ -1,4 linked D-glucose units) glucan chains (Tang and Copeland, 2007). Mechanism behind the enzyme susceptibility of amylose-lipid complex was proposed to be two-fold: 1) The starch-lipid complex decreases the extent of granule swelling and therefore, the enzyme may not easily reach inside of the starch granule or 2) The amylose-lipid complex is more resistant to enzymatic digestion in comparison to free amylose (Zhang et al., 2012; Okumus et al., 2018).

## Factors Effecting RS Formation

### Effect of Starch Composition on RS Formation

Granule structure, amylose-amylopectin ratio, starch source, etc. were previously reported to affect starch resistance to digestion (Finocchiaro et al., 2009). Total amount of amylose substitution was the main factor to determine the variations in resistant starch measurements. Since higher amylose content is expected to lower starch digestibility, positive correlation between amylose content and resistant starch exist and has been reported in many different studies (Leeman et al., 2006; Sang et al., 2008). This correlation may be associated with the digestion kinetics that defines two distinct limiting factors: 1) binding of enzyme to starch prevented by the barriers and 2) decrease in amylase activity because of starch structural features (Zhang et al., 2015). Therefore, depending on the structural differences in the amylose and amylopectin, small intestinal  $\beta$ -amylase access is limited by the straight chains of amylose (forms tightly-bound complexes) and the two terminal glucose units on the amylose chain. However, the highly-branched (porous) structure of amylopectin enabled  $\beta$ -amylases access (Ahuja et al., 2013).



## Effect of Processing on RS

Resistance to digestion derives from modifications in the specific physical state of starch such as the effects of heat treatments, amylose/amylopectin ratio, chain length of molecules, presence of amylose–lipid complex, etc (Demirkesen-Bicak et al., 2018). Sources of cereal grains, roots, tubers and legumes produce RS through the process of cyclic heating, autoclaving and extrusion methods. Different RS types are not specific to food samples. For example, RS1, RS2 and RS3 can be present in the same food. Both RS1 and RS3 can exist in beans, while RS1 and RS2 are usually found in bananas. Food processing, in most cases, decreases natural types of RS1 and RS2, but may lead to formation of RS3. Among all RS types, RS3 has been the most commonly studied particularly because its formation is dependent on various processes such as thermal treatment, gelatinization, extrusion etc. Generally, severity of the conditions such as moisture, temperature, number of heating/cooling cycles, autoclaving, storage, and drying will determine the amount of RS formed during processing (Fuentes-Zaragoza et al., 2010).

Starch has been reported to display more resistance to  $\alpha$ -amylase at higher temperatures (Zabar et al., 2008). This phenomenon has been related to starch gelatinization. During gelatinization, amylose molecules are leaching out of the swollen starch granules in coiled polymer structures while through cooling, they associate as double helices and form hexagonal networks, which resist digestion (Ahuja et al., 2013). Moreover, the method used for drying has also been reported to affect the RS content in green bananas (Tribess et al., 2009). Although the use of repeated heating–cooling cycles in the presence of moisture has attracted interest as an effective approach to increase the RS3 levels (Masatcioglu et al., 2017), heat-moisture treatments gave some contradictory results such as increases in corn, lentil and pea starches (Chung et al., 2009) but a decrease in corn starch (Brumovsky et al., 2009). Moisture or heat-moisture treatments followed by storage have been reported as a positive approach for increasing the RS content (Kim et al., 2006). Autoclave cycles followed by cooling were also promising (Hickman et al., 2009). Gelatinization alone has been considered as a negative factor for total RS formation (Chung et al., 2006), although gelatinization followed by cooling processes which are generally referred to as annealing procedures, are the common methods used to enhance the RS formation, in particular RS3 (Thompson, 2000; Chung et al., 2006). Among a number of different foods that were cooked, cooled and stored, the highest amount of total RS was obtained for the retrograded gelatinized starch (Rosin et al., 2002).

Acidic treatments applied as a pre-treatment before gelatinization using HCl at 40 °C (Koksel et al., 2008) and as a post-retrogradation treatment with citric acid (Zhao and Lin, 2009) was found to have increased the levels of RS. Gamma irradiation was also promising to increase the RS content of corn starch because the effect of gamma irradiation has commonly been related to increases in the degree of polymerization and/or branching of glucan polymers (Chung and Liu, 2009). Enzymes have roles in changing the amylopectin chain length distributions by means of increasing  $\alpha$ -1,6 linkages and decreasing  $\alpha$ -1,4 linkages. This structure aids in retrogradation which produces more resistant starch (mostly RS3). Starch becomes more resistant to digestion with an increase in the proportion of longer chains (Ahuja et al., 2013). Results revealed that the enzymatic hydrolysis by pullulanase followed by thermal processes and retrogradation increases the amylose contents and the formation of resistant starch (Reddy et al., 2014; Demirkesen-Bicak et al., 2018).

## Measurement of RS

Growing interest in resistant starch research (more than 200 SCI-indexed articles in the period of 2015–7) focusing on different perspectives, and underlines the significance of resistance starch measurements. However, resistance to digestion derives from specific physical states; therefore, different RS studies are not truly directly comparable at all times. In particular, the temperature of a RS assay may tremendously affect the measured value (Haralampu, 2008). Moreover, another significant point is that the measurement of RS compromises the sum of “added” and “naturally occurring” RS, as different types of RS may exist in the same food, although their susceptibility to various conditions are different. Digestion resistance might be affected from subjective physiological differences during the process of digestion, such as efficiency of chewing, time of gastrointestinal transit and the quantity of enzyme secretion. In addition, differences during preparation of ingredients, food processing and cooking conditions, food storage etc. might also affect the measured content of total RS (Brown et al., 2008). In particular, the method used for isolation of starch from food commodities and/or the extent of its purity might be significant. Calculation of RS is commonly made by subtracting the total amount of RDS and SDS generated from non-homogenized food samples by enzyme digestion, from the total starch obtained from homogenized and chemically treated sample.

Because of the difficulties during measurement, approaches such as comparison with both *in vitro* and *in vivo* RS measurement methods, use of a wide range of population and validation with large number of RS sources have been proposed (Champ et al., 2003). *In vitro* methods mimic the digestion process, which has been approved by AOAC as an Official Method 2002.02 or AACC Recommended method 32–40.01. In addition, several *in vivo* methods such as the hydrogen breath test, and direct collection of ileal effluent from healthy subjects or patients of ileostomy who had their colon removed have been reported (Champ et al., 2003). Besides, differential scanning calorimetry (DSC) is a common instrument used to identify mostly the RS3 produced from retrograded amylose, and has been proposed as a vehicle to display the time–temperature history between the glass transition temperature and the melting temperature of the starch micro crystallites (Haralampu, 2008).

## Conclusion

RS with its different sub-groups has gained increased importance recently. Increase in its broader use in food industry will probably direct future research more towards changes and responses during food processing. The relationship between food processing and RS may be considered as two-sided; both to determine its limits of susceptibility and to discover new potential mechanisms that encourage its formation. Therefore, the true determination and chemistry of RS will likely gain more interest in future studies.

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# Antimicrobial Peptides: The New Generation of Food Additives

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## Introduction

Increasing human population and the demand for safe and nutritious food impose food security challenges worldwide (Bajagai et al., 2016). It has been reported that an estimated 600 million, which means almost 1 in 10 people in the world, fall ill after eating contaminated food that can cause notably severe diarrhea and 420000 die every year (WHO, 2017). To face this threat it is critical to ensure food safety throughout the entire food chain from the production to the consumption of the end-products (WHO, 2014).

Foodborne illnesses are usually caused by bacteria, viruses, and parasites entering the body through microorganisms-contaminated foodstuffs (Pisoschi et al., 2018). Bacteria can account for both food spoilage and toxicity (Gram et al., 2002). Indeed, major foodborne illnesses are caused by *Salmonella* sp, *Campylobacter* sp, *Escherichia coli*, *Listeria* sp and *Vibrio cholera* (WHO, 2017). Administration of antibiotics is still the main practical strategy to treat these infections (Hassan et al., 2012). However, their overuse and misuse in veterinary and human medicine has been linked to the emergence of resistant foodborne pathogens, rendering the treatment of infectious diseases ineffective in animals and humans (Dong et al., 2007).

Microbial resistance development is one of the main fears to modern medicine and it has driven the search for new antimicrobial agents to replace conventional antibiotics (Hassan et al., 2012). Hence, both pharmaceutical and food industries are looking for safe new types of antimicrobials from natural sources (Boisvert et al., 2015). Besides, there is a growing consumer demand for natural alternatives to replace synthetic food preservatives such as nitrates, benzoates, sulfites, sorbates, and formaldehyde, which are known for their life-threatening side effects (Sultana et al., 2014). The search for an alternative to chemical preservations has led to defined novel methods like biopreservation that ensure food safety (Pisoschi et al., 2018). Plant and animal-derived antimicrobials, natural or controlled microflora, such as lactic acid bacteria (LAB) and their antibacterial metabolites such as lactic acid, bacteriocins demonstrated ability to prevent lipid oxidation, inhibited colour loss, prolonged shelf life and ensured food security (Pisoschi et al., 2018). Antimicrobial peptides (AMPs) such as bacteriocins have attracted particular attention as their producer organisms could obtain GRAS (generally recognized as safe) status and are naturally present in many food products (Tahiri et al., 2009). Either low toxicity, thermostability or high specificity make these molecules of clear interest to the food industries (de Castro and Sato, 2015).

Moreover, there is an urgent need for innocuous antimicrobials in food preservation, which has led numerous research activities towards AMPs (Boisvert et al., 2015; Beaulieu et al., 2015; Ennaas et al., 2015a, 2015b, 2016). AMPs are components of the innate immune defenses and exhibit high structural diversity (Reddy et al., 2018; Shabir et al., 2018). They generally have amphipathic structure facilitating their attachment and the formation of pores in the cytoplasmic membrane of microorganisms (Mor, 2000). This characteristic, unlikely to lead to the development of the microorganism's resistance, has stimulated their studies for alternative antimicrobial agents (Mor, 2000). Furthermore, the newly identified AMPs should be specifically active against foodborne pathogens, and they are expected not to influence negatively the consumer's own microbiome (Pisoschi et al., 2018). In addition, AMP-containing formulations have to exhibit the expected antimicrobial effects, with least chemical or biological degradation of peptides (Nordström and Malmsten, 2017).

This chapter will focus on the potential of AMPs as one of the key novel food preservatives for the future.

## Origins of Natural Antimicrobial Peptides (AMPs) and Their Different Functions

### Plant

Plant AMPs are small cysteine-rich peptides that have been isolated from roots, seeds, flowers, stems, and leaves of a wide variety of species (Terras et al., 1995). They are grouped into several families and many share general features, such as an overall positive charge, the presence of disulfide bonds, which stabilize the structure and a mechanism of action targeting outer membrane structures, such as ion channels. Based on amino acid sequence homology, AMPs were classified mostly as  $\alpha$ -defensins, thionins, lipid transfer proteins, cyclotides, snakins and hevein-like (Broekaert et al., 1997). In addition to their role in host defense and their appeal as simple models for studying the molecular mechanism of antimicrobial peptide action, AMPs have the potential to inhibit pathogens, including those showing increased resistance to conventional antimicrobial compounds. These peptides usually have a broad-spectrum of antimicrobial activity and therefore, representing promising candidates for plants applications (Nawrot et al., 2014). PhytAMP is a database that provides a very useful interactive platform that enables easy access to systematized information about all families of plant antimicrobial peptides. PhytAMP also provides useful tools that facilitate analysis of these peptides and search for new members of the plant AMP classes (<http://phytamp.hammamilab.org/>).

## Bacteria

Most families of bacteria are able to produce AMPs namely bacteriocins. Bacteriocins of gram-positive bacteria are more abundant and more diverse than those produced by gram-negative bacteria. Archaea are also able to produce their own bacteriocin-like-antimicrobials known as archaeocins (Riley and Wertz, 2002). Bacteriocins are ribosomally synthesized molecules and have bacteriostatic or bactericidal effects. Bacteriocins have narrow or broad spectra of activity that inhibit the growth of bacteria of the same species or other genera including pathogenic and antibiotic-resistant strains (Cotter et al., 2012). Bacteriocins represent a heterogeneous family of small, heat-stable peptides in terms of size, microbial spectrum of activity, modes of action and release and mechanism of bacterial immunity (Gordon et al., 2007). All this information has been assembled in a database named Bactibase, which is a web-accessible database that contains calculated or predicted physicochemical properties of several bacteriocins produced by both Gram-positive and Gram-negative bacteria. The information in this database is very easy to extract and allows rapid prediction of structure/function relationships and target organisms of these peptides and therefore, better exploitation of their biological activity in both the medical and food sectors (<https://www.ncbi.nlm.nih.gov/pubmed/17941971>).

## Fish and Marine Products

Many marine organisms such as crustaceans, crab, mollusks and fish are potential sources of bioactive peptides such as antimicrobial peptides (Kang et al., 2015; Cheung et al., 2015). Marine AMPs belong to a particular group of peptides. They have high cysteine content and are positively charged in their active forms. They play a crucial role as a first line of defence protecting the hosts against a broad range of pathogenic infections (da Costa et al., 2015). Marine AMPs differ in length, isoelectric point, secondary structure, hydrophobicity, and amphipathicity, and in their antimicrobial activities. Marine AMPs may be classified into four main categories, namely: (a) linear-helical peptides; (b) linear or helical peptides with abundance of one amino acid (proline, tryptophan, histidine or glycine rich peptides); (c) peptides forming hairpin-like-sheet or -helical/-sheet mixed structures stabilized by intramolecular disulfide bonding; and (d) cyclic peptides (Falanga et al., 2016). Most of the marine AMPs are described in APD (<http://aps.unmc.edu/AP/>), a database that contains information for 116 antimicrobial peptides identified from fish.

## Milk and Milk Products

Milk and milk products are shown to be good sources of a variety of bioactive compounds such as AMPs (Mohanty et al., 2016). AMPs have already been isolated and characterized from major milk proteins such as caseins, lactoferrin,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin (Meisel, 1998). They were shown to have a broad spectrum of activity against pathogenic organisms like bacteria, yeast, fungi, and some parasites (Mohanty et al., 2016). Milk-derived AMPs may be found in inactive form within the sequence of the precursor protein but may also be produced by enzymatic digestion especially during the gastrointestinal digestion (Gobbetti et al., 2007). Recently, a database, MilkAMP (<http://milkampdb.org/>), has been created to assembly all information about natural and artificial (synthetic or modified) antimicrobial peptides derived from amino acid sequences of dairy proteins of different origins and provides. This database contains most complete information on peptide structure/function relationships, inhibitory activity, spectrum of action and minimal inhibitory concentration (MIC) determined for each tested microbial strain. It also allows rapid prediction of structure/function relationships and target organisms and hence should lead to better use of the biological activities of peptides in both the pharmaceutical and food sectors.

## Algae and Mushrooms

Only a few studies concerning the inhibitory activities of macroalgal AMPs have been carried out. The lectin is an example of antibacterial algal protein reported in the literature (Liao et al., 2003; Holanda et al., 2005). In addition, a cyclic depsipeptide obtained from green seaweed *Bryopsis* sp. has demonstrated activity against *Mycobacterium tuberculosis* (Hamann and Scheuer, 1993; Hamann et al., 1996), while a dipeptide derivative originated from red delesseriacean macroalgae has displayed antibacterial effect against *Serratia marcescens* and *Salmonella typhi* XLD (N'Diaye et al., 1996). More recently, AMPs have been extracted from the brown macroalga *Saccharina longicuris* in order to recover antibacterial peptides by enzymatic hydrolysis with trypsin. The protein hydrolysate fraction exhibited activity against the bacterium *Staphylococcus aureus* (Beaulieu et al., 2015).

Mushrooms reported as producers of a large number of proteins and peptides with interesting biological activities, such as lectins, fungal immunomodulatory proteins (FIP), ribosome-inactivating proteins (RIP), antimicrobial proteins, ribonucleases, and laccases. These bioactive components have become popular sources of natural antimicrobial, antiviral, antioxidative, antitumor and immunomodulatory agents (Xu et al., 2013; Jose Alves et al., 2013; Lavanya and Subhashini, 2013). Some mushrooms-derived AMPs are described in APD (<http://aps.unmc.edu/AP/>), a database that contains information for 2 antimicrobial peptides identified from mushrooms.

## Mechanisms of Action

AMPs have diverse structures and functions and interact with cell membranes of invader cells by disturbing the membrane integrity. As shown in Fig. 1, adapted from Brogden (2005), AMPs may be divided into different groups based on their mechanisms of action. Some of them have the cell membrane as target and may act by inhibition of cell membrane synthesis, alteration of the cytoplasmic membrane and inhibition of cell septum formation or activation of the cell autolysis. Other peptides seem to act on intracytoplasmic targets. For example, arginine-rich peptides have been shown to act either by inhibiting the synthesis of the cell membrane, DNA, RNA or proteins. Other peptides such as histatins act rather by inhibiting enzymatic activity.

AMPs mechanisms of action could be classified in two main groups, AMPs targeting external processes or internal processes of microorganisms.

### Extracellular-Targeting AMPs

#### Outer Surface Lipids

AMPs must first be attracted to bacterial surfaces, and one obvious mechanism is electrostatic bonding between anionic or cationic peptides and structures on the bacterial surface such as lipids that constitute the outer membrane (de Leeuw et al., 2010). Defensins, nisin and other lantibiotics were able to interact with lipids constituting bacterial cells, and notably the lipid II that is a central component of the bacterial cell wall synthesis (de Leeuw et al., 2010; Sass et al., 2010; Hsu et al., 2004). The interaction of cationic AMPs is well understood and be explained by their initial electrostatic attraction with the outer leaflet of Gram-negative bacteria initiating the passage of AMPs across the outer membrane through a self-promoted uptake mechanism (Hale and Hancock, 2007). Such as the Cecropin P1, this attachment to the bacterial membrane could induce in many cases its disruption and membrane permeabilization, leading to cell-killing (Strauss et al., 2010).

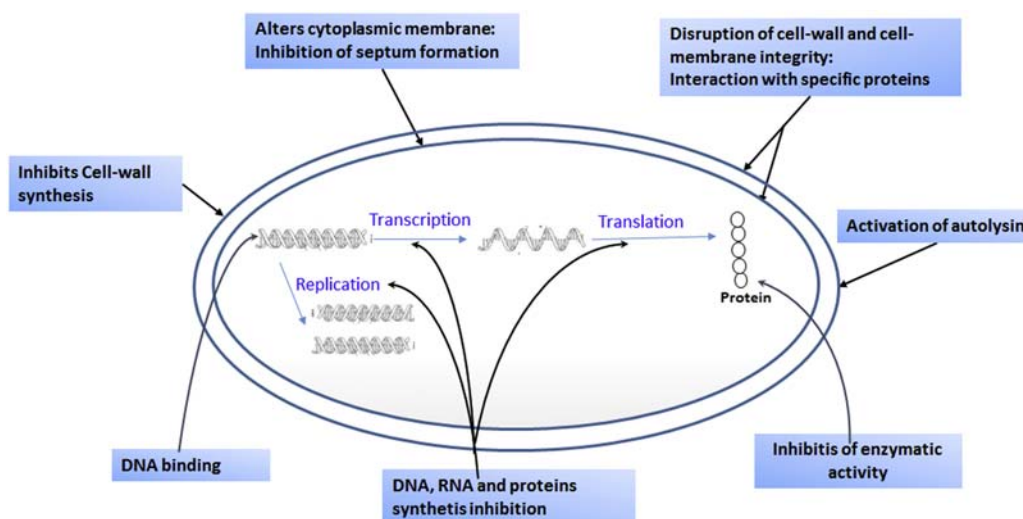
#### Outer Membrane Proteins

Inside the outer membrane of bacteria, it is known that several proteins play an important role as a receptor for AMPs. As describe by Lin et al. (2010), outer membrane protein I (OprI) plays a critical role in maintaining the integrity of the outer membrane of *Pseudomonas aeruginosa* and this protein serves as the receptor for the cationic-helical AMPs, SMAP-29 and CAP-18. In consequence, the bacterial membrane became permeable to metabolites. Other AMPs showed a non-membrane-lytic mechanism of action such as analogs of proteogrin I that are able to target the  $\beta$ -barrel protein LptD, a protein implicated in the outer-membrane biogenesis (Srinivas et al., 2010).

### Intracellular-Targeting AMPs

#### Inner Membrane

Membrane interactions remain important even for intracellular-targeting peptides because they must have a means of translocation (Nguyen et al., 2011). AMPs could create pores in the cell membrane, through different transmembrane pore-forming mechanisms such as toroidal pores, carpet or barrel stave (Hale and Hancock, 2007; Nguyen et al., 2011). All these mechanisms of action were directly correlated to the peptide structure and were the most common and studied among all the mechanisms of action known and



**Figure 1** Modes of action for cellular antimicrobial peptide activity. adapted from Brogden (2005).



identified from AMPs. Once in the cytoplasm, translocated peptides can alter the cytoplasmic membrane septum formation, and inhibit synthesis of the cell wall, nucleic acids, proteins or enzymatic activity.

### Nucleic Acids

While many AMPs kill bacteria through membrane permeabilization, a smaller subset of AMPs is believed to inhibit bacterial growth by interfering with essential intracellular functions (Hale and Hancock, 2007). For example, Buforin II and DesHDAP1 (histone-derived AMPs) are both able to target DNA of bacterial cells (Uyterhoeven et al., 2008; Sim et al., 2017) by electrostatic attraction to negatively charged phosphodiester bonds in the nucleic acid backbone (Sim et al., 2017). The AMP indolicidin interacted with DNA by covalent links with a basic site of single or double DNAs of the HIV-1 viral cDNA (Marchand et al., 2006). As they are able to bind DNA, AMPs such as the fish peptide pleurocidin, interact with RNA and thus inhibit macromolecular synthesis and all protein synthesis (Patrzykat et al., 2002).

### Intracellular Proteins

All the interactions with nucleic acids of bacterial cells lead indirectly to disruption of macromolecular process mechanisms, but AMPs also directly target macromolecules and proteins. The most detailed information on inhibition of protein synthesis derived from the studies of proline-rich AMPs, a group of AMPs with high content of proline and arginine (Scocchi et al., 2016). These AMPs exhibited in some cases, a polyproline helix II structure, which permits it to bind ribosome subunits and inhibit protein synthesis (Scocchi et al., 2011). Daptomycin is known as altering bacterial envelope architecture before killing but this lysis occurs as a secondary event, so it may be the late consequence of a general cell dysfunction and not the main action of this lipopeptide (Pogliano et al., 2012). However, in most other cases, the intracellular targets of AMPs are either unknown or need to be confirmed. All the mechanisms of action underline the complexity of interactions occurring at the most molecular level between AMPs and microbial structures.

## Applications of Natural AMPs as Food Additives

AMPs are gaining much attention as potential active components of food products. However, some limited application could be related to their selective intestinal uptake and physiological instability when consumed orally (Udenigwe and Fogliano, 2017). AMPs are subject to structural changes and to interact with the food matrix during product development and as a result possibly reducing their bioaccessibility, bioavailability as well as biological activities (Udenigwe and Fogliano, 2017). The selection of friendly processing methods and less reactive matrices is imperative to preserve AMPs integrity and stability.

Many approaches exist for preparation of AMP-containing formulations such as inoculating food products with LAB producing strains and using their antimicrobial metabolites to inhibit the growth of undesirable microorganisms (Tahiri et al., 2009), delivery systems by controlling AMP release rate (Nordström and Malmsten, 2017) or active packaging material incorporating AMPs to extend storage life (Meira et al., 2017).

### Lactic Acid Bacteria Producing Strains

The use of LAB with known antimicrobial activity as biopreservatives has been largely studied and reviewed in O'Bryan et al. (2015). Nisin was the first bacteriocin approved for use in food (FDA, 1998) and a commercial crude fermentation product of LAB containing pediocin has also been manufactured by Quest International (Alta 2341). LAB may be present as natural flora of the food, or be intentionally incorporated as starter cultures in an industrial fermentation process. Several applications of LAB in foods such as poultry and meat (Woraprayote et al., 2016), seafood (Calo-Mata et al., 2008), fruits and vegetables (Hanning et al., 2009) have been studied to increase safety or maintain quality in foods. For instance, the potential of bacteriocin-producing LAB of traditional Thai fermented meat has been studied in order to decrease risks due to *Salmonella* spp., *S. aureus*, and *Listeria monocytogenes* (Swetwiwathana and Visessanguan, 2015). In addition, it has been clearly demonstrated that *Carnobacterium divergens* M35 culture as well as divergicin M35 bio-ingredient could be applied to the inactivation of *L. monocytogenes* in ready-to-eat seafood (Tahiri et al., 2009), which have been enabled by Health Canada's Food Directorate as food additives. Recently, the effects of a starter culture for controlling kimchi fermentation, a traditional Korean lactic acid-fermented vegetable, have been determined to prolong shelf life (Moon et al., 2018).

### Active Packaging Material Encapsulating AMPs

Another strategy to control the microbial contamination in foods that have received much attention in recent years is the development of innovative films enriched with bacteriocins (Woraprayote et al., 2018). The important role of packaging system is maintaining the concentration of bacteriocins above the active concentration and constantly releasing bacteriocins to kill target contaminant microorganisms (Appendini and Hotchkiss, 2002). For instance, an antimicrobial biodegradable film containing bacteriocin to control the growth of *Aeromonas*, *Escherichia*, *Pseudomonas*, *Staphylococcus*, *Listeria* and *Salmonella* in pangasius fish fillets have been successfully developed (Woraprayote et al., 2018). Additionally, the inhibition of *L. monocytogenes* by liposomal

nanovesicles containing bacteriocins produced by *Lactobacillus sakei* has been shown to efficiently retard bacterial growth in UHT goat milk (Malheiros et al., 2016).

### Food Matrix Interaction and Bioavailability of AMPs

For evidencing the technological applicability of AMPs, tests on toxicity, functionality and consequences to sensorial properties when added to different food matrices are necessary. Compared to other antimicrobial compounds such as oil and phenolics, AMPs tend to introduce less negative influence on the sensory qualities of food (Gould, 1996). As peptides, AMPs could be easily digested in the stomach, avoiding residue build-up and some of them may even contribute benefits to health (Wang et al., 2015). In the food matrix, the stability of AMPs has to be recognized through evaluation of different parameters such as insensitivity to salt, adaptation of a wide range of pH, thermal resistance and low cytotoxicity or hemolytic activity (Wang et al., 2015).

### Food Additive Regulation and Approval of AMPs

In the food sector, each AMP must be subjected to a very rigorous evaluation by regulatory agencies, such as the Food and drug administration (<http://www.fda.gov/FoodGuidances>) or Health Canada (<https://www.canada.ca/fr/sante-canada/services/aliments-nutrition/legislation-lignes-directrices/documents-reference.html#add>) before being recognized as safe (GRAS status) and approved as a food additive. The applicant must provide the following information during the evaluation and the approval process:

1. Scientific name
2. Proposed name
3. Composition and distinctive characteristics
4. Method of manufacture
5. Quantity used, proposed uses and purposes thereof, detailed directions for use, and recommendations
6. Data on the effectiveness of the antimicrobial bio-preservative
7. Detailed report on product safety under recommended conditions of use including gastrointestinal stability, allergenic potential, effect of a long-term exposure
8. Data on the quantity of residue that might remain on or in the food product
9. Proposed limit for residue on or in the food product method of detecting and monitoring the bio-preservative in the food product
10. Example of a proposed label for the additive

### Conclusion

Identification of AMPs has attracted a lot of attention in recent years. Their biological activity as well as their GRAS status makes them very attractive and offers a very promising alternative for use in food, animal and human sectors. As described in this book chapter, AMPs are widely distributed in nature and are relatively well documented in the literature. However, very few AMP-based products are currently authorized by regulatory agencies for food, medical or veterinary use despite the increasingly urgent demand for this type of compound. Research on both fundamental and applied aspects to characterize AMPs, to understand their mechanisms of action, to study their behavior and biological activity in complex matrices, is still necessary to reach their true potential.

Future work should focus on the following priority targets:

- Evaluation of the extent of the spectrum of action of AMPs not only against bacteria but also fungi and viruses;
- Characterization of the nature of antimicrobial compounds and their complete physicochemical property. At present, only few AMPs have been relatively well studied;
- Study of the behavior and the biological activity of AMPs under the real conditions of use. Moreover, the interactions of these compounds with the endogenous microbial ecosystem (animal or food) must be well studied;
- Study of their safety and their potential undesirable effects for the animal and the human;
- The development of technological processes enabling large scale, efficient and cost-effective production and stabilizing of AMPs.

These studies will generate essential knowledge for a more efficient and better targeted use of AMPs in different sectors. This knowledge is crucial in the present context where the use of chemical additives is increasingly challenged in foods and that the problem of resistance to antibiotics as well as the emergence of new pathogens are increasing continuously in the veterinary and medical sectors.

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- APD (<http://aps.unmc.edu/AP/>).
- Bactibase (<https://www.ncbi.nlm.nih.gov/pubmed/17941971>)
- CAMP<sub>R3</sub> (Collection of Anti-Microbial Peptides) (<http://www.camp3.bicnirrh.res.in/>).
- MilkAMP (<http://milkampdb.org/>).
- PhytAMP database (<http://phytamp.hammamilab.org/>).

### Organizations

- FAO (Food and Agriculture Organization of the United Nations) (<http://www.fao.org/documents/card/en/c/18240EN>).
- Food and Drug Administration (<http://www.fda.gov/FoodGuidances>).
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- WHO (World Health Organization), 2017. Food Safety. Fact Sheet. <http://www.who.int/mediacentre/factsheets/fs399/en/>.

# INDEX

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## Notes

Cross-reference terms in *italics> are general cross-references, or refer to subentry terms within the main entry (the main entry is not repeated to save space).*

The index is arranged in set-out style with a maximum of three levels of subheading. Major discussion of a subject is indicated by bold page numbers. Page numbers suffixed by t, f, and b refer to Tables, Figures, and Boxes respectively. *vs.* indicates a comparison.

The index entries are presented in word-by-word alphabetical sequence in which a group of letters followed by a space is filed before the same group of letters followed by a letter. For example, entries beginning 'air density' are alphabetized before 'aircraft.' Prefixes and terms in parentheses are excluded from the initial alphabetization.

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